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ORIGINAL ARTICLE

A comparative investigation of H_2O_2-involved pretreatments on lignocellulosic biomass for enzymatic hydrolysis

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Abstract

An effective pretreatment to improve cellulose accessibility and facilitate glucose release is crucial in a cellulosic ethanol biorefinery. This work comparatively assessed four H_2O_2-involved pretreatments, i.e., concentrated H_3PO_4 plus H_2O_2 (PHP), H_2O_2–CH_3COOH (HPAC), alkaline-H_2O_2 (AHP), and Fenton chemistry (FC), for their pretreatment performances on wheat straw, poplar, and birch biomass. Substrate characteristics before and after pretreatment were assessed using SEM, XRD, and LSCM. The hydrolytic potentials of the pretreated substrates were compared by Simons' stain and cellulose–glucose conversion assessment. The results showed that acidic H_2O_2-involved pretreatments (PHP and HPAC) were more efficient in biomass delignification compared to AHP and FC. PHP pretreatment is more promising for cellulosic ethanol production due to its corresponding high glucose yield (368.0 mg g^{-1}) after enzymatic hydrolysis.

Keywords Lignocellulose · Delignification · Hemicellulose removal · Enzymatic saccharification · Enzyme accessibility

1 Introduction

There is an ongoing global tendency to develop biofuels and biochemicals that could replace petroleum-based and greenhouse-gas-emitting production processes due to the ever-growing demands on fossil resources and the gradually worsening environment caused by the consumption of fossil resources [1]. Lignocellulosic biomass such as sugarcane bagasse, straw, corn stover, cotton stalks, wheat straw, rice straw, rice husk, and wood chips is readily available at a relatively low cost and is suitable raw material for producing fuels, chemicals, and biobased materials [2–4]. However, the main challenge of utilizing lignocellulosic biomass, especially for the sugar-platform bioconversion route, lies in the crystalline nature of cellulose, high degree of polymerization of cellulose, large biomass particle size and recalcitrance, and lignin barrier, which allow a limited surface area for enzymatic hydrolysis [5]. Thus, an efficient pretreatment step to overcome biomass recalcitrance and increase cellulose digestibility is crucial in a typical bioethanol process.

Overall, the pretreatment process alters the macrostructure, microstructure, and chemical composition of lignocellulose. Moreover, it also alters the natural macromolecular structure of lignocellulose during decomposition to make it susceptible to the subsequent biological degradation. Currently, many pretreatment methods including physical, chemical, physicochemical, and biological approaches have been developed with various amounts of success; however, each method functions differently on deconstructing lignocellulosic biomass, resulting in distinct efficiency of subsequent conversion [6]. As a main component in lignocellulosic biomass, lignin actually acts as a biological cement that contributes to the

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formation of a highly recalcitrant lignocellulosic matrix. Moreover, it has been shown to have a negative effect on the enzymatic hydrolysis of cell-wall polysaccharides because it strongly reduces the access of enzymes and also binds to them, therefore weakening their activity [7, 8]. It has been reported that although an efficient delignification can be achieved in a short time, harsh pretreatment conditions are usually required [9]. In previous investigated pretreatment methods, delignification can be achieved by acid, alkaline, oxidation, AFEX, and organosolv pretreatment, in which oxidative delignification is an efficient path due to its selective lignin degradation with minimal effects on cellulose and hemicellulose [10]. Substantially, during the oxidative delignification, oxidizing agents release large amounts of free radicals, resulting in significant oxidative cleavage and removal of lignin [11]. The widely employed oxidants are hydrogen peroxide [12], ozone, oxygen [6], and peroxyacid (typically peracetic acid) [13], in which hydrogen peroxide embodies an ideal oxidant in terms of atom economy, availability, and green metrics [14], and is particularly attractive, both for its high oxygen content and the nature of its by-products (water and O_2 as the final products) [15]. Hydrogen peroxide itself can be used to pretreat lignocellulosic biomass [16]. It can also further improve the pretreatment effect by supplementing with other reagents [17]. Thereby, it is widely employed in alkaline or acidic conditions or catalyzed by transition metal ions to achieve oxidative delignification for pretreating lignocellulosic biomass [18–21].

Recently, at least four H_2O_2-involved pretreatment methods, i.e., alkaline hydrogen peroxide (AHP), hydrogen peroxide–acetic acid (HPAC), concentrated phosphoric acid plus hydrogen peroxide (PHP), and Fenton chemistry (FC), have been developed and reported for pretreating various lignocellulosic biomasses [20, 22–25]. As far as the reported characteristics were concerned, the delignification was the representative function of these pretreatment methods. The substantially occurred chemical reactions and the mechanisms on deconstructing biomass recalcitrance should be different. Besides, it could be found that the subsequent enzymatic hydrolysis of various methods ranged from 70 to 90% [20, 22–25]. In addition to the substantial breakdown of lignocellulosic recalcitrance for the subsequent hydrolysis, it should be noted that the employed biomass species, enzyme source and loading, use of auxiliary enzyme, and solids loading govern the overall efficiency of enzymatic hydrolysis [26]. Thus, comparing the efficiency of these H_2O_2-involved pretreatments should be investigated accordingly.

In these scenarios, a comprehensive comparison of these H_2O_2-involved pretreatment methods, AHP, HPAC, PHP, and FC, were performed to clarify their performance on removing biomass recalcitrance and the efficiency of cellulose hydrolysis, which helps to tailor the methodology for subsequent bioconversion. Typical agricultural residue wheat straw, hardwood poplar, and birch were employed for these H_2O_2-involved pretreatments. The main composition, cellulose recovery, hemicellulose and lignin removal, and cellulose hydrolysis were investigated to compare their technical features and performances. Meanwhile, structure changes, crystallinity, and enzymatic accessibility of the pretreated substrates were also evaluated to gain more insights into how H_2O_2 aids biomass delignification at various conditions.

2 Materials and methods

2.1 Feedstocks and chemicals

The employed wheat straw was harvested from the farm of Sichuan Agricultural University, Chongzhou, China, and the wood chips of poplar and birch were collected from the local furniture factory. The feedstocks were air-dried and milled into 40 mesh, and the main chemical components of the employed feedstocks are listed in Table 1. Phosphoric acid, hydrogen peroxide, sulfuric acid, ferrous chloride, sodium hydroxide, acetic acid, sodium acetate, glucose, and xylose used in this research are all provided by Chengdu Cologne Chemical Company. All these chemicals are analytical reagents. Cellulase (Cellic CTec2) is provided by Novozymes.

2.2 Pretreatment

Various H_2O_2-involved pretreatments, including AHP, HPAC, PHP, and FC, were performed on the mentioned feedstocks. The employed pretreatment conditions were based on the optimized conditions in the reported work [20, 23, 24, 27], in which AHP was carried out with 2% (*v*/*v*) H_2O_2 at a pH of 11.5 (using 10 mol L^{-1} NaOH for adjusting pH), and the pretreatment was performed at a temperature of 35 °C for 24 h. The HPAC pretreatment was performed at 80 °C for 2 h with the equal-volume mixture of H_2O_2 and CH_3COOH. As for the PHP, 79.6% H_3PO_4 and 1.91% H_2O_2 were employed for the pretreatment at 40.2 °C for 2.9 h. The FC was taken at 25 °C for 120 h, and the employed H_2O_2 was 176 mmol with 1.25 mmol Fe^{2+} as catalyst. These pretreatments were all carried out in 250-mL serum bottles with biomass loading of 10.0 g per 100.0 g pretreatment solution. The substrate-loaded bottles were fixed in a thermostatic shaker for pretreatment at the targeted temperatures and durations. When the pretreatment was completed, the pretreated substrates were washed with distilled water and filtered till the filtrate pH was close to 6.0. The washed substrates were stored in a 4 °C freezer for further analysis and enzymatic hydrolysis.

Table 1 Chemical compositions of raw wheat straw, poplar, and birch before pretreatment

Feedstocks	Chemical composition (%)					
	Cellulose[a]	Hemicellulose[b]	Acid–insoluble lignin	Acid–soluble lignin	Extractives	Ash
Wheat straw	31.6 ± 0.75	16.2 ± 0.40	18.5 ± 0.21	2.7 ± 0.19	11.3 ± 0.69	7.8 ± 0.45
Poplar	46.0 ± 0.10	16.7 ± 0.07	23.5 ± 0.34	3.0 ± 0.04	2.8 ± 0.38	1.0 ± 0.03
Birch	40.1 ± 0.62	17.5 ± 0.21	24.2 ± 0.08	3.2 ± 0.15	6.7 ± 0.15	1.8 ± 0.03

[a] Cellulose content was represented by glucan content

[b] Hemicellulose content was represented by xylan content

2.3 Enzymatic hydrolysis

The employed enzymes for saccharification were Cellic CTec2, which were gifted by Novozymes Beijing branch, in China. A relatively high enzyme input was 20 mg protein g^{-1} cellulose to ensure enough enzyme in assessing the hydrolysis efficiency, and the total working volume of the enzyme for hydrolysis was controlled at 12.0 mL of the 25-mL serum bottle with the solid loading of 2% (*w*/*w*, dry basis). The acetate buffer (0.05 M, pH 5.0) was supplemented in enzymatic hydrolysis to maintain a stable pH during the hydrolysis. All the hydrolysis experiments were duplicated. The incubation temperature and shaking frequency were controlled as 50 °C and 180 rpm, respectively. Prior to enzymatic hydrolysis, 60 μL tetracycline solution (40 mg L^{-1}) was added to inhibit the growth of microorganisms to prevent glucose consumption. Two hundred microliters of the slurry was sampled at 0, 4, 8, 12, 24, 48, and 72 h during enzymatic hydrolysis for the monomeric sugar determination. The withdrawn samples were heated at 100 °C for 5 min to inactivate the enzyme and then centrifuged at 1.0×10^5 rpm, 4 °C for 6.0 min. The supernatant was cryopreserved in the freezer (− 20 °C) for the further sugar analysis.

2.4 Analytic methods

2.4.1 Determination of lignocellulosic components

Klason lignin and carbohydrates in raw materials and pretreated substrates were analyzed according to referred work [28]. The typical carbohydrates of glucose and xylose in the hydrolysate from Klason lignin analysis were analyzed by high-performance liquid chromatography (HPLC) (Flexar, PerkinElmer, Inc., Waltham, MA, USA) equipped with a column (SH1011, Shodex, Showa Denko America, Inc., NY, USA) and a refractive index detector. The mobile phase was 0.05 mol L^{-1} H_2SO_4 with a flow rate of 0.8 mL min^{-1}, and the temperature of the column and the refractive index detector were fixed at 60 °C and 50 °C, respectively. The contents of glucan and xylan in the substrate were calculated by the determined glucose and xylose concentration. The obtained contents of glucan and xylan were employed to represent the cellulose and hemicellulose, respectively, in the substrates. According to the determined content of the mentioned composition and the solid recovery after pretreatment, cellulose recovery and hemicellulose and lignin removal were calculated according to Eqs. (1–4). Similarly, the released glucose from enzymatic hydrolysis was also detected by HPLC with the similar method, the enzymatic hydrolysis efficiency for each pretreatment was calculated by Eq. (5), and the glucose yield based on the feedstock (dry basis) was calculated by Eq. (6). All the testing samples were duplicated and a significance test ($P = 0.05$) was performed using SPSS software.

$$solid\ recovery(\%) = \frac{Wpretreated\ substrate}{Wfeedstock} \times 100 \tag{1}$$

$$cellulose\ recovery(\%) = \frac{Wcellulose\ of\ pretreated\ substrate}{Wcellulose\ of\ feedstock} \times 100 \tag{2}$$

$$hemicellulose\ removal(\%) = \left(1 - \frac{Whemicellulose\ of\ pretreated\ substrate}{Whemicellulose\ of\ feedstock} \times 100\right) \tag{3}$$

$$lignin\ removal(\%) = \left(1 - \frac{Wlignin\ of\ pretreated\ substrate}{Wlignin\ of\ feedstock} \times 100\right) \tag{4}$$

$$enzymatic\ hydrolysis(\%) = \frac{Mglucose\ of\ enzyme\ hydrolysate}{Mcellulose\ of\ pretreated\ substrate \times 1.1} \times 100 \tag{5}$$

$$\text{glucose yield (mg/g)} = \frac{Wpretreated\ substrate \times \alpha \times \beta}{Wfeedstock} \tag{6}$$

where $W_{\text{pretreated substrate}}$ is the dry weight of pretreated substrate; $W_{\text{feedstock}}$ is the dry weight of feedstock; $W_{\text{cellulose of pretreated substrate}}$, $W_{\text{hemicellulose of pretreated substrate}}$, and $W_{\text{lignin of pretreated substrate}}$ are the dry weights of cellulose, hemicellulose, and lignin in the pretreated substrate; $W_{\text{cellulose of feedstock}}$, $W_{\text{hemicellulose of feedstock}}$, and $W_{\text{lignin of feedstock}}$ are the dry weights of cellulose, hemicellulose, and lignin in the feedstocks; $M_{\text{glucose of enzyme hydrolysate}}$ is the glucose amount in hydrolysates; $M_{\text{cellulose of pretreated substrate}}$ is the cellulose amount in pretreated substrates; 1.1 is the coefficient of cellulose conversion to glucose; α is cellulose content; and β is enzymatic hydrolysis efficiency.

2.4.2 Crystallinity index

The crystallinity index (CrI) of raw feedstocks and pretreated substrates was recorded by an X-ray diffractometer (D8, Bruker, Ltd., Germany) with Ni-filtered Cu-K$_a$ radiation ($k = 0.1541$ nm) generated at 40 kV and 40 mA. The data were collected for 2θ from 5 to 85° with a step interval of 0.02°. The employed samples were dried and crushed through a 200-mesh sieve prior to the CrI determination. The CrI (%) was calculated according to the Segal method in Eq. (7) [29].

$$\mathrm{CrI}\ (\%) = \frac{I002 - Iam}{I002} \times 100 \tag{7}$$

where I_{002} is the maximum intensity of approximately 22.0° and I_{am} refers to the minimum intensity located at 2θ close to 18°.

2.4.3 Scanning electron microscopy

In order to directly check the structure changes of various H_2O_2-involved pretreatments, the microscopic images of raw feedstocks and pretreated substrates were captured by scanning electron microscopy (SEM) (Quanta650, Thermo Fisher Scientific America, Inc., USA). Dried samples were used for analysis, and ion sputtering was employed for spraying the surface. The image was taken at a voltage of 20 kV.

2.4.4 Simons' stain for determining enzyme accessibility

Simons' stain (SS) was employed to evaluate the pore information of the pretreated for enzyme accessibility, and the detailed protocol for SS was based on the modified procedure by Arantes [30].

2.4.5 Lignin distribution by laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) (A1-90i, Nikon Instruments, Inc., USA) was employed to check the lignin distribution on the pretreated substrates, and an excitation laser of Ar 488 nm was used under a fluorescent objective in the emission range of 490–560 nm. The samples were dispersed evenly in nanopure water, and a drop of dispersed solution was prepared on glass microscope slides for LSCM observation.

3 Results and discussion

3.1 Chemical composition of the pretreated substrates

After pretreatment, the main chemical composition of the pretreated substrates, including cellulose, hemicellulose, and lignin, were listed in Table 2. A decrease of lignin content was observed after AHP, HPAC, and PHP pretreatment. The residual lignin content in HPAC-pretreated biomass was 2.0–9.5% and 4.2–16.8% for PHP pretreatment. By contrast, the lignin content in the FC-pretreated feedstocks was much higher (24.1–28.8%). As for recalcitrant hemicellulose fraction, its content in the AHP-, HPAC-, and FC-pretreated substrates was in relatively higher levels; however, only 2.3–3.6% hemicellulose was detected for the PHP-pretreated substrates. According to the referred work on these pretreatments, almost similar results on hemicellulose and lignin content in the pretreated biomass can be achieved [20, 23, 24, 27]. In addition, it was documented that the low content of recalcitrant fraction, such as lignin and hemicellulose, benefits the subsequent enzymatic hydrolysis, in which lignin is related to the non-effective enzyme adsorption, while hemicellulose acts as the physical block for enzyme accessibility to substrate [31, 32]. Particularly, a high cellulose content of 66.4–83.0% was observed for PHP-pretreated substrates, which was beneficial for the subsequent enzymatic hydrolysis with high sugar concentration. The PHP-pretreated substrate was characterized by lower recalcitrant fractions (hemicellulose and lignin) and relatively higher digestible cellulose content, suggesting the corresponding high hydrolysis efficiency and glucose yield might be achieved.

Table 2 Substrates' chemical composition via various pretreatments

Pretreatment	Feedstocks	Cellulose	Hemicellulose	Lignin
PHP	Poplar	82.2 ± 0.69	2.8 ± 0.04	5.0 ± 0.13
	Birch	83.0 ± 1.44	3.6 ± 0.04	4.2 ± 0.12
	Wheat straw	66.4 ± 0.25	2.3 ± 0.12	16.8 ± 0.47
HPAC	Poplar	68.3 ± 0.19	15.7 ± 0.10	2.0 ± 0.01
	Birch	59.8 ± 0.29	19.7 ± 0.16	2.6 ± 0.08
	Wheat straw	43.0 ± 0.28	20.7 ± 0.19	9.5 ± 0.12
AHP	Poplar	48.2 ± 0.28	13.9 ± 0.06	22.5 ± 0.19
	Birch	43.4 ± 0.01	15.4 ± 0.07	24.1 ± 0.05
	Wheat straw	46.4 ± 0.75	21.8 ± 0.33	16.7 ± 0.71
FC	Poplar	43.5 ± 0.93	12.2 ± 0.17	26.4 ± 0.27
	Birch	41.1 ± 1.50	13.8 ± 0.52	24.1 ± 0.32
	Wheat straw	38.3 ± 0.94	17.0 ± 0.55	28.8 ± 1.11

3.2 Recalcitrant fraction removal and cellulose recovery after pretreatment

The removal of recalcitrant fractions, including lignin and hemicellulose, and cellulose recovery were calculated and compared (Fig. 1). The highest solid recovery after pretreatment appeared in the group after FC pretreatment with 67–88%, indicating its low efficiency of lignin and hemicellulose removal. As for AHP and HPAC pretreatment, their solid recoveries for hardwood poplar and birch were quite similar. Among the four testing pretreatments, PHP pretreatment exhibited the lowest solid recovery but much high cellulose recovery for all these three feedstocks, indicating its strong removal effect on the recalcitrant fractions (Table 2). When the hemicellulose removal was calculated, higher than 91% hemicellulose was removed by PHP pretreatment. It was reported that the high carbohydrate solubility in concentrated H_3PO_4 and strong deconstruction ability of PHP contributed to hemicellulose removal [33, 34]. On the other hand, hemicellulose removal for AHP, HPAC, and FC was lower than 42%. It was only 10.8% and 13.4% for AHP and HPAC pretreatment on wheat straw.

The highest delignification extent was observed for HPAC pretreatment (70.3–95.3%), followed by PHP pretreatment (64.3–93.9%). As for the AHP pretreatment, its lignin removal was quite low for these three feedstocks (~29 to ~49%). Moreover, only 12–30% lignin removal was observed for FC pretreatment. When LSCM was performed on these pretreated substrates (Fig. S1 in supplementary files), stronger fluorescence intensity was observed for FC pretreatment, indicating more lignin remained. On the contrary, rather low fluorescence intensity was captured for the HPAC- and PHP-

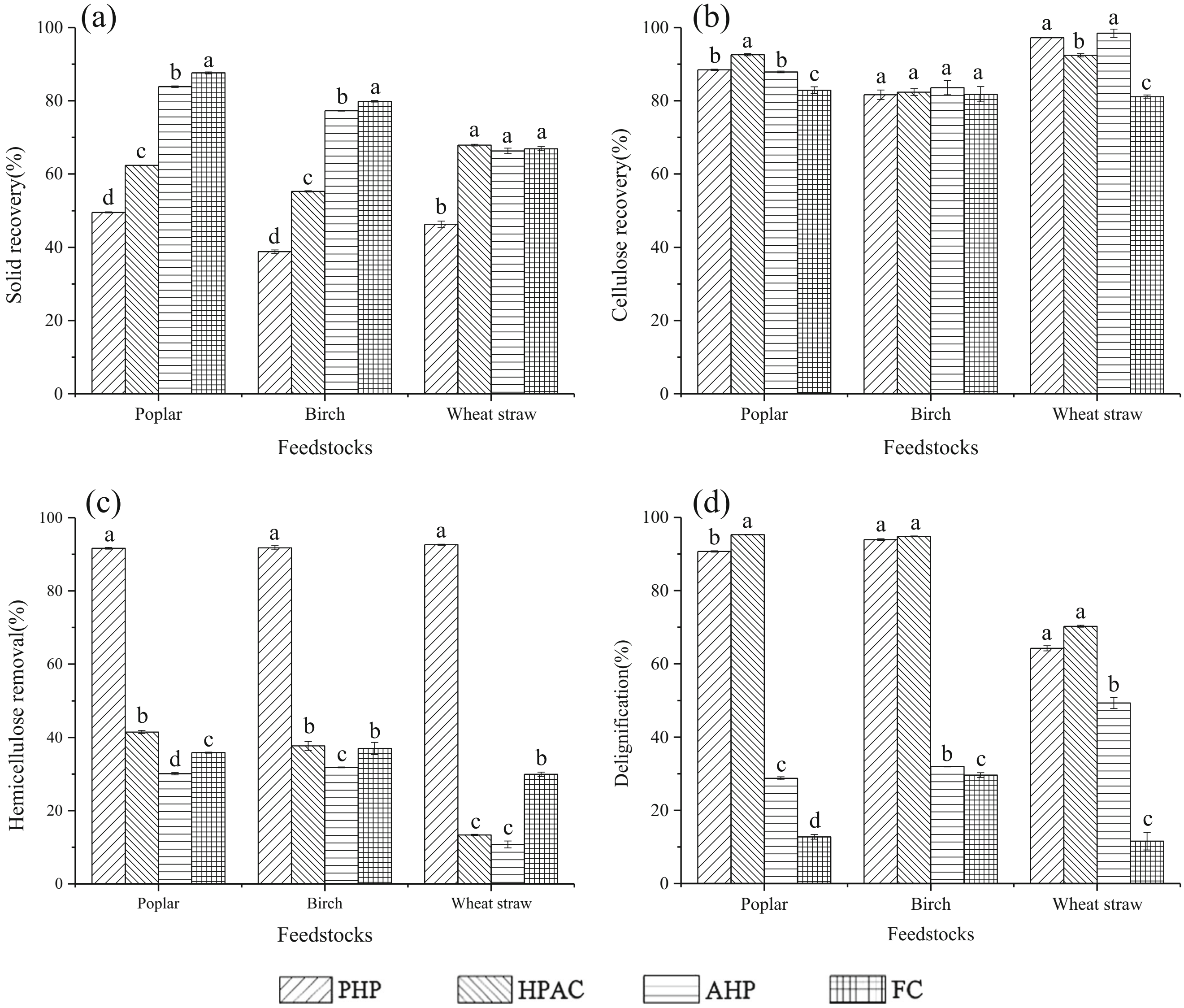

Fig. 1 Removal of recalcitrant fractions and cellulose recovery after pretreatment; **a** solid recovery, **b** cellulose recovery, **c** hemicellulose removal, **d** delignification. (The results were assessed using significance test, $n = 2$)

pretreated feedstocks, suggesting high lignin removal was achieved. These H_2O_2-involved pretreatments exhibited significant differences on their delignification efficiency, suggesting their delignification mechanisms were different from each other. The possible delignification mechanism of HPAC pretreatment was that the large amounts of peracetic acid was formed by mixing acetic acid and hydrogen peroxide, which further generated hydroxonium (HO^+) cations. Then, the electrophilic HO^+ selectively degraded biomass lignin through the oxidation process [35]. A series of reactions could occur between hydroxonium ion and lignin, which included displacing side chains, transforming guaiacyl and syringal lignin units into quinone methide, and converting aldehyde intermediates into carboxylic acids via Baeyer–Villiger oxidation [36]. Similarly, PHP pretreatment was also performed in acid conditions with H_2O_2, so peroxy acids were also produced accordingly. But depolymerizing guaiacyl units of lignin was more responsible for lignin degradation [22, 34]. As for the AHP and FC, the produced hydroxyl radicals were mainly responsible for lignin removal. It appeared that lignin was degraded by superoxides and then was esterified under the alkaline condition; thus, its delignification was intensified [23, 26, 37]. Although HO^+ could also be generated for AHP and FC pretreatment, it was not stable enough at such conditions; thus, their delignification efficiencies were still rather low. Besides, the delignification on woody biomass by these pretreatment methods was higher than that of grassy biomass of wheat straw, which relates to their lignin structure and the reactivity sites [37]. Generally, woody lignins are dominant in guaiacyl (G) and syringyl (S) units, while grassy lignins are dominant in G, S, and H (p-hydroxyphenyl) units [38]. S and G units can be degraded preferentially [39]; therefore, woody biomass lignin was more feasible to be removed using these pretreatments.

Cellulose recovery is highly related to cellulose hydrolytic potential. In this work, more than 81% cellulose was recovered in solid fraction after these H_2O_2-involved pretreatments. The average cellulose recovery of AHP was approximately 90.0%, followed by 89.1% for HPAC and PHP and 82.0% for FC. In the H_2O_2-involved pretreatment at acidic conditions such as PHP and HPAC, cellulose loss was mainly caused by the acid-catalyzed depolymerization and further oxidation. It appeared that cellulose was dissolved/swelled and regenerated by concentrated H_3PO_4 during PHP pretreatment; thus, cellulose degradation was enhanced [40]. Although AHP pretreatment did not cause detectable changes in the structure of highly crystalline cellulose [41], the oxidative degradation on the amorphous cellulose was considerable during the pretreatment. Moreover, cellulose degradation in FC pretreatment was more obvious than that in AHP, suggesting the formed cellulose-Na at alkaline conditions could hinder oxidative cellulose degradation by hydroxyl radicals.

Based on the above discussions, these H_2O_2-involved pretreatments were characterized by delignification, in which HPAC and PHP displayed higher efficiency. Moreover, high hemicellulose removal could be well integrated in PHP pretreatment. Consequently, the PHP-pretreated substrates exhibited lower hemicellulose and lignin contents, and a relatively higher cellulose content. By contrast, the HPAC-pretreated substrates were characterized by a rather low lignin content. These characteristics would potentially promote enzyme accessibility and hydrolysis efficiency.

3.3 Enzymatic saccharification of the pretreated substrates

To assess the hydrolytic potential of these pretreated substrates, enzymatic hydrolysis experiments were performed at the same conditions. As shown in Fig. 2, the highest cellulose–glucose conversion was achieved by PHP pretreatment, which was higher than 90%, even 100% for pretreated wheat straw and birch. Similar high cellulose–glucose conversions were also observed when PHP was conducted on other lignocellulose biomass, e.g., 94.3% for corn stover, 91.8% for Jerusalem artichoke stalk, 100% for oak, 92.3% for spruce, and 94.5% for bamboo [22]. Approximately 77.4–87.5% cellulose can be converted into glucose when the HPAC-pretreated feedstocks were employed, which was also comparable with the referred work [24]. However, their conversions were lower than that of PHP. Much lower conversion was observed for AHP-pretreated substrate, i.e., 67.8% for wheat straw, followed by birch (27.7%) and poplar (24.2%). By contrast, the enzymatic hydrolysis of FC-pretreated substrates was only in the range from 13.0 to 37.6%, which was close to that of the unpretreated substrates, indicating limited efficiency of FC pretreatment. The cellulose–glucose conversions for these two acid–H_2O_2 pretreatments (PHP and HPAC) were significantly higher than those for AHP and FC pretreatments according to the ANOVA results. Based on the obtained results, it appeared that substrates with low recalcitrant factions tended to give higher enzymatic hydrolysis. Particularly, lignin content was negatively correlated to enzymatic hydrolysis (correlation coefficient of 0.73) due to non-effective binding between lignin and cellulase [31]. It was interesting that although PHP- and HPAC-pretreated substrate exhibited similar lignin content, PHP pretreatment tended to give higher enzymatic hydrolysis (100%), likely due to lower biomass recalcitrance caused by higher hemicellulose removal (92.6%). Lignin removal was crucial to enhance cellulose hydrolysis, while hemicellulose removal could further promote it in a complementary fashion.

When glucose yield based on the dry weight of the employed biomass was considered, a high yield was observed in the group of PHP-pretreated biomass. It was 307.2, 368.0, and 327.5 mg g^{-1} feedstock for wheat straw, polar, and birch,

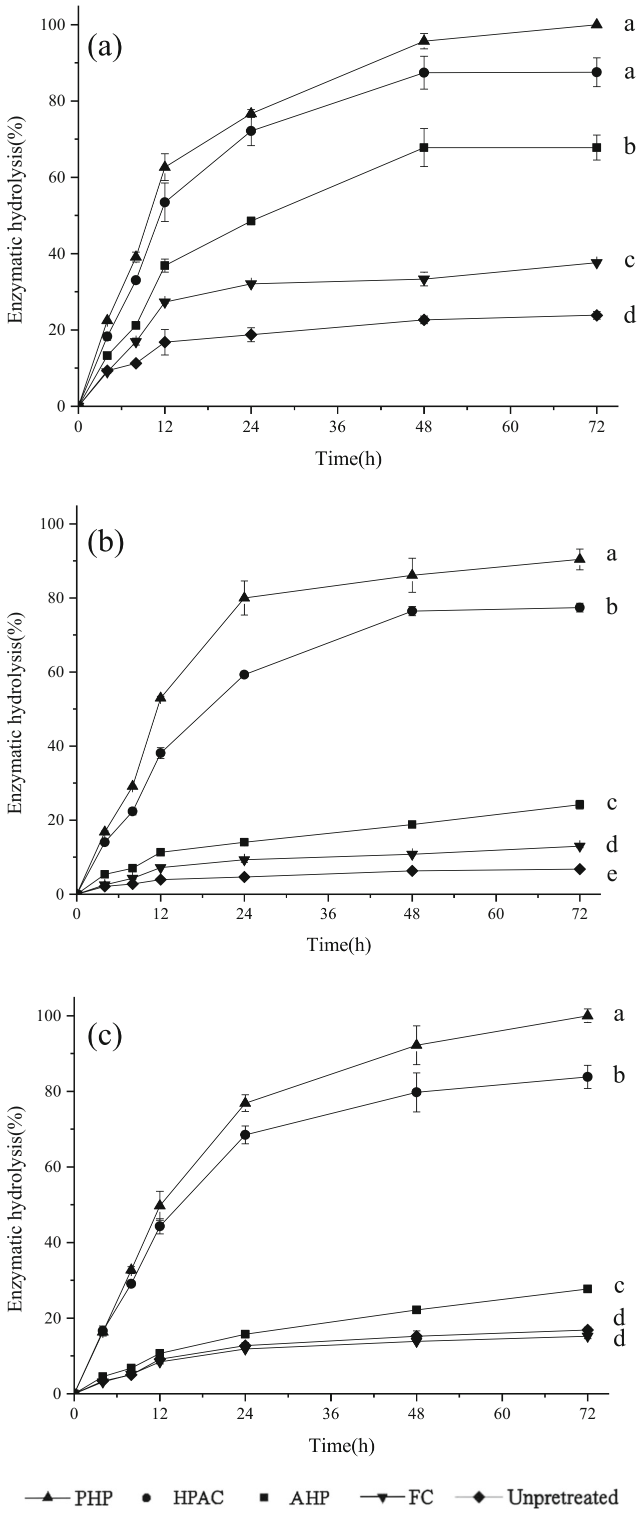

Fig. 2 Enzymatic hydrolysis efficiency of the pretreated substrates; **a** wheat straw, **b** poplar, **c** birch. (The results were assessed using significance test, *n* = 2)

respectively (Fig. 3). The corresponding glucose recovery based on the original cellulose content in the biomass feedstock was 88.4%, 72.7% and 74.3%, respectively, indicating cellulose enrichment was beneficial for glucose yield. A

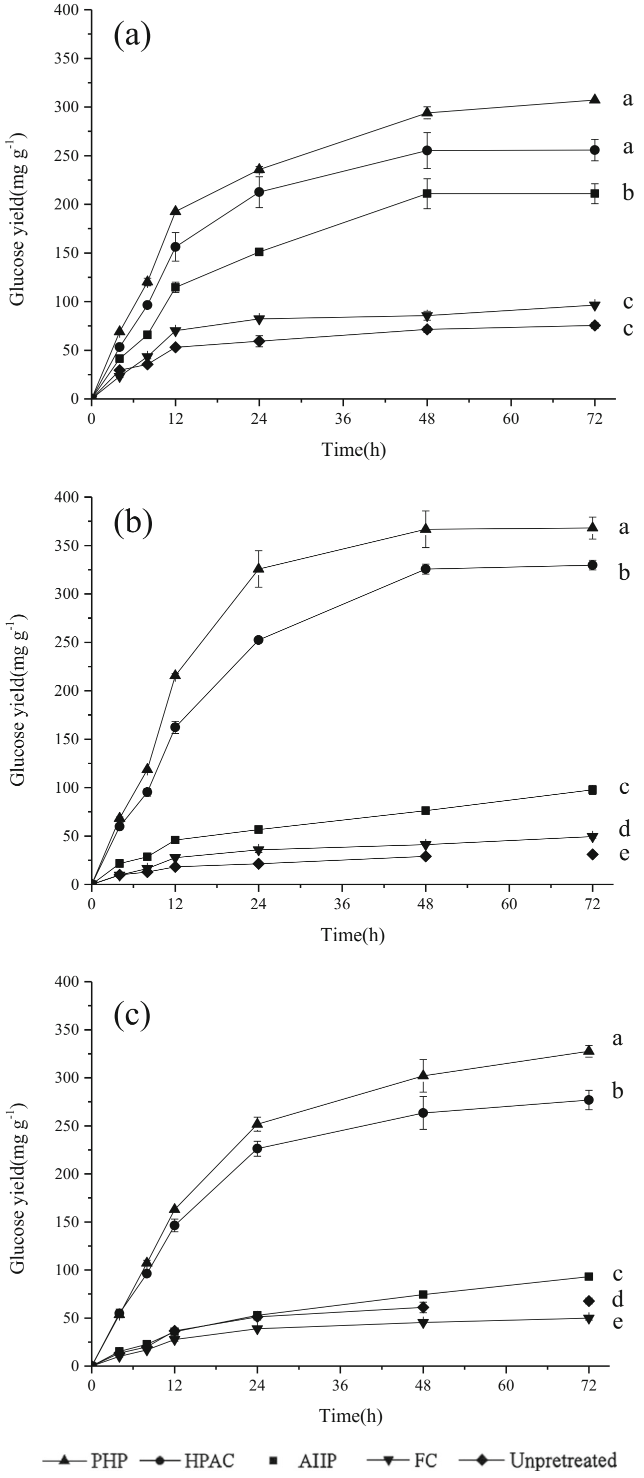

Fig. 3 Glucose yield based on feedstocks (dry basis); **a** wheat straw, **b** poplar, **c** birch. (The results were assessed using significance test, *n* = 2)

considerable glucose yield (255.7–329.7 mg g^{-1}) can be achieved by HPAC; however, their glucose recovery was significantly lower than that of PHP. A considerable glucose yield of 210.9 mg g^{-1} was obtained for AHP-pretreated wheat straw but was lower than 100 mg g^{-1} for hardwood poplar and

Table 3 Crystallinity index of raw material and treated biomasses

Pretreatment	CrI (%)		
	Wheat straw	Poplar	Birch
AHP	56.2	67.3	61.1
HPAC	56.1	70.9	65.1
PHP	62.3	73.4	71.3
FC	55.8	60.9	55.9
Unpretreated feedstocks	47.1	46.6	45.8

birch, indicating AHP was more suitable for agricultural residues. The glucose yield obtained from FC-pretreated wheat straw was 96.5 mg g^{-1}, which was close to that of raw feedstock without pretreatment. Based on the above results, PHP pretreatment was highly feasible to enhance cellulose hydrolysis, as demonstrated by its corresponding high glucose yield. Moreover, it could accept a wide range of biomass species (agricultural residues, hardwoods, softwoods, bamboo, and their mixture), while its enzymatic hydrolysis could be facilely conducted at high solids loading (20–25%) [22, 42]. In addition, HPAC was also a promising pretreatment method for recovering fermentable sugar from various biomasses. As for the AHP, it is more suitable for agricultural residues compared to woody biomass [37]. By contrast, FC was not encouraged for pretreatment due to its low efficiencies on removing recalcitrant fractions and recovering glucose. In addition, the resulting crude hydrolysate mixture composed of cellulolytic enzyme lignin and soluble glucose sugar fraction could be further processed for various downstream applications. Apart from ethanol production through fermentation, glucose could also be processed into platform chemicals such as hydroxymethylfurfural, butanone, and levulinic acid. Recent progresses have shown that nanofiltration and a sustainable separation technique potentially facilitate the sugar concentration process at a large scale and thus eases the downstream utilization of glucose. It has also been suggested that a high glucose yield was beneficial for the subsequent valorization [43–45].

3.4 Structure characteristics of the pretreated substrates

SEM images of the biomass before and after pretreatment were captured to assess their structural changes by various H_2O_2-involved pretreatments (Fig. S2 in supplementary files). Overall, the SEM images after FC pretreatment did not display obvious surface morphological changes compared to their corresponding unpretreated feedstocks. Although cellulosic fiber disintegration was observed for AHP-pretreated substrates, its cell wall structure was still existing, indicating considerable biomass recalcitrance. The surface of HPAC-pretreated substrates became smoother, suggesting the lignin that covered it was removed. More disintegrated fibers with a coarser surface were observed in the PHP-pretreated substrates due to its high deconstruction effect on the biomass recalcitrant structure.

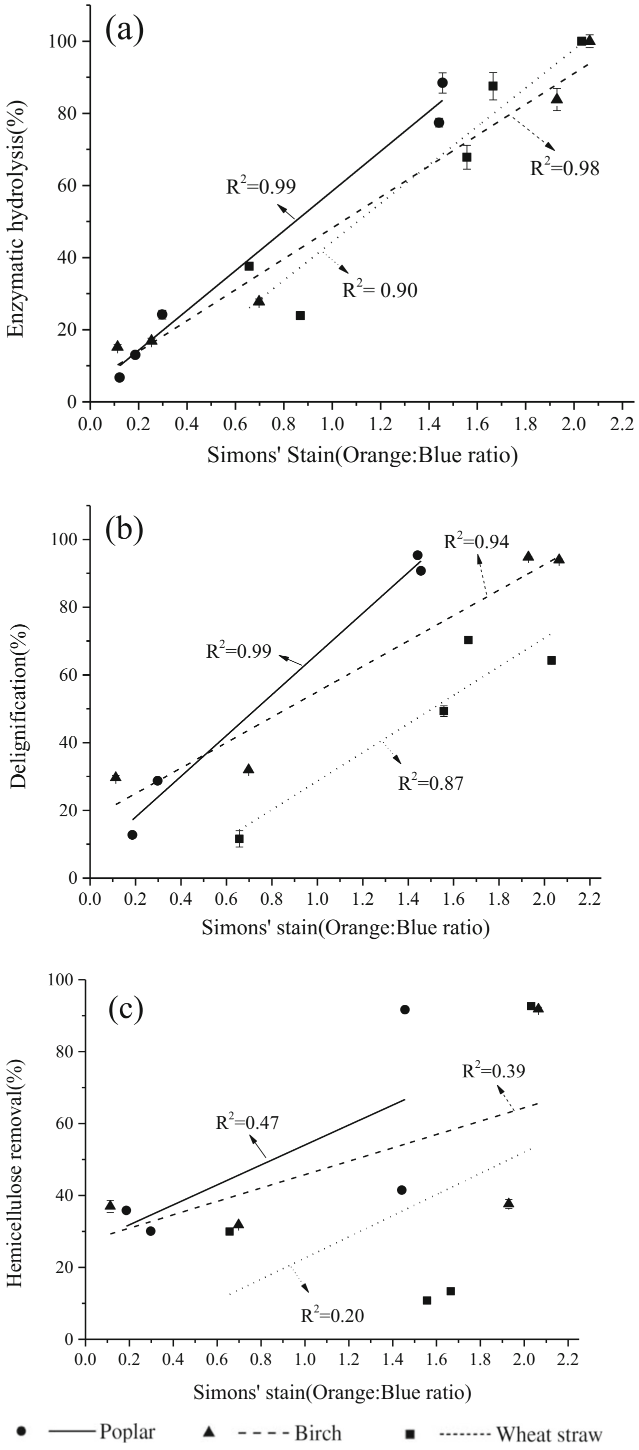

Fig. 4 Correlation analysis among the enzyme accessibility, enzymatic hydrolysis, and recalcitrant fractions' removal; **a** SS vs. enzymatic hydrolysis, **b** SS vs. delignification, **c** SS vs. hemicellulose removal

Table 4 Comparison of wheat straw SSF based on PHP and HPAC pretreatment

Pretreatment method	Pretreatment conditions	SSF conditions	Ethanol yield	References
PHP	79.6% H_3PO_4 and 1.91% H_2O_2, 40.2 °C for 2.9 h	Enzyme loading of 87.4 mg CTec2 g^{-1} cellulose, yeast loading of 3 g L^{-1}, 38 °C	112 g kg^{-1a} (208 g kg^{-1})^b	[42]
HPAC	The equal-volume mixture of H_2O_2 and CH_3COOH, 80 °C for 2 h	Enzyme loading of 0.13 μL cellulase g^{-1} mass with extra 33.78 μL xylanase g^{-1} mass, and yeast loading of 1 g L^{-1}, 37 °C	117 g kg^{-1c}	[24]

[a] The ethanol yield was calculated based on original wheat straw with 120 h SSF

[b] The ethanol yield was calculated based on PHP-pretreated wheat straw with 24 h SSF

[c] The ethanol yield was calculated based on HPAC-pretreated rice straw with 24 h SSF

Besides, the CrI of these feedstocks before/after pretreatment was also detected (Table 3). The increased CrI can be achieved after various pretreatments, which was consistent with previous work [29, 32, 46]. Moreover, it also can be found that the obtained CrIs were highly related to the removal of recalcitrant fractions of amorphous hemicellulose and lignin [29]. However, the CrIs were not indicative of the final hydrolysis efficiency, e.g., the highest cellulose hydrolysis was achieved in the group of PHP pretreatment, but their CrIs in this group were even higher than 70%, especially when Cellic CTec2 was used that was able to disrupt crystalline cellulose due to its polysaccharide monooxygenase content [47, 48].

Simons' stain can reflect the accessibility of the substrate to cellulase [30, 42], which helps to further assess the differences among enzymatic hydrolysis efficiencies. The highest DO/dB ratio was achieved for PHP pretreatment in the range of 1.46–2.06 among these three testing substrates, followed by HPAC and AHP. The Simons' stain value of FC-pretreated substrate was close to that of raw feedstock correspondingly. When Simons' stain values were plotted against their corresponding enzymatic hydrolysis values (Fig. 4a), a relatively high correlation coefficient (R^2) of 0.90 was obtained for wheat straw. Similar high correlation coefficient values were also obtained for poplar (0.99) and birch (0.98) (Fig. 4a). This result indicated the improved accessibility by pretreatment was consistent with enhanced enzymatic hydrolysis. Overall, the accessibility of pretreated substrates was positively correlated with lignin removal ($R^2 = 0.70$), rather than hemicellulose removal ($R^2 = 0.19$), indicating that delignification was a dominant variable for promoting substrate accessibility [49].

PHP and HPAC were more feasible options to pretreat various lignocellulosic biomasses for fermentable sugar release. According to the referred work (Table 4), the optimized conditions of PHP pretreatment can yield 112 g ethanol from 1.0 kg wheat straw through enzymatic hydrolysis and fermentation [42]. Therefore, the corresponding ethanol yield calculated for PHP-pretreated wheat straw was 208 g kg^{-1}. On the other hand, it was 117 g kg^{-1} ethanol for HPAC-pretreated rice straw [24]. This indicated these two pretreatment methods held tremendous potential applications in the cellulosic ethanol biorefinery.

4 Conclusions

PHP and HPAC pretreatments were more promising in the bioethanol process. The maximum glucose yield of the corresponding pretreated substrate was 368.0 mg g^{-1} and 329.7 mg g^{-1}, respectively. The acid–H_2O_2 pretreatments showed a higher delignification efficiency of 64.3–95.3% and could accept a wider biomass species compared to AHP and FC pretreatment. Particularly, PHP pretreatment removed more than 90% hemicellulose while the resulting cellulose showed the highest glucose yield. However, FC showed limited efficiency in overcoming the biomass recalcitrance due to its low extraction/degradation ability for hemicellulose and lignin fractions. It was shown that AHP pretreatment was more feasible for wheat straw substrate. The enhanced cellulose hydrolysis was well correlated with its higher extent of delignification, which was the main contributor to improve cellulose accessibility among those four H_2O_2-involved pretreatments.

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