Enhanced biochemical process for purifying xylo-oligosaccharides from pre-hydrolysis liquor

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Research

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Abstract

Background

Xylo-oligosaccharides (XOS) are promising biomass-derived chemicals that can be widely used in preparing medicines, food additives, and industrial chemicals. The pre-hydrolysis liquor (PHL) produced in the kraft-based dissolving pulp production process is rich in hemicelluloses and especially in XOS/xylan.

Results

In this study, a sustainable sequential process that included calcium hydroxide (CH), simultaneous laccase and xylanase (LX) and activated carbon (AC) treatments was proposed for more efficient recovery of XOS/xylan from PHL. Overall, the concentration of lignin, furfural and xylosugars decreased by 81.7 wt.%, 100 wt.% and 5.1 wt.%, respectively, but XOS concentration was increased by 36.6 wt.%. More importantly, the 2D-HSQC NMR and FT-IR were used in understanding the structure of sugar and lignin components in each step of the process and in the final products. The CH treatment mainly altered the chemical structure of XOS due to the release of acetyl groups, and downstream treatment steps have insignificant effect on XOS structures. Also, lignin carbohydrate complexes (LCC, i.e. PhGlc3) minimally existed in the purified XOS. The GPC and DSC results revealed that the molecular weight of the extracted lignin was 1768 ~ 2532 g/mol and it had a wide glass transition temperature (Tg) range (63 ~ 115 °C).

Conclusions

The results confirmed that a combination of sequentially treating PHL with calcium hydroxide (CH), simultaneous laccase and xylanase (LX) and activated carbon (AC) was effective in removing lignin and concentrating XOS in the PHL.

Background

Today, there is a great interest in utilizing natural based products [1, 2]. Derived from trees, xylo-oligosaccharides (XOS) products are sugar oligomers with 2 ~ 7 xylose units and their monomer (xylose) is connected by β-(1–4)-linkages [3]. Literature reports stated that XOS can improve bowel function by promoting the growth of beneficial intestinal bacteria, facilitate the absorption of calcium and reduce the risk of colon cancer in human [4–6]. In addition, XOS cannot be metabolized by human's digestive system, thus it can meet the need of sweeteners for diabetic and obese patients [7, 8]. XOS is a popular drug and food additive with high medical potential, and the market demand for XOS is steadily increasing due to its rich physiological functions [4, 9]. Although XOS derived from xylan-rich biomass, such as agricultural residues [10], researchers seek alternative routes to produce XOS from wood as woody materials are rich in XOS and use of wood do not interfere with the food chain.

As a by-product of the kraft-based dissolving pulping production process, the pre-hydrolysis liquor (PHL) contains hemicelluloses that can be used as abundant source of XOS production [11]. In this case, the preparation of XOS from xylan of PHL may be a highly effective approach to utilize PHL, which not only can widen the sources for XOS production and reduce the cost of XOS production, but also bring financial profits to the kraft pulping process [12].

Generally, XOS are mainly prepared from xylan by xylanase hydrolysis, which is one of the most attractive approaches due to its high yield of XOS production, less undesirable byproducts (e.g., furfural) and simple operation [13–15]. As reported early, the xylan obtained by alkaline pretreatment is more linear and easily digestible by xylanase than the xylan in raw materials as it contains fewer branched chains (especially acetyls) [10, 16]. Similarly, the dissolved xylan in PHL is rich in branched groups, such as acetyls, which maybe also suitable for alkaline treatment. Hence, to ensure the xylanase accessibility to xylan and enhance the efficient conversion of xylan to XOS in the PHL, an alkaline pretreatment before xylanase hydrolysis may be economical and technically feasible. Previously, calcium hydroxide (CH) and sodium hydroxide (SC) treatment has been reported in the purification of the PHL [11, 17]. Compared with SC treatment, the CH treatment was more effective in eliminating lignin with limited sugar loss from PHL [11, 18]. Hereby, in this paper, we further focused on investigating the effect of CH pretreatment on the enhancement of the subsequent xylanase hydrolysis efficiency for producing PHL-induced XOS.

To further facilitate the production of high quality XOS from PHL, an efficient purification process for removing the contaminants, e.g., lignin, from PHL needs to be carried out [19, 20]. Recently, laccase treatment, as an effective and environmentally friendly process for purifying PHL, has attracted attention [21, 22]. Generally, laccase treatment is considered to be a part of the purification process, while xylanase hydrolysis is regarded as value adding step for treating PHL [9]. However, both laccase treatment and xylanase treatment need a long process time, which may hamper their utilization at a commercial scale [23]. To optimize the process and reduce the complexity of the process, we investigated the simultaneous utilization of laccase and xylanase (LX) for synchronously removing lignin and producing purified XOS from PHL.

Although the influence of the purification methods on components of PHL has been studied [11, 19], to our knowledge, no study has reported how the structure of carbohydrates and lignin would be impacted by these pretreatment methods in PHL, which would eventually impact the quality of produced XOS and extracted lignin. For the first time, the structural changes of lignocelluloses of PHL were monitored during these treatment steps to better understand the mechanism of these processes and to design these processes for XOS production effectively.

Herein, a purified XOS preparation method consisting of CH, LX and AC adsorption treatments was investigated. The structural changes of the lignocelluloses passing through the pretreatment processes of PHL in producing XOS was studied in detail. With the data generated with high-performance anion exchange liquid chromatography (HAPEC), high-performance liquid chromatography (HPLC), Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) analyses, we were able to monitor

quantitatively the structural changes of lignocelluloses in PHL during these processes, for the first time. Subsequently, the molecular wight and thermal properties of isolated precipitates lignin were determined by gel permeation chromatography (GPC) and differential scanning calorimeter (DSC) to understand if the separated materials can be used as an energy source or biobased-material for other applications.

Methods

Materials

Poplar wood chips were received from Sun Paper Co. Ltd located in Shandong province, China. Calcium hydroxide ($Ca(OH)_2$, 99%), sulfuric acid (H_2SO_4, 99%) and phosphoric acid (H_3PO_4, 85%) were purchased from Tianjin Hengxing Company, China. Commercial activated carbon, AC (powder, 200 mesh particle size, wood-based) was supplied by Guangzhou Haiyan Company. Also, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and xylose were purchased from Sigma Chemical Company, XOS standards including xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexose were obtained from Megazyme, Ireland. Additionally, corncob based commercial XOS with 2～6 degree of polymerization (DP) were purchased from Shandong Longlive company. Laccase and xylanase were provided by Novozymes Biotechnology company and Longda biological engineering company, respectively. All chemicals were used without further purification.

Laccase and xylanase activities

Laccase activity (5000 U/mL) was determined by using a UV-vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) at 420 nm using 0.5 mM ABTS as the substrate at pH 4.5 and 20 °C [24]. One activity unit (U/mL) was defined as the amount of enzyme that oxidized 1 μmol of ABTS in a minute; xylanase activity (70000 U/mL) was determined based on the endo-xylanase activity [25]. One activity unit is defined as the quantity of enzyme that liberates 1.0 μmol of xylose per min under the conditions of pH 5.3 and 50 °C [16]. The activities of laccase and xylanase were expressed in U/mL.

PHL preparation

Figure 1 shows the flow chart of the experimental process conducted in this work to prepare purified XOS from pre-hydrolysis liquor (PHL) and the methods to analyze it. As seen, after hot water hydrolysis, the PHL was collected from digester and then it was treated by successive process for producing purified XOS.

At first, PHL, was prepared using an Electrically operated rotary pulping digester with a 15 L reaction vessel (KRK, No.2611, Kumagai Riki Kogyo Ltd., Tokyo, Japan). To prepare PHL, the water hydrolysis of wood chips was carried out with treating 1.0 kg of poplar chips at 170 °C, 1:6 wt./wt. wood chips/water for 1 h at 60 rpm. After the treatment, the system was cooled down at the rate of 60 rpm, and PHL was collected via filtering the treated wood chips from the digester. The treated wood chips were utilized for generating dissolving pulp. To obtain insoluble fractions, which mainly include lignin, the PHL was filtrated by qualitative filter paper. Then, the filtrate and separated precipitate lignin was collected, and the

filtrate was marked as F-PHL and kept at 4 °C for further steps. The precipitate lignin was thoroughly washed with deionized water and denoted F-lignin.

Synergetic Purification and preparation of XOS from F-PHL

Calcium hydroxide (CH) pretreatment of XOS

Calcium hydroxide (CH) pretreatment of F-PHL XOS was carried out at room temperature. In this set of experiments, different amounts (0.03 ~ 0.36 g) of $Ca(OH)_2$ were added to 50 mL Erlenmeyer flasks containing 30 g of PHL, then the mixture was stirred at 250 rpm for 10 min. After the CH pretreatment, the mixture was centrifuged at 4500 rpm for 3 min and the filtrate was collected and marked as CH-PHL for further analysis and enzymatic hydrolysis.

Laccase and xylanase (LX) treatment

In order to explore the synergistic influence of LX treatment on lignin and XOS of CH-PHL, laccase and xylanase hydrolysis was conducted simultaneously on CH-PHL. Also, laccase treatment and xylanase treatment were explored separately to optimize the dosage of enzymes and treatment time required for LX treatment.

Laccase treatment was conducted according to a previous report [11]. Briefly, 30 g of CH-PHL was placed in 50 mL Erlenmeyer flasks and treated with laccase under the conditions of 1 ~ 5 U/g laccase dosages, 0.5 ~ 5 h, 45 °C and pH 4.5, and 200 rpm. After completion, the mixture was boiled for 10 min to deactivate laccase and then centrifuged at 5000 rpm for 5 min. The filtrate was collected and marked as La-PHL; Also, xylanase hydrolysis was carried out in 50 mL Erlenmeyer flasks in a shaker at 200 rpm. The dosages of xylanase and retention time were varied from 2 to 20 U/g and 0.5 ~ 5 h in 30 g of CH-PHL at 55 °C and pH 5.5. Xylanase hydrolysis was stopped by boiling the treated CH-PHL for 10 minutes, then the mixture was centrifuged at 5000 rpm for 5 min, and the filtrate was collected and marked as Xy-PHL.

LX treatment was carried out in 50 mL Erlenmeyer flasks at 200 rpm for 3 h, and the dosages of laccase and xylanase were 1 U/g and 2 U/g (according to the above experimental optimization) respectively. The variables of this experiments were different temperatures (25 ~ 60 °C) and pH (3.5 ~ 6.5), Then, the mixtures were boiled and centrifuged at 5000 rpm for 5 min, the filtrate and precipitates lignin were collected and marked as LX-PHL and LX-lignin, respectively.

Activated carbon (AC) adsorption

In another set of experiments, 30 g of LX-PHL obtained under the optimal LX treatment was mixed with 0.2 ~ 1.2 wt.% of AC in 50 mL Erlenmeyer flasks for 10 min, 25 °C, 150 rpm and pH 5.5. In this process, the lignocelluloses of LX-PHL would adsorb on AC. After the treatment, AC was filtered from the mixture using 0.22 μm nylon membrane filter and the filtrated sample was denoted as AC-PHL. The above-mentioned PHL samples were all kept at 4 °C for further analysis.

The preparation of solid PHL derivatives

In order to accurately explore the influence of different treatment processes on the mass and structural changes of lignocelluloses in PHL, the PHL was filtrated firstly by qualitative filter paper to obtain the F-PHL (seen Fig. 1). Then, it was treated with CH and its filtrate was named CH-PHL. Next, LX-PHL was produced by treating CH-PHL with 1U/g of laccase and 2 U/g of xylanase at pH 5.5 and, 55 °C for 3 h. Finally, LX-PHL was purified with AC adsorption to acquire AC-PHL. To obtained solid sample, F-PHL, CH-PHL, LX-PHL, AC-PHL were concentrated using rotary evaporator under vacuum pressure at 60 °C followed by freeze-drying for 72 h under reduced pressure at -64 °C. These solid samples were denoted as S-F, S-CH, S-LX, S-AC, respectively and used for chemical and structural characterization (Fig. 1).

Chemical components of PHL

The concentration of xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose of PHL was measured by high performance anion exchange chromatography (HPAEC, ICS-5000+, Thermo Scientific, Inc., Sunnyvale, CA, USA), which was equipped with a Dionex CarboPac PA200 column (3 mm × 250 mm, Thermo Scientific, Inc., Sunnyvale, CA, USA) and an ED 40 electrochemical detector. The mobile phases were 0.1 M NaOH and 0.5 M NaOAc containing 0.1 M NaOH at a flow rate of 0.3 mL/min and column temperature of 30 °C. For determining total sugar concentration of PHL, the collected PHL samples were initially acid hydrolyzed with 4 wt.% of H_2SO_4 at 121 °C for 60 min. The concentration of xylosugars after this acid hydrolysis was determined using the aforementioned HPAEC as explained above.

Furthermore, the concentration of furfural and acetic acid before and after acid hydrolysis, which was conducted for sugar analysis, was also measured for all of these samples using HPLC [11]. The concentrations of acetic acid were measured using a Shimadzu LC-20T high performance liquid chromatography (HPLC) (Kyoto, Japan), which was equipped with a SUPELCOGEL C-610H column (30 cm × 7.8 mm, Sigma-Aldrich, St. Louis, MO, USA) and the SPD-20A detector. Samples were run at 30 °C and eluted at 0.7 mL/min, and mobile phase was 0.1% H_3PO_4 (vol/vol). The difference of acetic acid concentration in PHL before and after acid hydrolysis of samples (for sugar analysis) represents the amount of acetyl groups and thus acetic acid [26].

The concentrations of lignin in PHL solutions were determined at 205 nm wavelength using a UV/vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) according to the TAPPI UM-250 [27].

In addition, to ensure the experimental repeatability, five independent experiments were conducted, and the average values were reported in this work. The error bars show the variations in the experimental results.

Analysis of dried PHL samples

The surface functional groups and chemical structures of S-F, S-CH, S-LX, S-AC and commercial XOS sample were characterized by Fourier transform infrared spectrometer (FT-IR, VERTEX70, Bruker, Karlsruhe, Germany) and nuclear magnetic resonance (NMR, Bruker, Karlsruhe, Germany). In FT-IR analysis, the oven-dried samples were embedded in KBr pellets in a mixture of about 1 wt.% KBr. The

spectra were monitored in a transmittance band mode in the range 400 ~ 4000 cm^{-1}. Two-dimensional proton-detected heteronuclear single quantum (2D HSQC) spectrum of the samples was recorded to provide structural information of XOS [28]. First, 40 mg of sample were dissolved in 0.5 mL DMSO-d_6 and then added to 5 mL NMR tube to exploit the 2D-HSQC spectra of the samples using a standard Bruker HSQC pulse sequence. The acquisition parameters were 160 transients using 1024 data points in the F2 (^{1}H) dimension at 5000 Hz spectral widths and 256 data points in the F1 (^{13}C) dimension at 20000 Hz spectral widths. Total running time for each sample was 8 h and the coupling constant was 147 Hz. Data processing was performed by applying standard Bruker Topspin-NMR software [29].

The thermal stability of commercial XOS and purified XOS of AC-PHL was determined using a thermogravimetric analyzer, (TGA, Q50, TA instrument, Newcastle, DE, USA). About 10 mg of the samples was placed in a sample tray of the instrument and heated from room temperature to 600 °C at a rate of 10 °C /min under nitrogen environment.

Analysis of precipitates lignin samples

The molecular weights (weight average molecular (Mw) and number average molecular (Mn) weights) and glass transition temperature (Tg) of F-lignin and LX-lignin were characterized by a gel permeation chromatography (GPC, Agilent Technologies, Palo Alto, CA, USA) and differential scanning calorimeter (DSC, TA instrument, Q20, Newcastle, DE, USA) respectively. The acetylated lignin was obtained following the method described in the literature [11]. In brief, the lignin samples were acetylated in a mixture of pyridine/acetic anhydride (1/2 vol/vol) and stirred in the dark for 72 h under room temperature. Once the acetalization was finished, tenfold volume of ethyl ether was mixed with the solutions. The mixture was centrifugated at 8000 rpm, and the precipitate, i.e. acetylated lignin, was further purified by repeated washing with ethyl ether and dried in vacuum over P_2O_5. In one set of experiments, 5 mg of acetylated lignin samples was dissolved in 1 mL of tetrahydrofuran (THF), and its molecular weight was determined using a GPC, which was equipped with a Shodex KF-802.5 column and differential refractive index (RI) detection. THF was the eluent at the flow rate of 1 mL/min and column temperature of 30 °C. In the DSC experiments, about 5 mg of the dried lignin samples were placed in aluminum pans with sealed lids and heated from 20 °C to 300 °C at 10 °C /min and a nitrogen flow rate of 10 mL/min. Before testing, the samples were extensively dried at 60 °C under vacuum for 12 h to remove moisture. The Tg was defined as the mid-point of the temperature range at which the change in heat capacity occurred, and this point was determined by change in the signal slope.

Results And Discussion

Chemical compositions of the PHL

The chemical compositions of the original PHL are listed in Table 1. As can be seen, xylosugars, lignin, acetic acid and acetyl groups were the main components of the PHL from hardwood dissolving pulp production processes. The concentration of XOS was 14.5 g/L, which was the major components of

xylosugars (27.3 g/L). Also, it is generally considered that those XOS have the biological functions and are conductive to the growth of intestinal bifidobacterium [8, 28]. Hence, further enhancing the content of XOS with DP 2 ~ 6 from PHL will be of practical significance in this work. Additionally, acetic acid concentration was 3.1 g/L, while the content of acetyl groups was 3.7 g/L, implying that the dissolved xylan/XOS in PHL was still highly acetylated, as noted in the earlier reports [26].

Table 1
Main chemical compositions of original PHL (g/L)

Xylose	XOS[a]	Xylosugars	Lignin	Furfural	Acetic acid	Acetyl groups[b]
5.1 ± 0.22	14.5 ± 0.40	27.3 ± 0.43	7.1 ± 0.35	0.3 ± 0.02	3.1 ± 0.10	3.7 ± 0.12
[a] represents XOS with DP ranging from 2 to 6.						
[b] means the amount of acetyl groups linked to the dissolved xylan/XOS						

Process analysis

Effect of CH pretreatment on xylanase hydrolysis efficiency

The influence of CH dosages on the concentration of acetic acid, acetyl groups, XOS in CH treated PHL and subsequent xylanase hydrolysis PHL was investigated. As seen in Fig. 2a, with increasing the CH dosage, the concentration of acetyl groups bound to the dissolved XOS/xylan in PHL was obviously decreased. By contrast, the concentration of acetic acid in PHL was increased. This is due to the hydrolysis of acetyl groups under alkaline conditions and formation of acetic acid. When the CH dosage was 0.8%, around 80% of acetyl groups was released from backbone of XOS/xylan and acetic acid concentration became approximately doubled. Acetic acid can be successfully recovered in high purity to meet industrial needs [30, 31]. As can also be seen in Fig. 2b, when CH dosage was less than 0.8%, CH pretreatment had marginal effect on the XOS concentration before xylanase treatment, while further elevating CH dosage led to the obvious loss of XOS, as XOS were adsorbed on undissolved $Ca(OH)_2$ particles [11].

Interesting, when xylanase treatment was performed after CH pretreatment, the concentration of XOS in CH-PHL increased rapidly by 23% with the increase of CH dosage (Fig. 2b). It indicated that CH pretreatment improved the efficiency of subsequent xylanase hydrolysis of CH-PHL. This behavior is related to the significant reduction of branched chains groups, such as the acetyl groups on XOS, which made xylan/XOS more linear and accessible to xylanase [10]. In this case, with increasing the CH dosage (above 0.8%), more xylosugars were adsorbed on undissolved $Ca(OH)_2$ particles [11, 18], and it eventually reduced the concentrations of XOS at 1.2 wt..% dosage. In addition, results showed that 0.8 wt.% of CH could decrease the concentrations of lignin from 7.0 g/L to 4.5 g/L (Table 4). Based on these results, the CH pretreatment at 0.8 wt.% dosage was selected as the essential pretreatment step for purifying PHL, minimizing XOS loss and enhancing the efficiency of xylanase hydrolysis.

Single enzyme treatment of CH-PHL

The effect of laccase dosage and treatment time on the lignin removals of CH-PHL are shown in Fig. 3a. When the laccase dosage was increased from 0.5 U/g to 5 U/g, the lignin removal increased from 22.5–32.2%. The optimal lignin removal of 29.0% was achieved at 1 U/g of laccase dosage, indicating that a low usage of laccase could remove lignin efficiently, as noted in an earlier study [22]. The optimal treatment time for laccase treatment was 3 h, and further extension of the treatment time had a slight effect on the lignin removal. Laccase generally facilitate the polymerization of lignin via radical–radical coupling reaction of phenolic lignin [32]. The polymerized lignin has generally lower solubility than unpolymerized lignin, which promotes its removal from PHL [33].

Figure 3b showed the effect of xylanase dosage and treatment time on the concentration of xylose and XOS in CH-PHL. As can be seen, the concentration of XOS increased with the extension of treating time while the xylose concentration changed slightly at the xylanase dosages of 2 U/g and 5 U/g. The major increases in the XOS concentration of CH-PHL were 40.0% and 39.7% at the xylanase dosage of 2 U/g for 3 h, and at xylanase dosage of 5 U/g for 1 h, respectively. XOS concentration also changed slightly when the treatment time prolonged from 3 h to 5 h, and the reason for such behavior was due to the lower xylanase dosage. With increasing the xylanase dosage, the maximum increase of XOS concentration in CH-PHL was 41.8% and 44.2% at the xylanase dosages of 10 U/g and 20 U/g for 1 h, respectively. XOS concentration continued to decrease with prolonging the treatment time from 1 h to 5 h resulting in further degradation of XOS to xylose (i.e., a xylose concentration increase). In brief, the optimal conditions of xylanase treatment were the combination of high xylanase dosage and short treating time, or low xylanase dosage and long treating time. Thus, the long treatment time can be considered as a pathway for reducing the usage of xylanase and thus the cost of enzyme treatment. It can be concluded that the optimal xylanase dosage was 2 U/g at the treating time was 3 h (Fig. 3). Thus, the similarity in the optimal treatment time in both laccase and xylanase treatments can contribute to the treatment of laccase and xylanase (LX) to efficiently and simultaneously purify and prepare XOS from CH-PHL.

Laccase and xylanase treatment (LX) of CH-PHL

The simultaneous use of xylanase and laccase in CH-PHL at different pH and temperatures were evaluated for enhancing lignin removal and elevating XOS concentration. The lignin removal in single laccase treated PHL (La-PHL) and XOS concentration in single xylanase treated PHL (Xy-PHL) were also analyzed for comparison.

Influence of temperature on lignin removal and XOS concentration in different enzyme treatment processes was presented in Fig. 4. It can be seen that the lignin concentration of LX-PHL was similar to that of the single laccase treatment process. Both laccase and LX treatments for the lignin removal were effective in the temperature range of 45 to 55 °C (Fig. 4a). The lignin removal decreased rapidly with further elevating temperature, which was attributed to laccase deactivation at a high temperature (60 °C) [11]. In opposition to lignin, the XOS concentration of CH-PHL treated by xylanase and LX treatment gradually increased when the temperature increased from 25 °C to 55 °C. Moreover, with subsequently

increasing the temperature to 60 °C, the XOS concentration decreased owing to xylanase deactivation [34].

The effect of pH value on lignin removal and concentration of XOS in the LX-PHL are shown in Fig. 4b. The optimal pH range for the lignin removal in laccase and LX treating processes were 3.5 ~ 5.5. Additionally, the concentration of XOS in xylanase and LX treated PHL were similar, and the maximum increase in the XOS concentration of 40.0% and 41.4% was obtained at pH 5.5, respectively. The XOS concentration decreased when the pH value was further increased to 6.5. The optimal pH value and temperature were 5.5 and 55 °C, respectively, based on lignin removal and XOS concentration increase. Compared with laccase and xylanase treatment, LX treatment can simultaneously remove the lignin and concentrate XOS in PHL.

AC adsorption

Figure 5 shows the effect of activated carbon treatment on the concentrations of dissolved organics in the LX-PHL. It was observed that the residual lignin of LX-PHL can be removed more extensively than sugars, when AC dosage was lower than 0.6%. As compared to xylose, the loss of XOS was significant with concentrating AC in the system. As also shown in Fig. 5, the optimal dosage of AC was 0.6%, under which the concentration of lignin in LX-PHL could effectively decreased by 59.4 wt.%, while XOS loss was minimal. In the present study, the relatively high selectivity of lignin removal is very meaningful, and it can promote the downstream processing and utilization of the dissolved XOS.

Precipitate analysis

Molecular Weight analysis of lignin samples

The molecular weight and polydispersity (PDI, Mw/Mn) of lignin samples was determined using GPC and the results are listed in Table 2. The Mw, Mn and PDI of the F-lignin was similar to those reported in other studies [35, 36], suggesting that a substantial lignin with a low molecular weight was generated in the pre-hydrolysis process. Furthermore, the molecular weight of LX-lignin generated in the LX-PHL treated at 45 °C was higher than that at 25 °C, which confirmed that the higher temperature was more favorable for LX polymerization of lignin and thereby accelerated lignin removal (Fig. 4a). In addition, the similar Mw and Mn of LX-lignin at the pH range of 3.5–5.5 in the LX treatment suggested that laccase was reactive in this pH range. As compared to alkali lignin with a higher molecular weight (Mw = 3135, Mn = 1886), the PHL lignin may be suitable for production of platform chemicals such as epoxies, acrylates, polyurethanes and polymer blends [37].

Table 2
Molecular weight of lignin samples

Lignin Sample	Condition	M_w (g/mol)	M_n (g/mol)	M_w/M_n
F-lignin	-	2320	1152	2.01
LX-Lignin[a]	T = 25 °C	1768	890	1.98
LX-Lignin[a]	T = 45 °C	2532	1325	1.91
LX-Lignin[b]	pH = 3.5	2280	1142	2.00
LX-Lignin[b]	pH = 5.5	2465	1180	2.04
PHL lignin [35]	-	2975	794	3.75
PHL lignin [36]	-	2200	1294	1.70

Note: F-lignin obtained via filtrating the original PHL; (a): the lignin in CH-PHL obtained by LX treatment under the conditions of laccase dosage 1 U/g, xylanase dosage 2 U/g, 3 h, pH 5.5; (b): the lignin in CH-PHL obtained by LX treatment under the conditions of laccase dosage 1 U/g, xylanase dosage 2 U/g, 3 h, 55 °C.

DSC analysis of lignin samples

Glass transition has an important effect on the processing and mixing of lignin with other polymers [38]. To explore the glass transition temperature (Tg) of PHL-induced lignin, heat flow peaks were measured and shown in Fig. S1, and the Tg values are listed in Table 3. At different LX treatment temperatures of 25 and 45 ^{o}C, the Tg was different (63 and 114 ^{o}C, respectively), which was possibly attributed to the molecular weight of lignin (Table 2). However, Tg seems not to be affected by pH. In addition, the Tg of LX-lignin was similar to that of F-lignin and commercial alkaline lignin in Table 3. Overall, the Tg of lignin was not significantly changed when extracted via different methods from PHL [39, 40].

Table 3
Glass transition temperature (Tg) of lignin derivatives of PHL.

Lignin sample	Condition	Tg (°C)
F-lignin	-	92
LX-Lignin[a]	T = 25 °C	63
LX-Lignin[a]	T = 45 °C	114
LX-Lignin[b]	pH = 3.5	96
LX-Lignin[b]	pH = 5.5	102
Lignin [40]	-	109

Note: F-lignin: lignin obtained via filtrating from original PHL; LX-lignin: lignin obtained via LX treatment. Other experimental conditions: (a) laccase dosage of 1 U/g, xylanase dosage of 2 U/g, 3 h, pH 5.5; (b) laccase dosage of 1 U/g, xylanase dosage of 2 U/g, 3 h, 55 °C.

FTIR analysis of XOS

The FT-IR spectrum of PHL samples and commercial XOS are shown in Fig. 6. Evidently, the commercial XOS and PHL-induced XOS in the all PHL samples had similar absorption peaks at 800 to 1200 cm^{-1}, implying that the hemicellulose structures were not obviously changed in successive purification process. Specifically, the similar absorption peak at 1045 cm^{-1} assigned to the C–O–C stretching of glycosidic linkages is typical characteristic peak of xylan [15], the peak at 894 cm^{-1} is ascribed to the vibrations of the dominant b-glycosidic linkages between the sugars' units [6]. The presence of two low intensity shoulders at 1161 cm^{-1} and 987 cm^{-1} are the typical characteristic peaks of arabinosyl side chains [41, 42]. In addition, the band at 3420 cm^{-1} was ascribed to the O – H stretching vibration of OH groups, and the band at 2926 cm^{-1} originated from the methylene groups. However, compared to the peak intensity of lignin in F-PHL, the relative peak intensity of lignin in S-CH, S-LX, S-AC was weak or disappeared, indicating the high efficiency of the present purifying methods in removing lignin from XOS. For instance, the typical characteristic peaks of lignin appeared at 1600 $cm^{-1}$and 1515 cm^{-1} (aromatic ring), 1336 cm^{-1} (syringyl (S) ring), 1250 cm^{-1} (guaiacyl (G) ring breathing with C – O stretching) [43]. In addition, the major absence of absorption at 1730 cm^{-1} of PHL samples including S-CH, S-LX, S-AC, was due to the low abundance of acetyl groups, which was consistent with remarkable removal of acetyl groups of xylan in PHL (Fig. 2a).

2D-HSQC NMR Spectra analysis

To further understand the precise structural features of the associated carbohydrates and lignin fractions (LCC, side-chain regions and aromatic regions) in the PHL, 2D-HSQC NMR spectra of the S-F and the purified PHL solids (including S-CH, S-LX and S-AC) were analyzed. The obtained spectra and assigned cross-signals of these samples were identified according to published literatures [7, 9, 28, 44] and showed

in Fig. 7 and Table S1, respectively. The main substructures of carbohydrates and lignin fractions in 2D-HSQC spectra were also illustrated in Fig. S2 in Supporting Information.

As shown in Fig. 7a, the main substructures of carbohydrates and lignin fractions can be verified by their correlation signals in the 2D-HSQC spectra at δ_C/δ_H 51–109/2.6–5.6. In general, the signals of carbohydrates and lignin fractions in spectrum of the S-F were stronger and more complete than that in purified PHL solids, and the spectra of the purified PHL were considerably similar. Firstly, the signals for the carbohydrates were interpreted as follows: (a) β-(1→4)-D-xylopyranoside of internal xylan (X, δ_C/δ_H i.e., X2 (73.0/3.03)); (b) 2-O-acetyl-β-D-xylopyranoside ($X2_2$, δ_C/δ_H 73.9/4.50), 3-O-acetyl-β-D-xylopyranoside ($X3_3$, δ_C/δ_H 75.4/4.80); (c) a-(1→4)-D-xylopyranoside with reducing end (aXR1, δ_C/δ_H 92.8/4.86); (d) β-(1→4)-D-xylopyranoside with reducing end (XR1, δ_C/δ_H 98.1/4.22); (e) β-(1→4)-D-xylopyranoside with non-reducing end (XNR, δ_C/δ_H i.e., XNR1 (102.3/4.26) and XNR3 (77.0/3.07)); (f) a-(1→4)-L-arabinofuranoside (Ara, δ_C/δ_H i.e., Ara5 (61.1/3.52)); (g) β-(1→4)-D-mannopyranoside (Man3, δ_C/δ_H 71.4/3.51); (h) β-(1→4)-D-glucopyranoside (Glc6, δ_C/δ_H 62.0/3.52)) and 4-O-methyl-a-D-glucuronic acid (UA, δ_C/δ_H i.e., UA4 (82.1/3.11), UA-OMe (59.8/3.35)) and X-UA-OMe4 (77.2/3.62). It can be noted that the cross-signals of xylosugars (xylan/XOS) in the S-F and purified S-CH, S-LX and S-AC were clearly observed through the C_2-H_2, C_3-H_3, C_4-H_4, and C_5-H_5 correlations from β-(1→4)-D-xylopyranoside of internal xylan (X2, X3, X4, and X5). Obviously, these signals associated with xylan were the prime cross-signals in all spectra, suggesting that the degraded carbohydrate in the PHL was mainly β-(1→4)-D-xylan. In addition, the presence of other sugars (Ara, Man, Gal, Glc and UA) with intensive cross-signals, such as Ara5, Man3, Glc6 and UA, were detected in both non-purified and purified PHL, which suggested that there were little structure changes for those sugars. Also, the correlation signal of X-UA-OMe4 unit (δ_C/δ_H 77.2/3.62) is assigned to the C_4-H_4 of 4-O-methyl-α-D-glucuronic acid linked to O-2 position of xylan backbone. However, the intensive cross-signals of O-acetyl-β-D-xylopranoside in the CH-PHL, such as the cross-signals of C_3-H_3 from 3-O-acetyl-β-D-xylopranoside ($X3_3$), respectively, cannot be detected after CH pretreatment because of their low frequency. It suggest that CH-PHL contained XOS/xylan with less acetyl groups due to its alkaline hydrolysis, which was consistent with the analysis of acetyl groups in Fig. 2a and 6. In brief, the above results suggested that the polysaccharides in PHL were mainly made up of linear XOS/xylan decorated with a few side chains, such as 4-O-methyl-D-glucuronic acids, and acetyl groups.

Secondly, the LCC linkages of phenyl glycoside (PhGlc) was found in all PHL spectra in Fig. 7a, indicating that lignin of the PHL was linked with different carbohydrates by phenyl glycoside linkages to form LCC structures [28, 45]. It was revealed that LCC can be released into PHL under mild pre-hydrolysis conditions [9]. However, the correlation signal of PhGlc2 (δ_C/δ_H 99.4/4.70) was disappeared in S-CH spectrum, which was probably due to the removal of lignin in CH pretreatment process. It demonstrated that the removed lignin fractions containing LCC linkages were mainly PhGlc2. On the contrary, the LCC linkages of PhGlc3 (δ_C/δ_H 102.3/4.92) was present in all PHL spectra, implying that the soluble lignin fractions

existed in the form of LCC structures before and after purifying PHL. These results are in accordance with those of a previous report stating that purified-XOS contained LCC structures [28].

Thirdly, the side-chain regions of all PHL solids spectra were also shown in Fig. 7a. Considering the basic substructures of lignin fractions, β-O-4 (A), β-β (B), and β-5 (C) were observed by their interunit correlation signals, such as β-O-4 (A): Cα-Hα (Aα) at δ_C/δ_H 71.6/4.62, Cβ – Hβ (Aβ(G)) at δ_C/δ_H 83.8/4.30 and Cγ – Hγ (Aγ) at δ_C/δ_H 59.8/3.35; β-β (B): Cγ – Hγ (Bγ) at δ_C/δ_H 71.2/4.16, 3.80; β-5 (C): Cγ – Hγ (Cγ) at δ_C/δ_H 63.5/3.46. The above results indicated that the common lignin substructures were preserved during the hot water pre-hydrolysis process [46, 47]. Also, these signals became weak or absent with continuous purification steps. Interestingly, only the side-chain signals: Aγ at δ_C/δ_H 59.4/3.54 and Cγ at δ_C/δ_H 63.5/3.48 were found in the purified PHL samples (S-CH, S-LX and S-AC) spectra. The results revealed that a part of residual lignin in the purified PHL, especially in the AC-treated PHL, possessing at least a dimeric form, might be also linked with carbohydrates by the β-O-4 and β-5 linkages.

As shown in Fig. 7b, prominent correlation signals for syringyl units (S), guaiacyl units (G) and *p*-hydroxyphenyl (H) lignin in the PHL samples can be observed in the aromatic region (δ_C/δ_H 100–131/6.0-7.8). Overall, the signals about lignin fractions in spectrum of the S-F were stronger than those in the purified PHL solids (S-CH, S-LX and S-AC).

In the S lignin units, the correlation signals at δ_C/δ_H 104.3/6.60 was attributed to the $C_{2,6}$-$H_{2,6}$, and the signals of oxidized S lignin units occurred at δ_C/δ_H 106.4/7.30 ($C_{2,6}$-$H_{2,6}$). In addition, the signals at δ_C/δ_H 111.3/6.89, 115.2/6.74, and 119.3/6.78 were attributed to the C_2-H_2, C_5-H_5, and C_6-H_6 in G lignin units. The signals of these units in S-AC spectrum were much weaker than those in the S-F spectrum, indicating only a small quantity of lignin in purified PHL. Noticeably, the lignin in F-PHL appeared to have the typical condensed S units, which was also reported by others in the extraction of lignin from Eucalyptus via γ-valerolactone/water/acid system [43].

As compared to S-F, S-CH had limited condensed S units as seen from the low frequency of condensed S signals, demonstrating that CH pretreatment was effective in removing lignin with condensed S unit structure. In addition, the monomeric lignin-degraded fractions, such as *p*-coumaric acid (PCA) and ferulic acid (FA) structures, were identified in the F-PHL spectrum. While PCA structures (PCA8 and PCA2,6) could not be found, and the signals of FA structures (FA2 and FA6) was obviously lower in all purified PHL spectra.

Thermal stability analysis

To better understand the thermal behavior of the final purified XOS, the mass loss and mass loss rate of S-AC were analyzed, and the results are compared with those of commercial XOS in Fig. 8. When the temperature increased to 600 °C, approximately 80 wt.% of the XOS was decomposed. In this decomposition process, the mass loss process could be divided into three stages. The temperature of the first stage was lower than 200 °C. During this stage, a small weight loss of XOS appeared, which was mainly caused by the removal of moisture and the generation of noncombustible gases (e.g., formic acid

and acetic acid) [41]. It is noted that the major weight loss of XOS sample was occurred in the second stage (temperature of 200 to 450 °C), which was due to the degradation reactions forming the combustible gases when heating (e.g., depolymerization, hydrolysis) [41, 48]. The third stage of weight loss ranging from 450 to 600 °C had no significant changes with the combustion of few volatile components [49]. Furthermore, the DTG curve of XOS (Fig. 8) showed a wider decomposition temperature range for the commercial XOS, which may show that the decomposition performance of XOS samples were different.

Overall performance

Table 4 shows the concentration of xylose, XOS, xylosugars, lignin and furfural at each purification step and the overall changes in the compositions of the chemicals in the process. Firstly, F-lignin was obtained as insoluble lignin by filtrating the original PHL which had non-obvious influence on the removal of other components as the concentration of lignin and furfural in F-PHL was basically similar to original PHL. Next, CH pretreatment led to 35.7 wt.% lignin and 66.7 wt.% furfural removals with an insignificant sugar loss, which showed the high selectivity in impurity removals. Additionally, LX treatment was effective in eliminating lignin and producing XOS, and it was noted that the XOS concentration remarkably increased by 40.7 wt.%. The recovered lignin including F-lignin and LX-lignin possessed relative lower molecular wight and Tg. Overall, the concentration of lignin, furfural and xylosugars decreased by 81.7 wt.% 100 wt.% and 5.1 wt.%, respectively, but XOS concentration was increased by 36.6 wt.%. In addition, lignin carbohydrate complexes (LCC) existed in the finial purified XOS. In previous reports, the application of poly ethylene imine led to 20.2% xylosugars loss and 58.4% lignin removal [50]. In another work, the treatment of CH and AC resulted in 6.8% xylosugars loss and 66.9% lignin removal [18]. Comparatively, the combination of CH, LX and AC treatment process not only acquires a satisfying purification effect, but also enables a substantial increase in XOS content simultaneously.

Table 4
Effect of different treating steps on the concentration of PHL components and the final increase or removal of each component after purification

PHL (g/L)	Xylose	XOS	Xylosugars	lignin	Furfural
Original PHL	5.1 ± 0.22	14.5 ± 0.40	27.3 ± 0.43	7.1 ± 0.35	0.3 ± 0.02
F-PHL	5.1 ± 0.20	14.5 ± 0.35	27.2 ± 0.41	7.0 ± 0.28	0.3 ± 0.02
CH-PHL	5.0 ± 0.24	14.4 ± 0.28	26.9 ± 0.36	4.5 ± 0.33	0.1 ± 0.01
LX-PHL	5.3 ± 0.29	20.4 ± 0.38	26.8 ± 0.32	3.2 ± 0.18	0.1 ± 0.01
AC-PHL	5.2 ± 0.16	19.8 ± 0.25	25.9 ± 0.29	1.3 ± 0.20	0
Change (wt.%)	**Xylose**	**XOS**	**Xylosugars**	**lignin**	**Furfural**
Present work	+ 2.0	+ 36.6	-5.1	-81.7	-100
[18]	-4.0	-	-6.8	-66.9	-70.1
[50]	-	-	-20.2	-58.4	-

Conclusions

The results confirmed that a combination of sequentially treating PHL with calcium hydroxide (CH), simultaneous laccase and xylanase (LX) and activated carbon (AC) was effective in removing lignin and concentrating XOS in the PHL. Overall, 80.4% of lignin, 100% of furfural and 5.1% xylosugars were removed while XOS concentration was increased by 36.6% in this approach. The structural analysis of PHL revealed that S (especially condensed S units) and G types lignin with β-O-4 and LCC (PhGlc2) linkages were removed by CH treatment, and about 80% of acetyl groups linked to XOS/xylan (O-acetyl-β-D-xylan) was hydrolyzed and formed acetic acids in the CH treatment process. LX and AC treatments further reduced S, G and H type lignin as well as lignin-degraded fractions, such as p-coumaric acid and ferulic acid, however, these two steps had little effect on XOS structures. Also, the separated PHL lignin and LX-lignin had resemble Mw and Tg. Additionally, PhGlc3 as main LCC linkages between lignin and carbohydrate existed in the final purified XOS. The description of chemical and structural properties of PHL passing through these steps would help develop enzyme based biorefining processes for the generation of altered products.

Declarations

Ethical approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

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Authors' Contributions

GY, FX, YN and JC were the main authors of this work and designed the experiments; GY, FX, JD and KZ performed the experiments; GY, FX, XJ and PF analyzed the data; JC and YN were the lead supervisors of the group on these projects; GY, FX and PF wrote the paper; PF and YN provided an overall assessment of this work. All authors read and approved the final manuscript.

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Figures

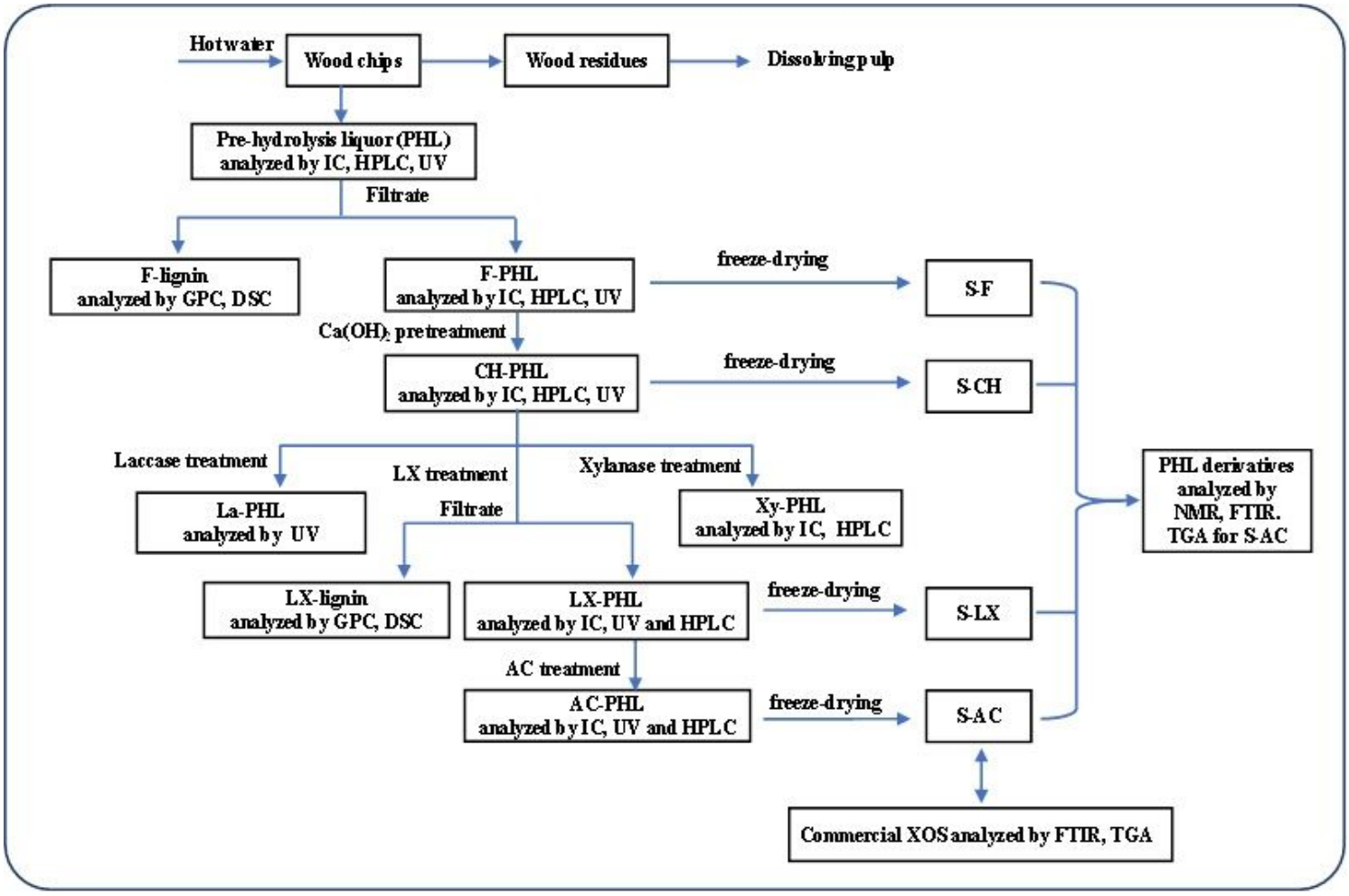

Figure 1

Experimental procedure conducted for purifying and producing XOS

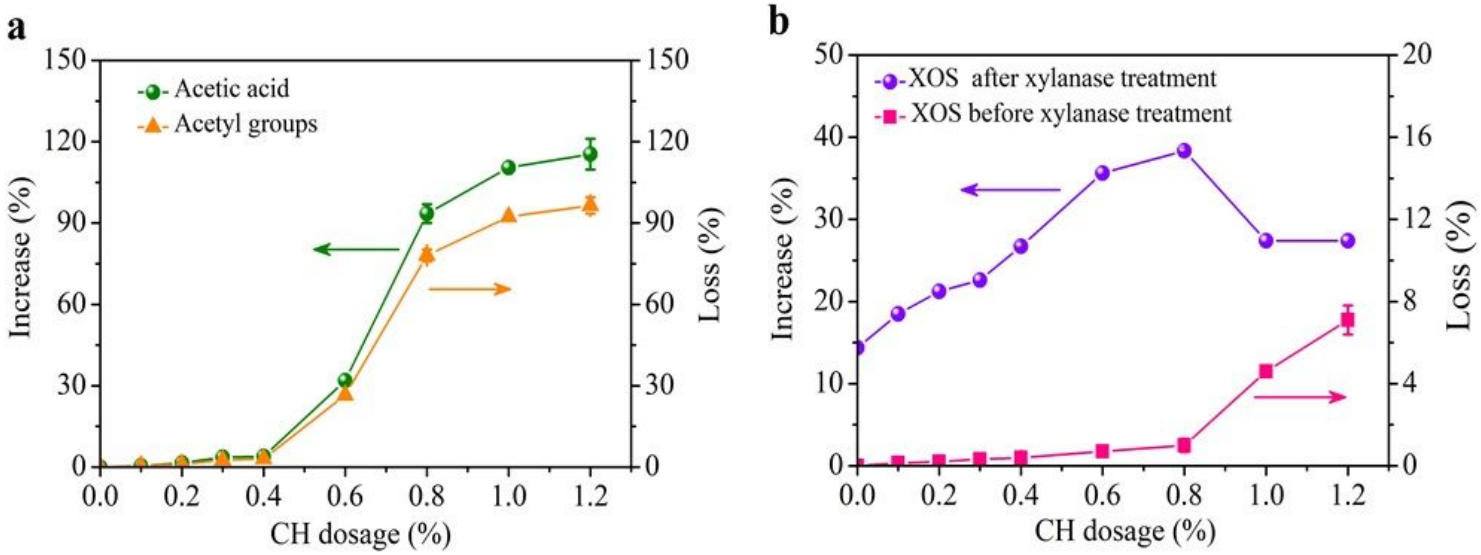

Figure 2

Effect of CH pretreatment on the concentration of a acetic acid, acetyl groups and b XOS in the PHL and in subsequent of xylanase hydrolysis PHL (conditions: xylanase dosage 2 U/g, pH 5.5, temperature 55 °C, time 3h)

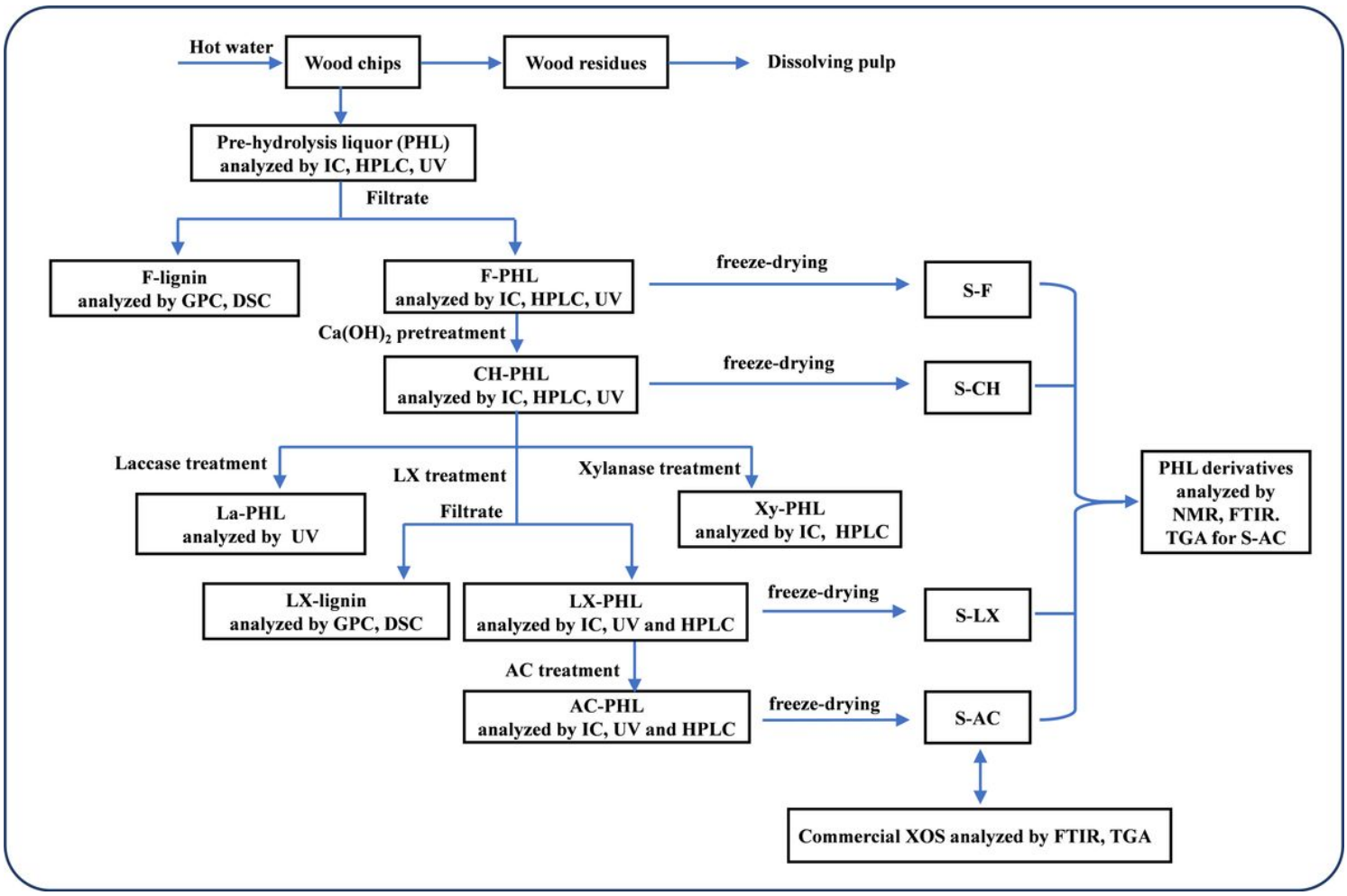

Figure 3

a Effect of laccase treatment on the lignin removal of CH-PHL b Effect of xylanase treatment on the concentration increases of xylose and XOS of CH-PHL. a ( ): The other treating conditions were pH 4.5, temperature 45 °C, time 3 h; ( ): laccase dosage 1U/mL, pH 4.5, temperature 45 °C. b The other treating conditions were pH 5.5 and temperature 55 °C.

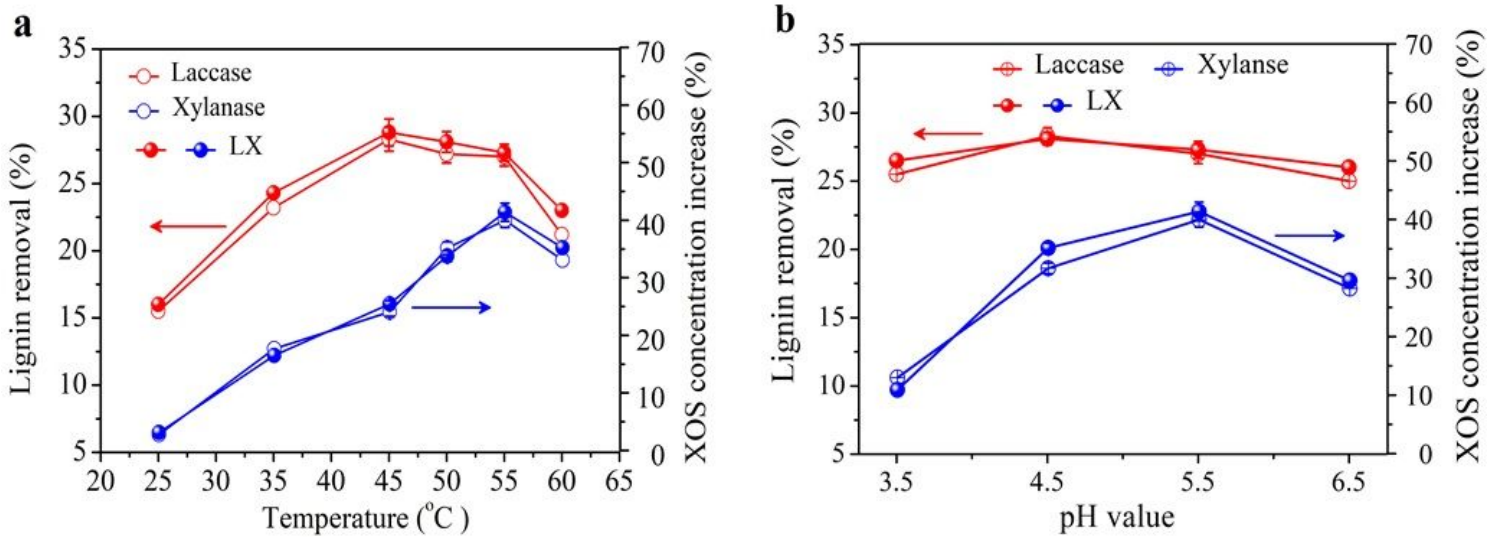

Figure 4

Effect of temperature a and pH b on lignin removal and XOS concentration in LX-PHL by various treatment conditions: a laccase dosage 1 U/g, xylanase dosage 2 U/g, 3 h, pH 5.5; b laccase dosage 1 U/g, xylanase dosage 2 U/g, time 3 h, temperature 55 °C.

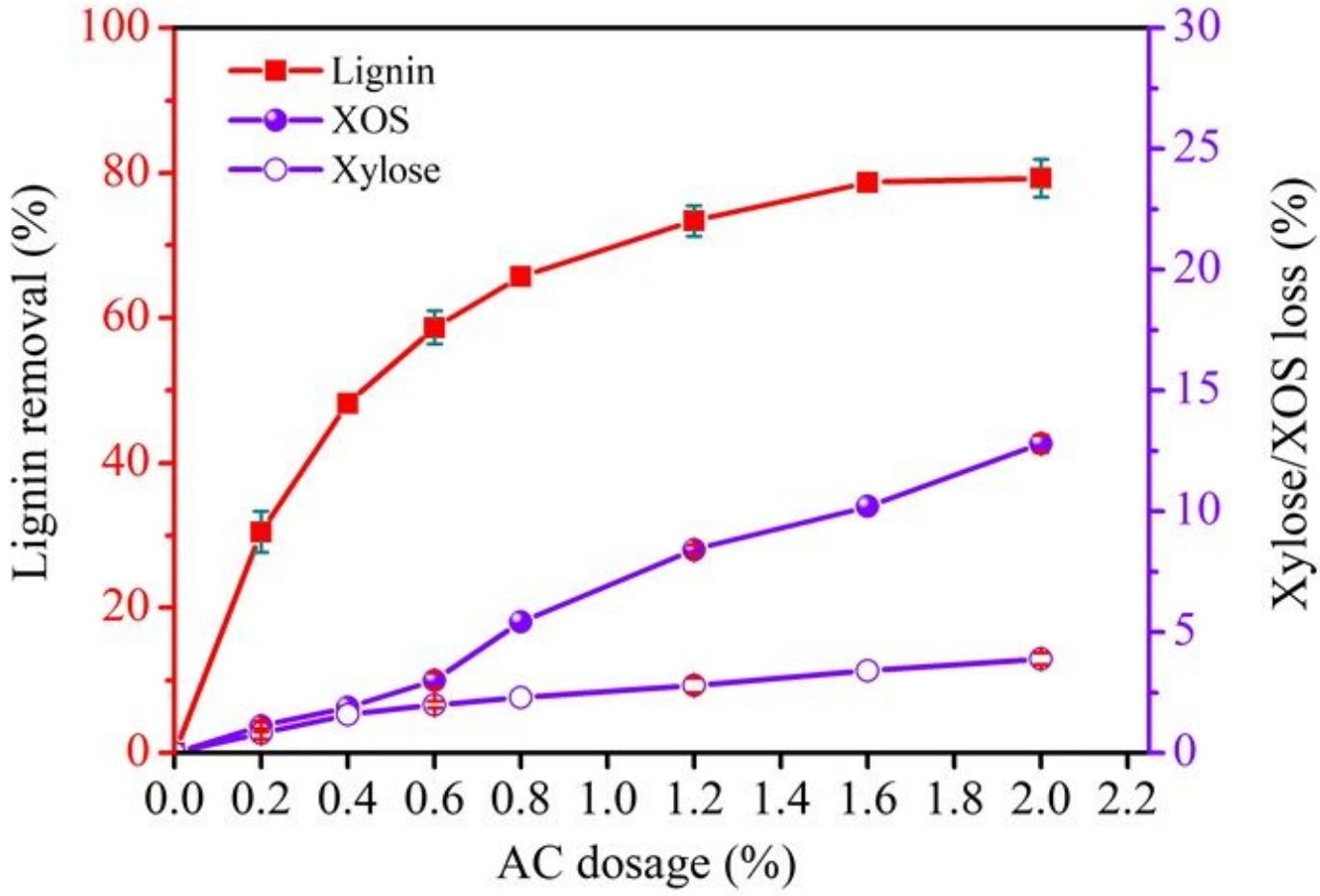

Figure 5

Effect of AC dosage on the removal of lignin and the loss of xylose and XOS.

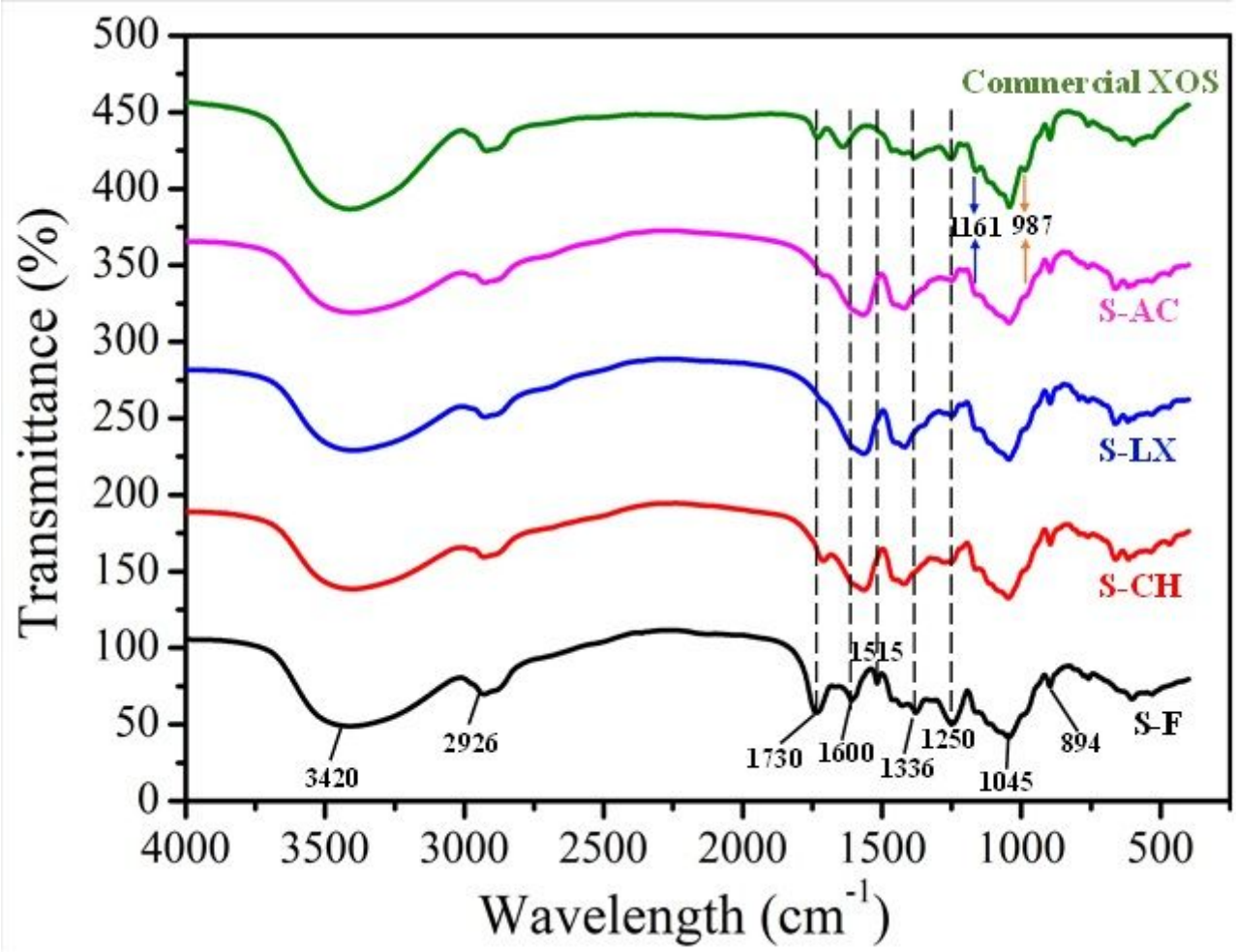

Figure 6

FTIR spectra of S-F, S-CH, S-LX, S-AC and commercial XOS. S-F, S-CH, S-LX, S-AC were obtained from F-PHL, CH-PHL, LX-PHL and AC-PHL, respectively. F-PHL obtained via filtering original PHL with qualitative filter paper; CH-PHL obtained via treating F-PHL with CH particles; LX-PHL obtained via treating CH-PHL using laccase and xylanase; AC-PHL obtained via treating LX-PHL with activated carbon.

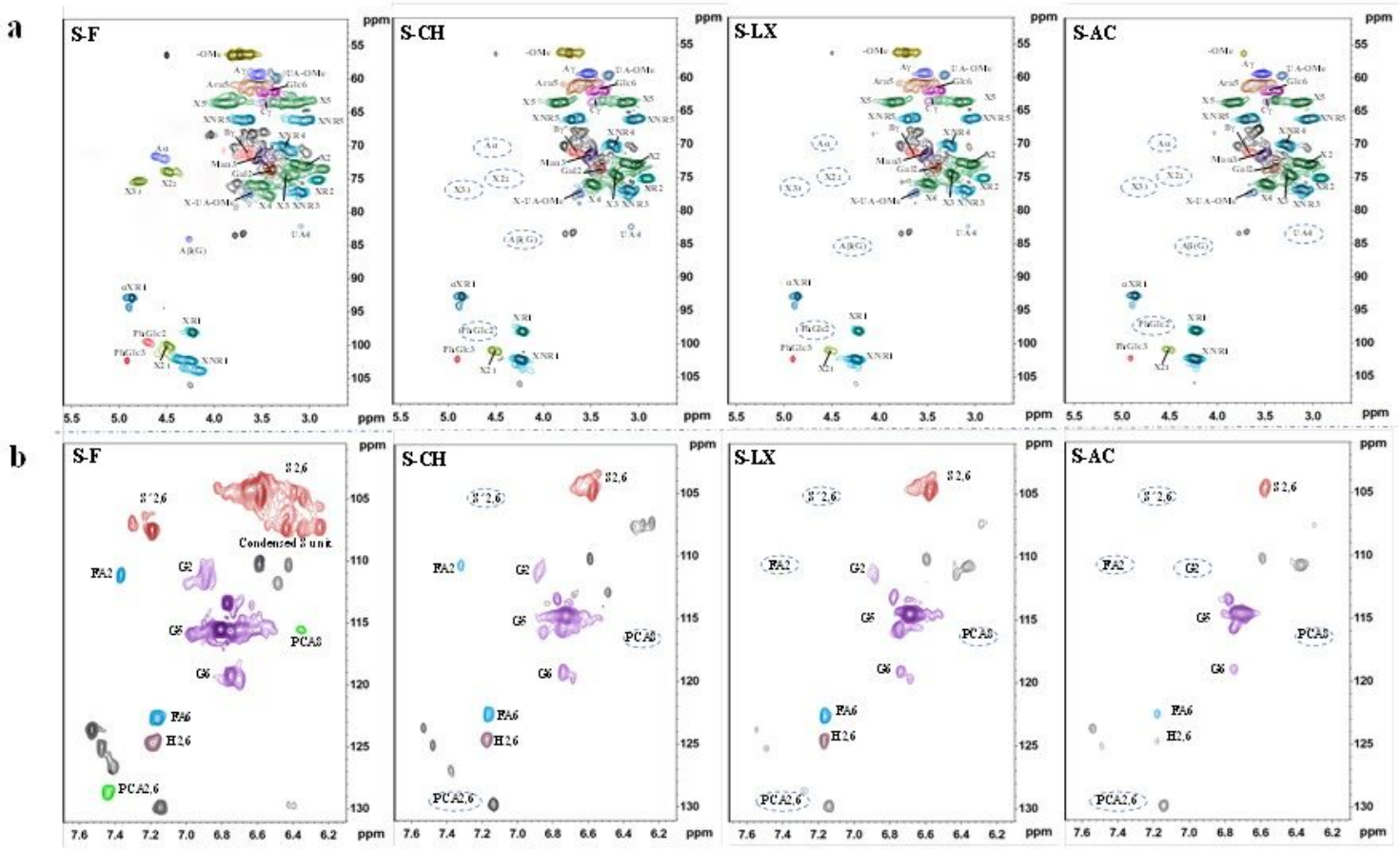

Figure 7

2D HSQC NMR spectra of main substructures of associated carbohydrates and lignin fractions (LCC and side-chain regions) a and aromatic regions b in the S-F and the further purified PHL solids (S-CH, S-LX and S-AC).

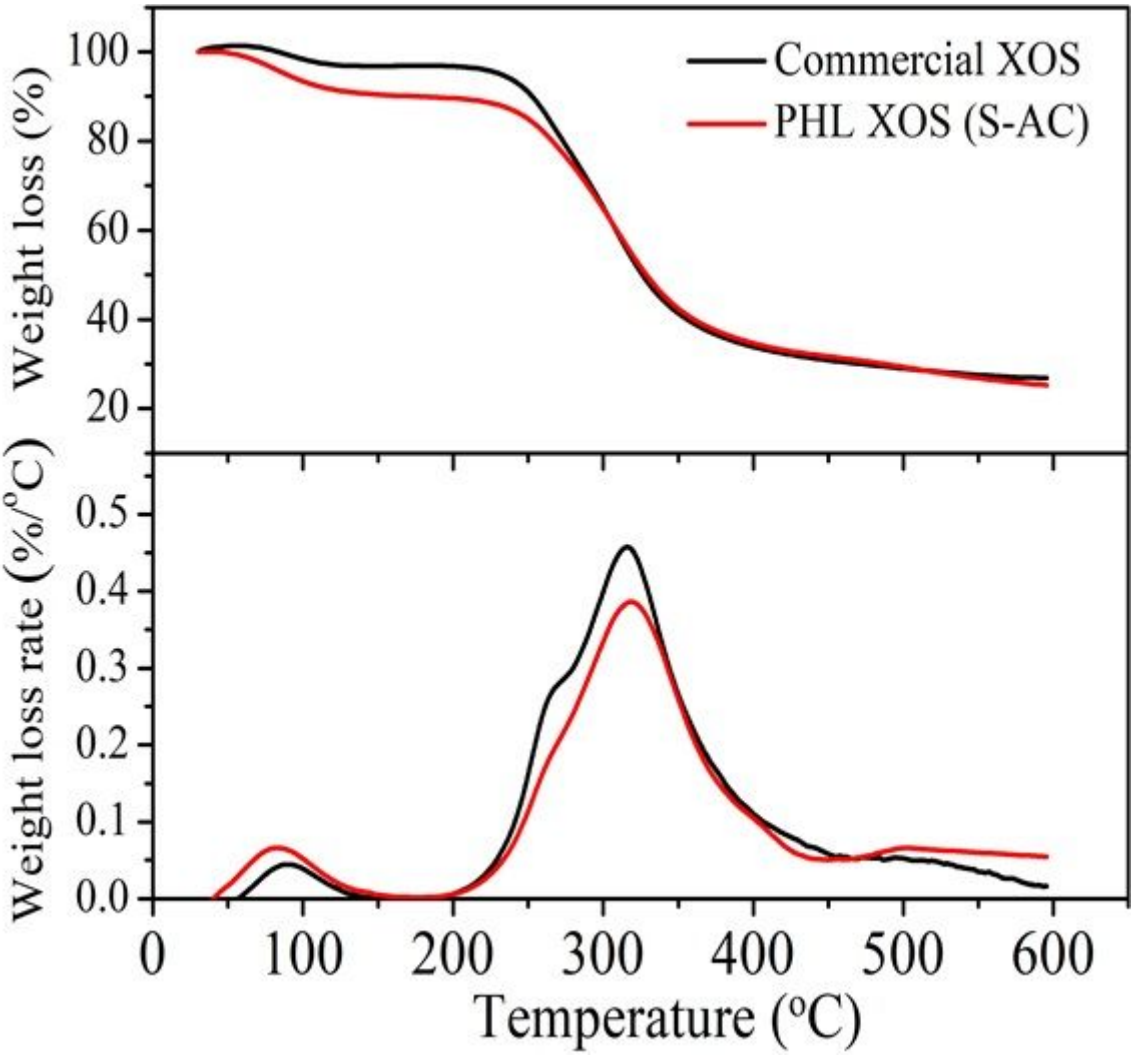

Figure 8

Thermal degradation of the commercial XOS and PHL XOS (S-AC).

Supplementary Files

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- Supportinginformation.docx