# Modified alkaline peroxide pretreatment: an efficient path forward for bioethanol production from bamboo

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**Abstract**

To overcome the typical delignification saturation point of alkaline peroxide pretreatment and further facilitate lignin removal, a novel modified alkaline hydrogen peroxide pretreatment (MAHP) was proposed by introducing ethanol into the reaction

system. The dosages of $H_2O_2$, ethanol, and pretreatment temperature were optimized, and the results revealed that a maximum lignin removal as high as 79.25% could be achieved at only 100 °C, 3 wt% $H_2O_2$ concentration and 1 wt% ethanol concentration. Meanwhile, 76.5% of glucan and 56.0% of xylan were preserved at this pretreatment condition. By overcoming the delignification saturation point, enzymatic hydrolysis efficiency was remarkably enhanced, achieving 96.76% and 97.38% of glucan and xylan conversion, respectively, which are 7.4 and 11.4 times as compared to that of the untreated bamboo. Furthermore, the simultaneous saccharification and fermentation (SSF) result indicated an identical ethanol yield of ~75% when elevating the SSF solid loading from 5% to 30%. Based on the sequential SSF and xylose fermentation results, about 5.6 tons of bamboo would be consumed to produce 1 ton of ethanol. Finally, the energy balance revealed that a positive balance of 1255.4 KJ could be generated via processing 1 kg bamboo. The results demonstrate that the MAHP is a promising high-efficiency pretreatment technology for bamboo due to the mild pretreatment severity and robust ethanol yield.

**Keywords:** modified alkaline hydrogen peroxide pretreatment; lignin removal; enzymatic hydrolysis; simultaneous saccharification and fermentation

## 1. Introduction

There is a growing consensus that the global climate is changing due to the anthropogenic activities, one of which is the overuse of fossil fuels, such as petroleum, natural gas, and coal as primary energy resources which discharge a huge

amount of greenhouse gases [1],[2],[3]. Several approaches have developed to address global warming effects, including blending mandates, tax incentives, purchasing policies, and others. While in the USA, a framework of using renewable energy as a substitute for gasoline has been implemented [4]. Bioethanol is proposed as the leading candidate to replace or supplement petroleum-based liquid fuels, as it can be used in motors in a mixture up to 10% with gasoline with no need to modify the engines [5]. USA and Brazil are currently the two biggest bioethanol producers globally, accounting for 89% of the total global ethanol production [6]. Although the 1$^{st}$ generation of biofuels, such as corn- and sugarcane-based ethanol, is a promising substitute, their production is under scrutiny because it competes with the food market, which affects global food security.

Second-generation bioethanol, which uses lignocellulosic biomass as its feedstock, could alleviate the above-mentioned issues because the raw materials are nonfood, abundant, renewable, and inexpensive [7],[8]. Lignocellulosic biomass is rich in cellulose and hemicellulose, which can be depolymerized into fermentable sugars *via* cellulolytic and hemicellulolytic enzymes, and further fermented to ethanol [9]. However, the plant has a rigid and compact cell wall structure, in which the cellulose fibrils interact with intermolecular hydrogen bonds, and are wrapped and sealed by the polymeric matrix of hemicellulose and lignin [10]. This intrinsic recalcitrance restricts the availability of the polysaccharides for conversion into biofuels and chemicals. Therefore, a pretreatment step is often deemed a prerequisite for deconstructing the plant cell wall and enhancing the accessibility of

polysaccharides to enzymes [11],[12]. This effect is typically realized by the removal or redistribute of hemicellulose and/or lignin during the pretreatment under severe conditions (i.e., high temperatures, pressure, and chemical loadings). Nevertheless, the physical and chemical structures of the plant cell walls are highly dynamic in nature, and thus the efficiency of pretreatments can vary significantly from biomass to biomass. For example, autohydrolysis have been proven to be feasible in improving the enzymatic hydrolysis efficiency of grass and hardwood by removing a large amount of hemicellulose [13],[14], but it seems to be inefficient in softwood due to the abundance of lignin [15]. In another report, DeMartini and the co-workers have proposed that hemicellulose is the key recalcitrance-causing factor in switchgrass, while lignin likely plays an important role hindering enzymatic digestion of the highly lignified woody biomass [16].

To date, a number of bioresources have been investigated for their potential in bioethanol production, including agricultural residues [17], energy crops [18], woody biomass [19], and industrial waste solid residues [20]. China has the world's most abundant bamboo resources, with an area occupying 33000 $km^2$, about 3% of the global forest area [21]. Previous studies have demonstrated bamboo as a promising feedstock for bioethanol production due to its abundance, rapid growth, and high productivity [22]. Typically, bamboo is a very recalcitrant biomass with high lignin content, and thus most of the relevant pretreatments applied on bamboo are targeting at removing the lignin and thus increasing its carbohydrate accessibility. Mohan et al. pretreated bamboo with ionic liquid at 150 °C for 3 h, which removed 74.4% lignin

and resulted in a 80% cellulose hydrolysis yield [23]. In another work, it was indicated that using a kraft pulping pretreatment (i.e., 26% effective alkali and 24% sulfidity at 160 °C for 70 min) could remove more than 95% bamboo lignin, while the cellulose hydrolysis yield was only 79% [24]. Recently, Yuan et al. conducted sequential alkaline pre-extraction (8 wt% NaOH at 100 °C for 3 h) and alkaline peroxide pretreatment (4 wt% $H_2O_2$ at 75 °C for 3 h) on bamboo, which removed ~85% lignin and resulted in a cellulose hydrolysis yield of 92.6% [25]. Nevertheless, these pretreatments were conducted at severe conditions with high chemical loadings, which would significantly increase the cost in the biorefinery, and mild pretreatment at low temperature and low chemical loadings are imperative to be developed.

Alkaline hydrogen peroxide pretreatment (AHP) has been widely adopted in pulp bleaching to remove the chromophores and whiten the pulp. Researches have also implied that the AHP is also applicable in removing bamboo lignin, thus improving cellulose accessibility. However, a "delignification saturation point" was reported in our previous publication when using the AHP to pretreat the bamboo, which means that a certain amount of lignin could not be removed even with the increase of the chemical charge and temperatures [26]. Our work further found that with ethanol's addition to the AHP system, this delignification saturation point could be partially overcome at a mild pretreatment condition. To determine the potential and practical feasibility of the proposed modified AHP (MAHP), in this study, the effects of $H_2O_2$ and ethanol concentration on degrading the bamboo cell wall and changing the chemical and elementary composition were systematically investigated with an

objective to explore the role of the main components variations in affecting the enzymatic hydrolysis efficiency of bamboo. Finally, the mass and energy balances were estimated quantitatively to determine the feasibility of our proposed biorefinery concept.

## 2. Materials and methods

### 2.1. Materials

Bamboo (*Neosinocalamus affinis*) culms in this study were harvested in 2017 in Guizhou Province, China. Cellulase (*Cellic CTec2,* Novozymes) and xylanase (X2753-50 g) were obtained from Sigma (Shanghai, China), with an enzyme activity of 250 FPU/mL and 3490 U/g, respectively. Glucose and xylose fermentation strains of *S. cerevisiae* and *P. stipites* were kindly provided by the Biochemical Engineering Lab of Nanjing Forestry University. All other chemicals, of analytical grade, were purchased from Sinopharm Chemicals Reagents Co. Ltd (Beijing, China).

### 2.2. Bamboo size reduction and pretreatment

Prior to the pretreatment, the obtained bamboo culms were mechanically treated to reduce the size by being sequentially subjected to a twin-screw extruder (JWP50, Jiangsu Jinwo Mechinery Co., Ltd, Jiangsu, China) and disk mill (GNM300, Chunhui Machinery Co., Ltd, Beijing, China), in an attempt to enhance the heat transfer during the pretreatment. In detail, the bamboo culms were first immersed in tap water overnight to adsorb water. The wet sample was then separated and subjected to the twin-screw extruder to cause fibrillation. Next, the bamboo fibrils were loaded into the

disk mill to reduce the particle size. Disk milling was conducted in a 30 cm disk refiner at atmospheric pressure. The bamboo was subjected to the disk milling at a 25% consistency with the disk gap of 0.5 cm. The disk milling operation was performed two times.

Bamboo pretreatment was conducted using a modified alkaline hydrogen peroxide pretreatment (MAHP) by introducing ethanol into the AHP system. Briefly, the pretreatment liquor was prepared by dissolving NaOH, $Na_2SiO_3$, diethylenetriaminepentaacetic acid (DTPA), $H_2O_2$, and ethanol into DI water, in which their final concentrations were 2.2 wt%, 0.4 wt% and 0.1 wt%, 0-3 wt%, and 1-3wt%, respectively. The liquor was then poured into a 1 L beaker containing 20 g dry weight of bamboo at the solid to liquid ratio of 1:10 (w:v), and then vigorously stirred for 2 min. The mixture was then loaded into a 350 mL pressure glass flask and sealed with a screw cap. The flasks were then put in a water bath with temperature ranging from 40 to 100 °C, and maintained for 60 min. Upon the completion of the MAHP, the pretreated solid and liquid were separated with a cloth bag and the solid was washed substantially with tap water until the effluents were pH neutral. The pretreated and washed bamboo was then stored at 4 °C for the following experimentations. Notably, the pretreatment liquid was separated, but not used for any tests in this study.

### 2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 150 mL glass flasks with a working volume of 20 mL and a consistency of 5% (w/w). In detail, 1 g pretreated bamboo (dry weight) was weighed into the glass flasks, followed by the addition of cellulase and

157 xylanase at the dosages of 25 FPU/g-glucan and 150 U/g-xylan, respectively. Next, 1
158 M acetate buffer was added to control the enzymatic hydrolysis system pH around 4.8.
159 Tetracycline (0.10 g/L) was used in all enzymatic hydrolysis runs to inhibit the
160 microbial contamination. Finally, DI water was supplemented to set the final volume
161 of 20 mL. The flasks were then incubated at 50 °C and 150 rpm for 72 h. After
162 enzymatic hydrolysis, 1 mL enzymatic hydrolysate was withdrawn, centrifuged, and
163 diluted for fermentable sugars analysis.

164 2.4. Microorganisms cultivation

165 Prior to the fermentation test, the strains were first cultured to proliferate the cells.
166 In detail, *S. cerevisiae* was inoculated into the medium containing 20 g/L glucose, 5
167 g/L peptone and 3 g/L yeast extract, and cultured at 30 °C and 150 rpm for 24 h. After
168 that, the cells were transferred to the same fresh medium for further proliferation. After
169 culturing 3 rounds, the cells were centrifuged and then washed with excessive DI water
170 to remove any residual sugars. Finally, 50 mL sterilized water was added to re-suspend
171 the cells and the OD (optical density) value of the microorganism suspension was
172 measured at 600 nm with a spectrophotometer.

173 The cultivation and collection of *P. stipites* were conducted with the same
174 procedure of *S. cerevisiae* except the medium which was composed of 30 g/L xylose,
175 30 g/L glucose, and 3 g/L peptone.

176 2.5. High-solids simultaneous saccharification and fermentation (SSF)

177 SSF was conducted in 150 glass flasks with a working volume of 20 mL. To
178 achieve high solid loadings in the SSF, pretreated and washed bamboo was air-dried at

RT until constant moisture content (7.48% in this study). The dried bamboo was then weighed into the flasks at the solid loadings ranging from 5% to 30%. To alleviate the mass transfer problem induced by the high solid loadings, a fed-batch strategy was adopted when the initial SSF substrate loading was higher than 10%, which was achieved by supplementing the remaining sample at a 5% loading per 12 h until reaching target solid loading. Notably, the cellulase (25 FPU/g-glucan), xylanase (150 U/g-xylan), and yeast (OD of 5.0 in the SSF system) were added at the beginning of the SSF, different from the substrate. Citrate buffer was also used in the SSF to control the pH round 4.8. Afterwards, nutritive salts were added at final concentrations of 0.24 g/L urea, 0.08 g/L $ZnCl_2$, 0.08 g/L $MgSO_4$ and 0.20 g/L $CaCl_2$. After adding sterile water to 20 mL, the flasks were sealed with rubber stoppers with a syringe needle and then incubated at 36 °C and 150 rpm for 168 h. Samples were withdrawn during the course of the SSF. All the tests were conducted in duplicate, and the results represented an average value.

After the SSF, the flasks were subjected to a 50 °C water bath to evaporate the ethanol, and they were then sterilized at 121 °C for 15 min. After that, an additional volume of water was supplemented to compensate the water loss in the aforementioned process. Next, *P. Stipitis* was inoculated into the system at the OD of 5 to further convert xylose to ethanol, along with various nutritive salts of 0.25 g/L $MgSO_4$, 0.25 g/L $CaCl_2$, 0.25 g/L $KH_2PO_4$ and 0.24 g/L urea.

Ethanol yields during the SSF ($Y_{SSF}$) and xylose fermentation ($Y_X$) were calculated by the following equations:

$$Y_{SSF}\ (\%)=\frac{\text{ethanol in the broth (g)}}{\text{initial glucose in the pretreated substrate (g)}\ \times 0.51}\times 100\%$$

$$Y_X(\%)=\frac{\text{ethanol in the broth (g)}}{\text{initial xylose in the broth (g)}\times 0.46}\times 100\%$$

2.6. Analytical procedure

The crystalline structure of the samples was tested with a Bruker XRD instrument (D8 advanced instrument, Bruker, Germany), using Cu Kα as X-ray source at a voltage of 40 kV and a current of 30 mA. The scanning 2θ was from 10° to 40° with a scanning speed of 2°/min. Thermogravimetric analysis (TGA) was performed with a TG209F1 instrument (Netzsch, Germany) under a high-purity nitrogen atmosphere. The surface atomic composition and chemical environment were analyzed with the X-ray photoelectron spectroscopy (XPS, Shimadzu AXIS UltraDLD, Shimadzu, Japan) using Al K$\alpha$ X-ray radiation as the X-ray source. Elemental atomic percentages (molar percentage) were calculated by integrating the intensities of the XPS peaks with the XPSpeak41 software. The oxygenated to unoxygenated carbon ratio was calculated as follows [27]:

$$C_{ox/unox}=\frac{C_{oxidized}}{C_{unoxidized}}=\frac{C_2+C_3}{C_1}$$

The compositions of all the samples in this study were measured according to the two-step sulfuric acid hydrolysis procedure developed by NREL, including glucan, xylan, arabinan, acid-soluble lignin, and acid-insoluble lignin [28],[29]. The concentrations of monosaccharides and ethanol in this study were quantified with an Agilent HPLC (1260 Ⅱ, Agilent, USA). The details of the HPLC testing process can be referred to our previous research [9], .

The following equation calculated the enzymatic hydrolysis yield:

223 $$\text{Enzymatic hydrolysis yield (\%)}=\frac{\text{glucose or xylose in enzymatic hydrolyzate (g)}}{\text{initial glucose or xylose in the substrate (g)}}\times 100\%$$

224 2.7. Energy balance assess

225 The energy balance was estimated by including the energy input for biomass
226 milling, pretreatment, SSF, xylose fermentation, and ethanol distillation, and the energy
227 output recovered in the form of ethanol. The detailed energy balance is shown as
228 follows:

229 2.7.1. Energy consumption in the size reduction process

230 Size reduction mainly includes two steps from the twin-screw extruder and disk
231 mill. The energy consumed by the twin-screw extruder is negligible compare to that in
232 the disk mill. Thus it was excluded for the energy consumption in this study. As to the
233 disk mill's energy consumption, it is not stable because the amount of loaded bamboo
234 is low in the initial and ending period. Therefore, we conducted several batch tests with
235 a high amount of 5 kg bamboo (dry weight), and the averaged energy consumption was
236 measured to be ~553 Wh/kg dry bamboo (i.e., 1990.8 KJ/kg).

237 2.7.2. Energy consumption during the pretreatment.

238 Energy consumption in the pretreatment heating process was evaluated based on
239 the following equation according to the report by Kaur et al. [30]:

240 $$E_{heat}=W\times C(T_F-T_I)$$

241 Where W is the dry weight of the substrate (kg), C is the specific heat capacity of
242 bamboo (1.5 kL/kg °C) [31], $T_F$ is the final pretreatment temperature, and $T_I$ is the initial
243 temperature (25 °C in this study).

244 2.7.3. The energy input in the fermentation

Energy input in the SSF was estimated to be 266.7 KJ/kg biomass based on the results of Zhu et al. [32]. Energy input in the following xylose fermentation was also based on this value as the operating temperature of these two processes is close.

2.7.4. Energy consumption for ethanol distillation

Energy consumption in the distillation process is estimated using the average distillation and dehydrating energy of 6500 MJ/t ethanol [30].

2.7.5. Energy output

Energy output is the recovered energy from the total ethanol in the whole process, and it was calculated based on the theoretical average energy value of 27 MJ/kg [33].

## 3. Results and discussion

### 3.1 Biomass composition before and after MAHP

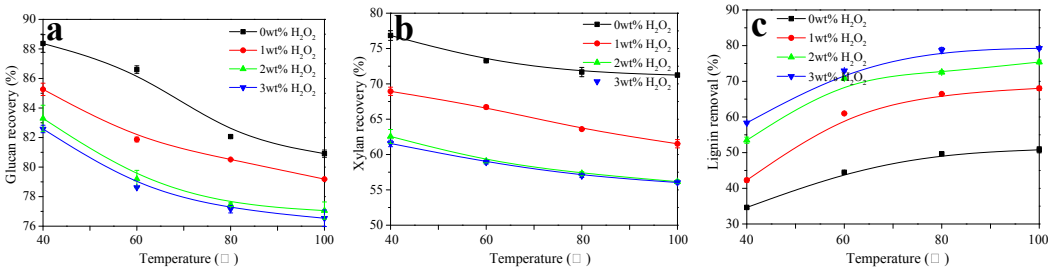
It is well known that bamboo is a highly lignified biomass, and severe pretreatment conditions are normally needed to break down its structure and increase the cellulose accessibility to enzymatic digestion. The research by many other colleagues has demonstrated lignin as a key factor hindering the enzymatic digestion of bamboo which is different from other herbaceous plants (such as wheat straw and switchgrass) where hemicellulose is the main inhibiting factor. Although some of the previous researches have realized high enzymatic hydrolysis yield of pretreated bamboo, those studies are based on pretreating bamboo at high temperatures with high alkaline loadings. AHP has been verified to be an efficient technology to delignify the bamboo and enhance the enzymatic digestibility of bamboo [34],[35]. We conducted the AHP using bamboo as

267 substrate, and found that lignin removal increased from 36.4% to 74.6% as the
268 temperature increased from RT to 80 °C ($H_2O_2$ concentration of 3 wt% and NaOH
269 concentration of 2.2 wt%, as shown in Fig. S1). However, further delignification degree
270 was not observed as the temperature was increased to 100 °C. In addition, it was also
271 found that the lignin removal cannot be enhanced by introducing more $H_2O_2$ into the
272 reaction system, as almost the same extent of delignification was observed at the $H_2O_2$
273 concentration of 7 wt% (data not shown here). Thus the highest level of delignification
274 a pretreatment technique that could achieve under particular conditions was defined as
275 the delignification saturation point, which is about 75% in the traditional AHP. The
276 existence of this saturation point suggests that a certain amount of lignin cannot be
277 removed. However, the enzymatic digestibility of the pretreated substrate is
278 significantly governed by the amount of lignin presented in the solid residue. In a most
279 recent study, we showed that when ethanol was introduced (as high as 15 wt%) into the
280 AHP system, lignin removal further reached 80.04% (Fig. S1), subsequently resulting
281 in a near 100% of cellulose digestibility [36]. Despite the dramatic improvement in
282 sugar release during enzymatic hydrolysis, the MAHP system has not been optimized,
283 and the effect of ethanol addition on the fermentation was not employed either.

284 In this study, the effects of $H_2O_2$, ethanol addition, and the temperature was
285 systematically investigated based on the pretreated solid residue (without accounting
286 the liquid fraction), and the results are shown in Fig. 1 (1 wt% ethanol concentration),
287 along with the detailed chemical compositions shown in Table S1. As can be seen in
288 Fig. 1a and b, the recoveries of glucan and xylan were remarkably decreased with the

289 increase of both the temperature and $H_2O_2$ dosage. Specifically, the recovery of
290 glucan was gradually decreased from 88.38% (0 wt% $H_2O_2$), 85.27% (1 wt% $H_2O_2$),
291 83.29% (2 wt% $H_2O_2$) and 82.58% (3 wt% $H_2O_2$) to 80.91%, 79.18%, 77.05% and
292 76.54%, respectively, as elevating the pretreatment temperature from 40 °C to 100 °C.
293 The similar trends were also observed for xylan recovery which decreased from
294 76.85% (0 wt% $H_2O_2$), 68.95% (1 wt% $H_2O_2$), 62.56% (2 wt% $H_2O_2$) and 61.57% (3
295 wt% $H_2O_2$) to 71.25%, 61.53%, 56.16% and 56.04%, respectively. These results are
296 consistent with previous studies showing that high temperatures and high chemical
297 loadings contribute to the degradation of the carbohydrates [37]. Besides, the
298 recoveries of glucan and xylan became very close as the $H_2O_2$ dosage increased from
299 2 wt% to 3 wt%, which indicated that the solubilization of carbohydrates in the
300 MAHP plateaued with the addition of 2 wt% $H_2O_2$ (Fig.1 a and b). Furthermore, the
301 delignification was found to be improved with the increase of pretreatment
302 temperature and $H_2O_2$ dosage, which increased from 34.6% (0 wt % $H_2O_2$), 42.3% (1
303 wt% $H_2O_2$), 53.5% (2 wt% $H_2O_2$) and 58.4% (3 wt% $H_2O_2$) to 55.8%, 68.0%, 75.4%,
304 and 79.3%, respectively, as the pretreatment temperature was elevated from 40 °C to
305 100 °C. It should be noted that the highest lignin removal in this study reached
306 79.25% at only 100 °C with low chemical loadings, superior to the traditional alkaline
307 pretreatments such as green liquor pretreatment and kraft puling which were
308 performed at much more severe conditions [24],[38]. Moreover, the effect of ethanol
309 concentration was studied by increasing its concentration from 0 wt% to 3 wt% (Fig.
310 S2), and it can be observed that the delignification was significantly improved by

311 introducing 1 wt% ethanol into the system, while further increasing the ethanol
312 dosage from 1 wt% to 3 wt% only resulted in a slight increase in the lignin removal,
313 with the highest value of 81.55%. As a result, 1 wt% ethanol addition was used in the
314 following MAHP experiments.




315

316 **Fig. 1.** Compositional variations during the pretreatment. a. glucan recovery; b. xylan
317 recovery; c. lignin removal.

318

319 3.2. Elements analysis of the pretreated bamboo samples

320 **Table 1** Detailed atomic information from the XPS spectra (pretreatment at 100 °C).

| | O/C | C1 s total=100% | | | $C_{ox/unox}$ |
|---|---|---|---|---|---|
| | | C1 (%) | C2 (%) | C3 (%) | |
| Raw | 0.46 | 41.78 | 46.69 | 11.53 | 1.39 |
| 0wt% $H_2O_2$+1wt%ethanol | 0.54 | 34.06 | 53.62 | 12.32 | 1.94 |
| 1wt% $H_2O_2$+1wt%ethanol | 0.64 | 24.15 | 60.10 | 15.75 | 3.14 |
| 2wt% $H_2O_2$+1wt%ethanol | 0.64 | 22.92 | 61.16 | 15.92 | 3.36 |
| 3wt% $H_2O_2$+1wt%ethanol | 0.66 | 20.63 | 62.89 | 16.48 | 3.85 |
| 3wt%$H_2O_2$+3wt%ethanol | 0.69 | 18.25 | 65.51 | 16.24 | 4.48 |

321

The chemical environment and atomic concentration of the pretreated bamboo were investigated with XPS since it is very sensitive to the surface state of the samples and can be used to qualitatively and semi-quantitatively analyze the element distributions of a solid surface. The results are shown in Fig. S3, S4, and Table 1. Bamboo is mainly composed of cellulose, hemicellulose, and lignin; thus, its primary element components are carbon, hydrogen, and oxygen. Fig S3 shows the typical XPS survey spectra of the raw and pretreated bamboo with strong signals arising for carbon and oxygen centered at about 284 eV and 532 eV, respectively. In addition, a small amount of nitrogen can be found in the spectrum of raw bamboo at 396 eV, which disappeared after the pretreatment, indicating that the proposed pretreatment is capable of dissolving protein during the pretreatment.

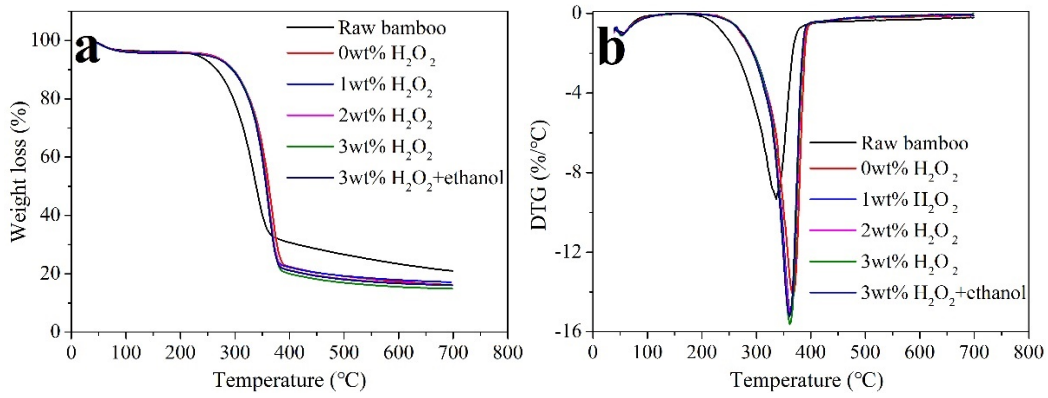
It is well known that lignin is enriched in carbon element, while the carbohydrates contain a relatively high content of oxygen. Therefore, the oxygen to carbon molar ratio (O/C) can be used to evaluate the content variations of these compositions during the pretreatment, and the results are shown in Table 1. The O/C ratio for raw bamboo is 0.46, higher than that of moso bamboo (*Phyllostachys edulis (Carr.) H.de Lehaie*) [39]. It has been reported that cellulose possesses a higher O/C ratio (0.83) than hemicellulose, and lignin has the lowest O/C ratio (0.33). Therefore, a high O/C ratio indicated low coverage of lignin. After the pretreatment (1 wt% ethanol addition), the O/C ratio increased dramatically from 0.46 to 0.54, 0.64, 0.64 and 0.66, respectively, with the $H_2O_2$ dosage of 0 wt%, 1 wt%, 2 wt%, and 3 wt%. This result is consistent with the lignin removal in Fig. 1, which shows that a small amount of $H_2O_2$ addition

344 could cause significant delignification. The MAHP performed with 3 wt% ethanol led
345 to the biomass showing the highest O/C ratio of 0.69 among all the tested samples,
346 indicating that delignification is further facilitated by extra ethanol.

347 Generally, the high-resolution C1s spectrum of the sample can be deconvoluted
348 into four types of carbon atoms, *i.e.*, C1 (C-C/C-H), C2 (C-O), C3 (C=O) and C4 (-
349 COOH). However, only C1, C2, and C3 carbon atoms were distinguished from each
350 other in our samples (see Fig. S4), which is in agreement with other lignocellulosics
351 [40]. The C1 peak is mainly derived from lignin and extractives, and the C2 and C3
352 peaks are associated with the carbohydrates in biomass, including cellulose and
353 hemicellulose [41]. The variations of peak area contributions are shown in Fig. S4, and
354 Table 1. Results showed that the C1 contribution considerably decreased from 41.78%
355 to 20.63% after the MAHP pretreatment when 1 wt% of ethanol was used, and this
356 value further decreased to only 18.25% in the 3 wt% ethanol assisted MAHP. In
357 contrast, C2 and C3 contributions increased correspondingly from 46.69% and 11.53%
358 to 65.51% and 16.24%, respectively. These results collectively revealed that the
359 pretreatment proposed in this study is quite effective in removing lignin, while
360 simultaneously retaining most of the carbohydrates. An increase in the $C_{ox}/C_{unox}$ was
361 observed after the pretreatment, as shown in Table 1, indicating that there were surface
362 oxidation and hydrolysis reactions during MAHP pretreatment [39].

363 3.3. Thermal stability of the samples

364




365

366 **Fig. 2.** TGA (a) and DTG (b) curves of raw and pretreated bamboo (at 100 °C).

367

368 It is important to explore the pretreated samples' thermal properties, which are
369 associated with their chemical structures. TGA and DTG analysis were performed to
370 directly reflect the thermal information of different samples, and the results are shown
371 in Fig. 2. The moisture evaporation and sample dehydration mainly occurred below 120
372 °C, with a weight loss of ~4% for all the samples. The primary degradation of the
373 samples was observed between 200-400 °C, including hemicellulose decomposition at
374 180-320 °C, cellulose degradation at 320-400 °C. In the case of lignin, it has a broad
375 degradation temperature, ranging from 170 °C to more than 600 °C [34],[42]. At a
376 temperature higher than 400 °C, the weight loss is mainly attributed to the breakdown
377 of charred fractions into gas compounds [43].

378 From the TGA and DTG curves, it can be found that there is only one main weight
379 loss peak, which is different from some literatures which reported two peaks [34]. This
380 difference could be because the initial degradation temperatures of hemicellulose,
381 cellulose, and lignin are very close to each other in bamboo. Increased thermal stability
382 was observed for all the pretreated bamboo substrates, evidenced by the higher

decomposition temperature of the pretreated samples (see DTG curve). The maximum weight loss temperature of raw bamboo was 336 °C, while the pretreated bamboo samples have a higher weight loss temperature of ~361 °C. The $H_2O_2$ and ethanol addition were found to have a negligible impact on the maximum weight loss temperature. This result is mainly attributed to the removal of a certain amount of hemicellulose and lignin during the pretreatment, which has a random amorphous structure and is therefore easily degraded. In the case of cellulose, which has a crystalline structure and long chain with a high degree of polymerization, it is more refractory during the heating. In addition, the residual lignin in pretreated bamboo also increased the degradation temperature in the TGA test as lignin owns a broad degradation temperature.

### 3.4. The effect of MAHP on the enzymatic hydrolysis efficiency of pretreated bamboo.

Enzymatic hydrolysis yield is the pivotal criteria for assessing the efficiency of pretreatment technology, and the MAHP treated bamboo samples (water washed samples) were subjected to enzymatic hydrolysis at a consistency of 5% for 72 h, and the results are shown in Fig. 3. The enzymatic hydrolysis yield of raw bamboo without pretreatment was 13.00% and 8.55% for glucan and xylan, respectively. After the MAHP, the bamboo's enzymatic hydrolysis efficiency was significantly improved, even at low temperatures, without the addition of $H_2O_2$. For example, the glucan and xylan hydrolysis yields increased to 52.65% and 62.77% at 40 °C without $H_2O_2$ addition, and these yields further reached to 68.33% (glucan) and 83.05% (xylan) at 100 °C (without $H_2O_2$). In addition, it was found that the enzymatic hydrolysis

efficiency was remarkably increased with the addition of $H_2O_2$. Take the samples pretreated at 100 °C for example, the glucan hydrolysis yield was increased from 68.33% to 83.66%, 91.01%, and 96.76%, as the $H_2O_2$ concentration increased from 0 wt% to 1 wt%, 2 wt%, and 3 wt%, respectively. The xylan hydrolysis yield was also increased as the $H_2O_2$ concentration increased and finally reached a maximum of 97.38%. Moreover, when increasing the system ethanol concentration from 1 wt% to 3 wt% (as shown in Fig. S5), the glucan and xylan hydrolysis yield slightly increased to 99.84% and 100%, respectively. An ideal biorefinery should be based on a looped process in which the end-products can be used in the processing steps, thus the ethanol addition should be as low as possible to make the biorefinery cost-effective, thus 1 wt% ethanol addition was considered as the optimized charge.

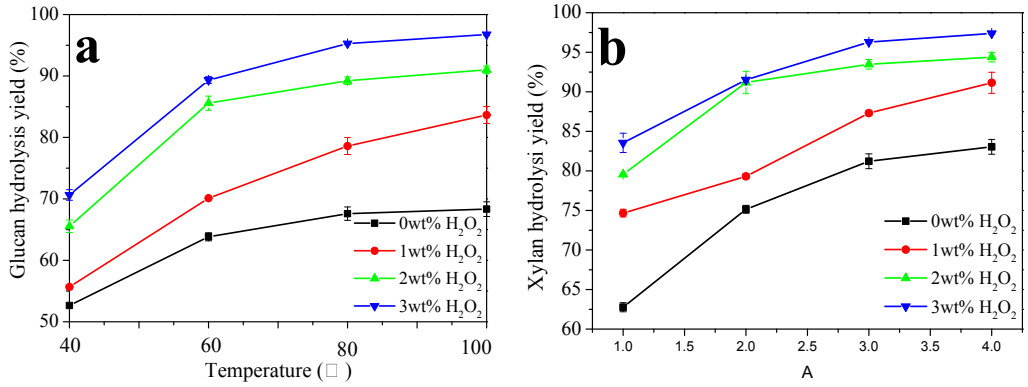
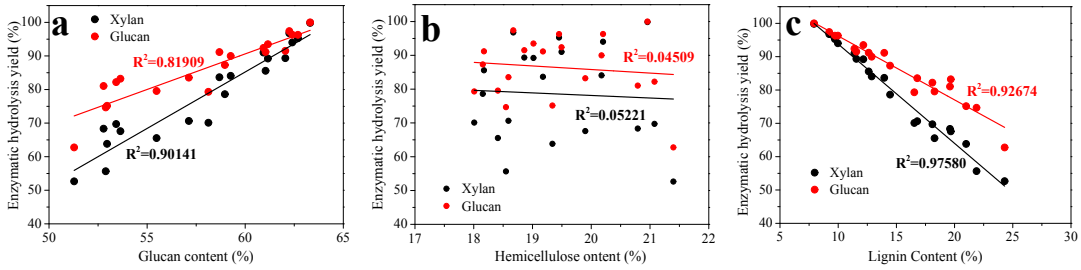



**Fig. 3.** Enzymatic digestibility of the pretreated bamboo with 1 wt% ethanol addition.

Although a comprehensive understanding of the biomass cell wall's architecture and recalcitrance is still unclear, it is generally acknowledged that cellulose in the plant cell wall is wrapped and crosslinked with other non-cellulosic compounds, forming the compact chemical structure of cellulosic biomass. The relative

423 importance of the three main chemical composition on the aforementioned enzymatic
424 hydrolysis yields was evaluated, and the results are shown in Fig. 4. It can be found
425 that the glucan content showed a positive correlation with the hydrolysis yield of both
426 glucan ($R^2$=0.90) and xylan ($R^2$= 0.82). Whereas the relationship between hydrolysis
427 yield and hemicellulose content was not apparent (Fig. 4b), although the
428 hemicellulose is widely reported to be detrimental to the enzymatic digestion. One
429 possible explanation is that the MAHP only removed a small portion of hemicellulose
430 and the part that shows recalcitrance retained in our MAHP treated substrate. In
431 addition to cellulose and hemicellulose contents, lignin seems to play a crucial role in
432 restricting the enzymatic hydrolysis yield. As shown in Fig. 4c, a strong negative
433 correlation ($R^2$=0.93 and 0.98 for glucan and xylan hydrolysis yields, respectively)
434 between lignin content and enzymatic hydrolysis yield was observed, indicating that
435 the lignin is the main factor inhibiting the enzymatic digestion of bamboo. The
436 adverse effects of lignin include a physical barrier to the carbohydrate and a non-
437 productive binding to enzymes, which have been illustrated previously [44].



438

439 **Fig. 4.** Correlation of glucan hydrolysis yield with (a) glucan content, (b)
440 hemicellulose content, and (c) lignin content.

441

Cellulose crystallinity is another factor that may influence the enzymatic hydrolysis efficiency. The relationship between the crystallinity index (CrI) and the enzymatic hydrolysis yield was shown in Fig. S6. As can be seen, cellulose CrI increased (from 57.04% to 66.24%) when the pretreatment temperature and chemicals loading increased, mainly caused by the removal of amorphous hemicellulose and lignin components [14]. Interestingly, a positive correlation between the enzymatic digestibility and CrI was observed ($R^2$=0.80 and 0.86 for glucan and xylan enzymatic hydrolysis yield, respectively). Generally speaking, amorphous cellulose is expected to be hydrolyzed at a much faster rate than crystalline cellulose [45]. However, many studies also reported that no straightforward relationship between enzymatic digestibility and CrI was observed, especially when using natural biomass as substrate [46],[47]. This result is because the substrate's enzymatic digestibility is interactively influenced by many factors, such as the specific surface area of cellulose, biomass particle size, cellulose accessibility, surface hydrophobicity and others [48],[49]. The cellulose CrI variations during the MAHP are inevitably accompanied by the changes of these factors, which consequently improved the enzymatic hydrolysis efficiency of the pretreated bamboo.

### 3.5 High-solid SSF of the pretreated bamboo

After enzymatic hydrolysis, fermentation is an essential step to finally convert the obtained fermentable sugars to liquid fuels such as ethanol. Nevertheless, the sequential enzymatic hydrolysis and fermentation, which is termed as separate hydrolysis and fermentation, has its limitations. One of the drawbacks is the limited

solid loadings which is caused by the unavoidable sugar product feedback inhibition at high solid loadings, accompanied by the high hydraulic loads, high energy demand for heating and agitating, and the risk of contamination. SSF protocol, in which the sugars from enzymatic hydrolysis can be immediately converted to ethanol, can help to address the aforementioned problems [50]. In this study, we elevated the solid loading in the SSF to 30%, which is the highest as reported to our knowledge, and the results are shown in Fig. 5. It was found that the majority of the glucose accumulated within the initial 6 h, which indicated that the glucose was accumulated at a higher rate than that it was consumed by the strain (Fig. 5a) [13]. After 6 h of microorganism adaption, the glucose concentrations in all the runs started to decrease and were finally lower than 1 g/L for all the solid loading runs after 24 h SSF. These results indicated that *S. cerevisiae* could efficiently metabolize the glucose, avoiding the undesired product feedback inhibition effects.

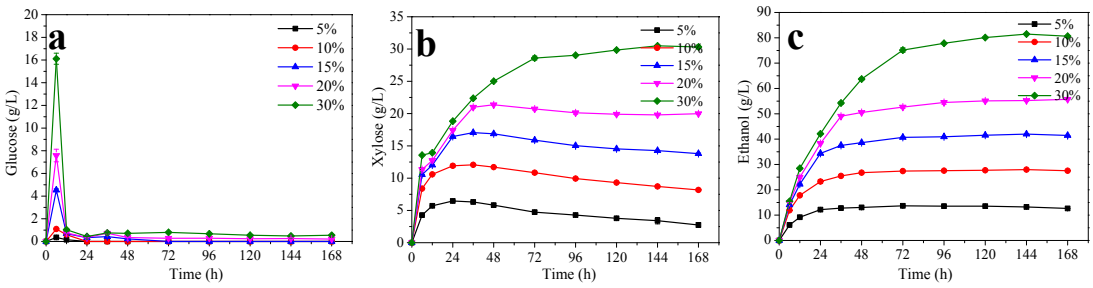



**Fig. 5.** Glucose (a), xylose (b) and ethanol (c) concentrations during the SSF experiments at different solid loadings (5-30% indicates solid loading ranging from 5% to 30%).

With the consumption of glucose, ethanol accumulated, and its concentration was increased with both higher solid loading and longer SSF time, as shown in Fig.

5c. In addition, the ethanol concentrations plateaued after 72 h SSF for 5%, 10%, and 15% solid loading runs. It took 96 h and 120 h for the 20% and 30% solid loading runs, respectively, to reach the plateau, which is probably due to the fed-batch addition of the pretreated solid. After the SSF, maximum ethanol concentrations of 13.67, 27.40, 41.99, 55.69, and 81.47 g/L were obtained with solid loading ranging from 5% to 30%, corresponding to the ethanol yields of 75.18%, 75.35%, 76.98%, 76.57% and 74.68% (calculated by the glucan in pretreated solid). As to ethanol productivity, it increased from 0.08 to 0.16, 0.25, 0.33, and 0.48 g/(L·h) accordingly. This result suggested that the yeast utilized glucose at a greater rate under the high solid loading, thus leading to high ethanol productivity. It has been widely reported that higher solid loading could result in high system viscosity and uneven slurry distribution, thus decreasing the ethanol yield [51]. Therefore, the high enzyme costs and low yields at high solid fermentation significantly challenge the overall competitiveness of the biorefinery, and how to overcome the negative effect of high solid fermentation remains a big problem. In this study, the ethanol yields were around 75% for all SSF runs, including the highest 30% solid loading SSF. We ascribed this phenomenon to the adoption of the modified SSF strategy, which greatly helped to alleviate the mass and heat transfer problem by adding the remaining fresh substrate when the system viscosity has been decreased.

In addition to glucose and ethanol, a certain amount of xylose was also quantified in our SSF system, as shown in Fig. 5b. It is noticed that the xylose concentrations increased first during the first 24/48 hours (depending on the solid loading) and then slightly decreased (excluding the 30% solid loading run). For example, in the 20% solid loading SSF, the xylose concentration increased from 0 to 21.36 g/L after 48 h SSF, and then decreased to 19.98 g/L at the end of the SSF. Li et

al. reported similar trends when conducting SSF using organic solvent pretreated Loblolly pine and sweetgum as substrate. The authors ascribed it to the interaction between xylose and ethanol that generated a compound named ethyl xyloside, which, as a result, decreased the xylose concentration [52]. In the case of the 30% solid loading SSF experiment, the xylose concentration continued increasing until 144 h, which was due to the saccharification of the fed-batch added substrate. Finally, at the end of the SSF, 2.75, 8.18, 13.80, 19.98, and 30.32 g/L xylose was obtained in the SSF broth. These parts of xylose need to be further converted because the wild *S. cerevisiae* is incapable of utilizing xylose.

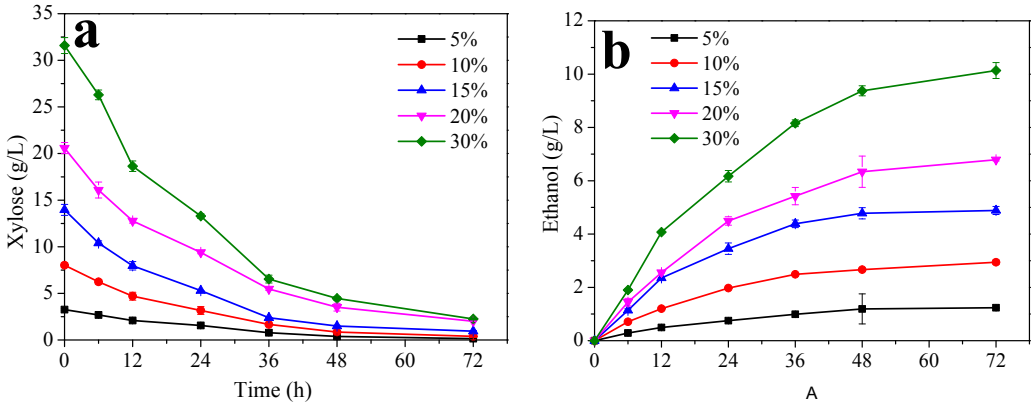



**Fig. 6.** Time curse for the conversion of the residual xylose in the system.

Till now, high-efficiency fermentation of xylose remains a challenge due to the lack of appropriate microorganisms. *P. stipites* is one of the few strains that can utilize xylose to produce ethanol. However, *P. stipites* has a low ethanol tolerance and tend to re-assimilate the ethanol in the surroundings [53]. To address the problem, the fermentation broth from the above-mentioned SSF was subjected to a water bath to remove the ethanol from the SSF and then inoculated with the *P. stipites*. The time course of the xylose and ethanol during the fermentation is shown in Fig. 6. It can be seen that the xylose was gradually consumed during the fermentation, which was

529 decreased from 3.25, 8.02, 13.96, 20.59 and 31.59 g/L to 0.14, 0.39, 0.95, 2.01 and
530 2.26 g/L after 72 h of fermentation, indicating 95.75%, 95.14%, 93.22%, 90.25% and
531 92.86% xylose was metabolized by *P. stipites*, respectively, upon the various solid
532 loadings. These results indicated that the *P. stipites* in this study could efficiently
533 utilize the xylose, which is even superior to the engineered strains [54]. At the same
534 time, ethanol concentration increased accordingly and finally reached the highest
535 values of 1.24, 2.94, 4.89, 6.79, and 10.14 g/L, respectively, corresponding to the
536 ethanol yields 86.14%, 83.76%, 81.63%, 79.43%, and 75.12%. Although the xylose-
537 fermenting is less efficient than that of the glucose, this sequential SSF and xylose
538 fermentation still outperform the conventional SSCF (simultaneous saccharification
539 and co-fermentation) process which has a compromise in the glucose fermentation
540 with less ethanol tolerance when using the engineered strain [55].

541 3.6 Mass and energy balances of the biorefinery process

542 The overall mass balance, based on 1 kg raw bamboo, of the whole biorefinery
543 process was estimated, and the results are summarized in Fig. 7. As shown, there were
544 442.4 g glucan, 189.5 g xylan, and 260.8 g lignin in 1 kg raw bamboo accordingly to
545 the biomass compositional analysis. After the MAHP, only 586.5 g solid was
546 recovered, including 365.2 g glucan, 106.2 g xylan, and 54.1 g lignin. In
547 accompaniment, we modeled 30% solid loading SSF at cellulose loading of 25 FPU/g
548 glucan, xylanase loading of 150 U/g xylan, and *S. cerevisiae* OD of 5, which resulted
549 in 159.3 g ethanol. Besides, an additional fermentation of the residual xylose further
550 generated 19.8 g ethanol. These results indicated that MAHP is an efficient
551 technology towards bamboo biorefinery, enabling about 5.6 t raw bamboo producing
552 1 t of ethanol.

The biorefinery process's energetic feasibility was further appraised to confirm its competence as a sustainable model. The energy assessment was based on comparing the energy input in bamboo size reduction, pretreatment, fermentation (including SSF and xylose fermentation) and distillation, and the energy output from recovered ethanol, and the result is also shown in Fig. 7. The scenario depicts that most of the energy input took place in the size reduction and ethanol distillation steps, which was 1990.8 KJ and 1164.2 KJ, respectively, based on 1 kg raw bamboo. In the pretreatment process, only 112.5 KJ energy was consumed due to the low processing temperature. Besides, a total energy input of 312.8 KJ was observed in the fermentation process, including SSF and the following xylose fermentation. Moreover, the biorefinery sequence generated an energy output of 4835.7 KJ from ethanol, indicating a positive balance of 1255.4 KJ, which is the primary concern as it benefits the energetic and economic feasibility of the lignocellulosic biorefinery. Finally, it should be noted that the energy input in milling and distillation is the most energy-intensive step. Thus, advanced size reduction technology should be developed, which can help to decrease the energy input. As to the distillation, novel ethanol concentration technology, such as membrane separation, would reduce the downstream energy further, thereby enhancing overall energy efficiency.

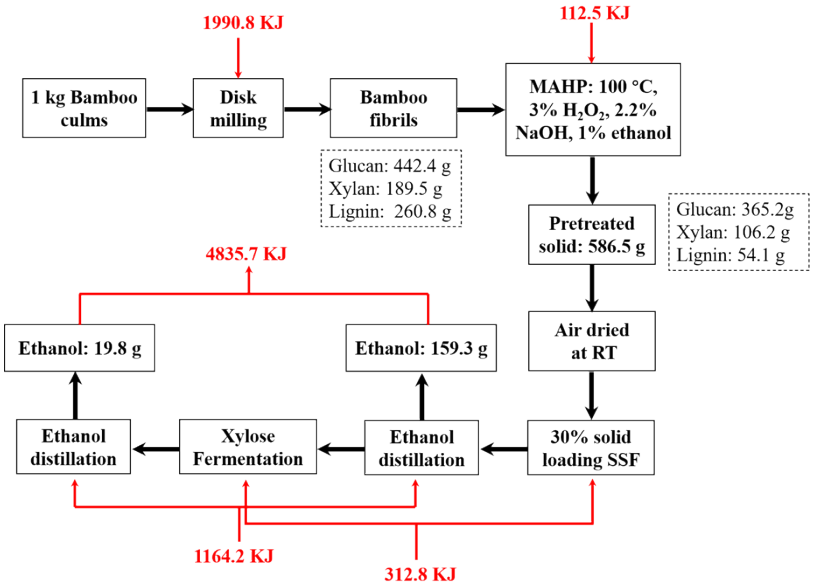



**Fig. 7.** Mass and energy balances of the proposed biorefinery process.

## 4. Conclusion

The MAHP was proven to be a powerful technology to degrade the lignin in bamboo, and 79.25% lignin was removed at a mild pretreatment condition of 100 °C, 3 wt% $H_2O_2$ with 1 wt% ethanol concentration, in addition to preserving a large amount of carbohydrates. The pretreated solids were readily to be enzymatic digested, with the hydrolysis yields as high as 96.76% (glucan) and 97.38 % (xylan). Interestingly, the SSF test showed an identical ethanol yield of ~75% as increasing the solid loadings from 5% to 30%. The mass and energy balances results suggested that 5.6 tons of raw bamboo could produce 1 ton of ethanol, with a positive energy balance of 1255.4 KJ during the whole process.

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