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A comparison of different oxidative pretreatments on polysaccharide hydrolyzability and cell wall structure for interpreting the greatly improved enzymatic digestibility of sugarcane bagasse by delignification

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Abstract

In order to confirm the contribution of delignification to the increase in lignocellulosic cellulose digestibility, several laboratory oxidative pretreatments under mild conditions, including alkaline-hydrogen peroxide (AP), two-step alkaline/peracetic acid (APAA) and sodium chlorite (SC) pretreatments were employed to achieve selective delignification of sugarcane bagasse and retained most of the hemicelluloses (xylan) in the pretreated solids. Four commercial cellulase cocktails were used to test the enzymatic hydrolyzability of pretreated substrates. Results revealed that delignification indeed could greatly improve the final (120 h) cellulose hydrolysis with relatively high final (120 h) glucan conversion (> 90%) by different cellulase cocktails even if the substrates still had a high hemicelluloses content. However, the xylan conversion seemed to be more greatly dependent on the pretreatments and cellulase cocktails used. AP and APAA pretreatments resulted in the disappearance of middle lamella and liberation of cellulose fibers with significant etching, deformation and fracture of cell wall structure. SC pretreatment greatly modified the sugar bagasse surface morphology to make the surface much coarser. The cell wall also underwent serious fracture and deformation with some middle lamella disappearing. However, no significant alteration on the structure of pure cellulose was observed by SC oxidative pretreatment of filter paper. Oxidative pretreatment might also modify lignin structure and surface properties thus greatly reducing the non-specific adsorption of enzymes. The obtained results strongly support the conclusion that delignification under mild pretreatment condition can be very helpful to improve the enzymatic hydrolysis of lignocellulosic cellulose by commercial cellulase cocktails even if the substrates has a high hemicelluloses content.

Keywords: Lignocellulosic biomass, Enzymatic hydrolysis, Oxidative pretreatment, Delignification, Surface morphology, Cell wall structure

Introduction

Lignocelluloses is one of the most promising renewable resources for biorefining to produce various biofuels, biochemicals and biomaterials. However, the presence

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of non-cellulose components has become a challenging limitation for efficient enzymatic hydrolysis of lignocellulose to release fermentable sugars (Zhao et al. 2012a, b). Lignin is amorphous and complex aromatic biopolymer naturally existing in plant cell wall. It provides terrestrial plants mechanical support, stress response, pathogen resistance, and plays an important role in water transport (Vanholme et al. 2010). Lignin has been found to greatly contribute to the cell wall recalcitrance to biological degradation of cell wall cellulose. This is because lignin not only plays as a physical barrier to "protect" cellulose from the attack by microorganisms and enzymes, but also can non-productively adsorb cellulase proteins, limiting the enzymatic hydrolysis of cellulose (Zhao et al. 2017a, b). Many works have corroborated that delignification of sugarcane bagasse could well improve cellulose accessibility. One of the most effective delignification approaches refers to alkaline pretreatment, which can saponify lignin–carbohydrate complex (LCC), depolymerize lignin macromolecule and remove lignin from cell wall (Zhang et al. 2020). Organosolv pretreatment also can achieve good delignification as well as fractionation of biomass, thus well exposing cellulose surface for enzymatic hydrolysis (Zhao et al. 2017a). Actually, most of the leading pretreatments with removal of a part of lignin, such as hydrothermal, steam explosion or acid coupled with alkaline pretreatments, sulfite pretreatment, ionic and organosolv pretreatments, etc., also simultaneously remove a considerable part of the hemicelluloses. However, removing hemicelluloses has also been proved to be effective to increase cellulose digestibility. Therefore, when samples prepared by these pretreatment methods were used to investigate the negative effect of lignin on cellulose enzymatic digestibility, it would be equivocal to judge the "contribution" of delignification to the increase in cellulose digestibility because delignification is usually positively related to the degree of hemicellulose removal during these processes. Sometimes, the "contribution" of delignification is underestimated or neglected. For example, Yang and Wyman (2004) found that removal of xylan seemed to be more important than removal of lignin to increase the enzymatic digestibility of corn stover cellulose by batch and flow-through pretreatment between 160 and 220 °C. Mussatto et al. (2008) employed a sequence of dilute acid and dilute alkali for pretreatment of brewer's spent grain. They also concluded that the contribution of removal of hemicellulose was larger than that of removal of lignin. However, Chen et al. (2016) concluded that the relative significance of the negative impacts of hemicelluloses on enzymatic hydrolysis of lignocellulosic cellulose was dependent on lignin content. The structural features of lignocellulosic substrates are complicated, and it is really difficult to distinguish which factor is the most important to limit the cellulose accessibility. In some cases, one factor has the most important influence, but in some other cases, another factor may become the predominant one. It means that strong interactive effects exist between hemicelluloses and lignin to construct the cell wall recalcitrance. Therefore, to analyze the effect of lignin on biomass recalcitrance, it is necessary to obtain samples under mild conditions to achieve selective removal of lignin, but minimizing removal of hemicelluloses and modification of cellulose. In this aspect, sodium chlorite (SC) pretreatment and combination of alkaline and oxidative delignification, such as alkaline-oxygen (Kallioinen et al. 2013), alkaline-hydrogen peroxide (AP) (Dutra et al. 2018) and alkaline/peracetic acid (APAA) (Zhao et al. 2009) seems to be good choice, because they usually can be operated under mild conditions to retain most of hemicelluloses in the pretreated solids and achieve a high degree of delignification (*DD*). Actually, SC and peracetic acid (PAA) have been used as standard methods to determine the holocellulose content of lignocellulosic biomass (Rabemanolontsoa and Saka 2012), indicating that selective delignification can be obtained by SC and PAA treatment. However, whether the oxidative pretreatment is routinely effective for different commercial cellulase cocktails, and how oxidative pretreatment modifies biomass surface morphology and alters the cell wall structure have not been well elucidated. Therefore, the objective of this work is to compare different oxidative delignification pretreatments of sugarcane bagasse under mild conditions, including AP, APAA and SC pretreatments. The efficiency of pretreatment was analyzed in terms of the chemical compositions of the pretreated solids, the cellulose enzymatic hydrolyzability and the structural features of the substrates. The obtained findings would provide insightful information to understand the negative effects of lignin on cellulose accessibility for fermentable sugar production.

Materials and methods

Chemicals and materials

Air-dried sugarcane bagasse harvested in Guangxi Zhuang Autonomous Region, China, was used as the lignocellulose feedstock. It was produced by a local sugar mill and used as it was received without pre-processing to reduce the particle size and remove the pith. The main chemical compositions of the bagasse were determined to be 41.3 ± 1.3% glucan, 25.9 ± 0.9% xylan, 24.0 ± 1.2% total lignin and 2.4 ± 0.3% acetyl group contents. The chemicals, mainly including 30 wt% hydrogen peroxide, glacial acetic acid, sodium chlorite, 98% sulfuric acid and Tween-80, were reagent grade and purchased locally.

Standard chemicals used in high-performance liquid chromatography (HPLC) analysis, mainly including glucose, xylose and arabinose were purchased from Sigma-Aldrich (Shanghai branch, CN). The xylan used for xylanase activity analysis was purchased from Shanghai Rhawn Chemical Technology Co., Ltd (Shanghai, China), which was isolated from corncob. PAA was prepared by reaction of acetic acid and 30 wt% hydrogen peroxide at volume ratio of 1.5:1 at room temperature for 72 h with addition of 1.5% (w/w) sulfuric acid as the catalyst according to our previous kinetic and optimization studies (Zhao et al. 2007, 2008a, b). The PAA concentration in the obtained solution was 16 wt% as determined by titration method (Zhao et al. 2008b). Four commercial cellulase cocktails were used for comparison experiments, namely Cellic® CTec2 (Novozymes A/S, Denmark), Celluclast 1.5L (Novozymes A/S, Denmark), Vland cellulase (Qingdao Vland Biotech Inc., China) and Habio cellulase (Mianyang Habio Biotech Ltd, China), which had cellulase activity (filter paper activity) of 119.4, 29.6, 21.9 and 132.1 FPU/ml, and xylanase activity of 408.1, 90.0 299.9 and 72.5 U/ml, respectively.

Oxidative pretreatment

Three oxidative pretreatments were employed to remove lignin under mild conditions, namely alkaline hydrogen peroxide (AP), alkaline/peracetic acid (APAA) and sodium chlorite (SC) pretreatments. AP pretreatment was performed under the optimal condition for sugarcane bagasse as reported by Dutra et al. (Dutra et al. 2018), namely 2.98 wt% H_2O_2 concentration, 8.6% (w/v) solid loading, pH 11.5, with stirring at 250 rpm in a 35 °C air-bath shaker for 20 h. APAA pretreatment was performed according to previous optimization work (Zhao et al. 2009). This was a two-step pretreatment comprising alkaline treatment and PAA delignification steps. In the first step, alkali, i.e., NaOH, was used to partly remove lignin and swell the fibers. This step was called alkaline pre-pretreatment and conducted with 10 wt% NaOH loading based on dry bagasse weight at liquid-to-solid ratio of 3:1 at 90 °C for 1.5 h. After that, the solid was washed with running water until neutrality and pressed till the liquid content was about 75%, and then PAA was used for further delignification. In the second step, the obtained solid from alkaline pre-pretreatment was treated with PAA solution for further delignification at 70–75 °C with PAA loading of 10wt% based on the initial dry bagasse weight for 2.5 h. SC pretreatment was performed according to the procedure described in the work of Chen et al. (2016) which was used as a standard method to determine the holocellulose of lignocellulosic biomass (Rabemanolontsoa and Saka 2012). 10 g of air-dried bagasse solid was added to 300 mL (including moisture of the sample) deionized water with 2.5 mL glacial acetic acid and 3 g sodium chlorite (80wt%) in a 500-mL triangle bottle at 75 °C for 0.25–2 h. 2.5 mL glacial acetic acid and 3 g sodium chlorite were supplemented at the first hour when the pretreatment was performed for more than 1 h. The pretreated solid was then thoroughly washed with water until neutrality and stored at 4 °C for further analysis. When used for enzymatic hydrolysis, the wet cake was directly used without drying. However, prior to the instrumental analysis of the biomass structure, conventional oven-drying may cause hornification of fibers and change of structure (Luo and Zhu 2011). Therefore, in this work, acetone was used for dewatering of the wet cake followed by freeze-drying in order to minimize the change in the biomass structure (Beecher et al. 2009).

Enzymatic hydrolysis

The wet solid substrates were incubated at 50 °C and 150 rpm in 50 mM sodium acetate buffer (pH 4.8) in an air-bath shaker. All experiments were performed in duplicate in 10 mL working volume at initial solid consistency of 2.5% (g/100 ml). It should be noted that in this work we investigated the effects of substrate structural features on enzymatic hydrolysis of cellulose, thus the condition of enzymatic hydrolysis should not be a limiting step for glucan conversion. Therefore, a relatively high cellulase loading (15 FPU/g solid) and low initial solid consistency (2.5%, w/v) were used. However, for comparison, lower cellulase loading of 5–10 FPU/g solid was also employed. It should be noted that the wet cake of pretreated solid and cellulase cocktail can bring liquid into the hydrolysis system. Therefore, the volume of buffer should be carefully calculated by the total volume needed subtracting the volume of water of wet cake and the cellulase cocktail used. Enzymatic digestibility was characterized by glucan and xylan conversion, defined as the percentage of glucan or xylan converted to glucose or xylose, respectively, as the following equations:

$$\text{Glucan conversion (\%)} = \frac{C_G}{s \times 1.05 \times G_{\text{In}}} \times 100\%, \quad (1)$$

$$\text{Xylan conversion (\%)} = \frac{C_X}{s \times 1.13 \times X_{\text{In}}} \times 100\%, \quad (2)$$

where C_G and C_X are the concentrations of glucose and xylose in the enzymatic hydrolysate, respectively, g/L; s is the initial solid loading in the system, g/L; G_{In} and X_{In} are the glucan and xylan contents in the pretreated substrates.

Analytic methods

Chemical compositions

About 5 g wet pretreated bagasse was weighed exactly and dried at 105 °C for 6 h to determine moisture content. The main compositions of raw sugarcane bagasse and pretreated solids, including cellulose (glucan), xylan, acid-soluble lignin and acid-insoluble lignin were determined according to standard methods provided by NREL's Laboratory Analytical Procedure (Sluiter et al. 2008). The xylan removal or degree of delignification (*DD*) was defined as the dry weight percentage of xylan or lignin removed after pretreatment, which was calculated based on mass balance with the xylan or lignin content of the raw biomass, the solid yield and xylan or lignin content of the pretreated substrates. The monosaccharide (glucose, xylose and arabinose) concentrations were determined by Shimadzu (Tokyo, Japan) HPLC (LC-10AT) system equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a differential refraction detector using 5 mM H_2SO_4 as an eluent at flow rate of 0.8 ml/min.

Enzyme activity assay

The cellulase activity was determined by hydrolyzing filter paper (filter paper activity, FPA) to release glucose according to NREL's Laboratory Analytical Procedure with some modification, and expressed as filter paper unit (FPU) (Adney and Baker 2008). Glucose concentration was determined with HPLC instead of reducing sugar concentration determined by DNS method. One FPU was defined as the amount of cellulase enzyme required to hydrolyze filter paper to release exactly 2 mg glucose in 1 min at 50 °C and pH 4.8. The xylanase activity was determined with the same protocol for cellulase activity determination, but using corncob xylan as the substrate instead of filter paper. One unit of xylanase was defined as the amount of enzyme required to hydrolyze xylan substrate to release exactly 1 μmol xylose in 1 min at 50 °C and pH 4.8.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were recorded with a Thermo Scientific Nicolet iN10 FTIR Microscope (Thermo Nicolet Corporation, Madison, WI) equipped with a liquid nitrogen-cooled MCT detector. About 1 mg acetone-dried samples were embedded in 100 mg KBr pellets. Scans were conducted at 400–4000 cm^{-1}. Background scanning was performed for correction prior to recording the spectra.

Crystallinity analysis

The crystallinity of the samples was determined by X-ray diffraction (XRD) using XRD-6000 instrument (Shimadzu, Japan). The X-ray diffractograms were recorded from diffraction angle (2θ) of 5° to 50° at a scanning speed of 5°/min with Ni-filtered Cu Kα radiation ($\chi = 1.54$ Å) at 40 kV and 40 mA. The crystallinity index (*CrI*) was calculated as $CrI = (I_{002}\text{-}I_{am})/I_{002} \times 100\%$, where I_{002} is the intensity of peak at a 2θ angle close to 22.5° and I_{am} is the scattering intensity of amorphous fraction at a 2θ angle close to 18°.

Surface morphology

Surface morphology analysis of the raw bagasse and pretreated solids was performed using scanning electron microscope (SEM). A Hitachi S-3400N II (Hitachi, Japan) instrument operated at 15 kV was used. Prior to imaging, the samples were sputter-coated with a thin layer of gold to make the fibers conductive, avoiding degradation and buildup of charge on the specimen. Images were obtained at magnifications ranging from 45× to 30,000× depending on the feature to be traced.

Cell wall ultrastructure

The cell wall ultrastructure was observed by transmission electron microscope (TEM). Untreated and pretreated bagasse were first fixed in 2.5% glutaraldehyde and then dehydrated successively in gradient ethanol for 15 min each (50, 70, 80, 90, 3 × 100% (v/v) ethanol) and subsequently treated by 50% (v/v) acetone–ethanol and 100% acetone. The samples were further incubated in resin successively with increasing concentration overnight (diluted in acetone with a ratio of 1:1, 2:1, 3:1, and pure resin). The samples were then transferred to 60 °C oven, with resin changed twice (2 h for each) before cooled down. Embedded samples were sectioned with a Leica EM UC6 ultramicrotome (Leica, Wetzlar, Germany) and sections were collected on formvar–carbon-coated copper grids. An H-7650B transmission electron microscope (Hitachi, Japan) operated at 80 kV was used to observe the ultrastructure of the sections.

Results and discussion

Chemical compositions of pretreated sugarcane bagasse by oxidative delignification

The chemical compositions of pretreated sugarcane bagasse by different oxidative pretreatments are shown in Table 1. Relatively high solid yields were obtained for all of these pretreatments. Particularly, SC pretreatment obtained solid yield as high as 80%. This was mainly because the pretreatments were performed under mild condition, and thus the solubilization of hemicelluloses (xylan) was low, while the degradation of glucan (cellulose) was even much lower. AP and APAA pretreatments removed about 40% of hemicelluloses, while glucan solubilization was lower than 6%. SC pretreatment obtained

Table 1 Chemical compositions of pretreated sugarcane bagasse by different oxidative pretreatments

	Solid yield (%)	Glucan (%)	Xylan (%)	Lignin (%)	Acetyl group (%)	Glucan removal (%)	Xylan removal (%)	Lignin removal (%)
Raw bagasse	100	41.3 ± 1.3	25.9 ± 0.9	24.0 ± 1.2	2.40 ± 0.3	0	0	0
AP	60.2 ± 1.5	64.8 ± 0.1	24.5 ± 0.004	5.1 ± 0.2	1.13 ± 0.03	5.55 ± 0.21	43.1 ± 0.01	87.2 ± 2.7
APAA	57.5 ± 1.7	70.5 ± 1.2	26.5 ± 0.9	1.15 ± 0.08	0.35 ± 0.07	4.01 ± 1.8	40.4 ± 2.5	97.2 ± 3.6
SC (2 h)	80.0 ± 0.8	47.5 ± 1.02	29.5 ± 0.2	2.80 ± 0.1	0.19 ± 0.01	7.99 ± 1.97	8.88 ± 0.62	90.7 ± 5.8

much less removal of xylan (< 9%), which was the major reason why it could obtain much higher solid yield. However, all of these pretreatments could obtain very high extent of lignin removal with degree of delignification (*DD*) of 87–98%. APAA pretreatment obtained the highest *DD* of 97.2% with lignin content in the pretreated substrate of only 1.15%. AP pretreatment retained a relatively high lignin content in the pretreated substrate (5.1%), which was primarily due to the mildest pretreatment condition (35 °C). However, the results definitely indicated that these pretreatments could achieve highly selective delignification, especially for the SC pretreatment.

In terms of the chemistry of delignification, AP pretreatment to remove lignin is primarily due to the generation of hydroxyl radicals ($OH^{\bullet}$) and superoxide ($O_2^{-\bullet}$) by reaction between hydroperoxide anion and H_2O_2, which play the most important roles for delignification (Dutra, Santos et al. 2018). Oxidation of the side chain structures and cleavage of aryl-ether bonds (mainly β-O-4) in lignin are the major reactions resulting in the depolymerization of lignin macromolecule (Li et al. 2012; Mittal et al. 2017). PAA delignification is mainly mediated by the formation of hydroxonium ion, HO^{+}, from PAA in acidic media which attacks the electron sites of lignin, causing electrophilic substitution with ring hydroxylation, oxidative demethylation, oxidative ring-opening, displacement of side chains, cleavage of β-aryl ether bonds and epoxidation of olefin structure (Zhao et al. 2017a). SC pretreatment to remove lignin is ascribed to the oxidation action of chlorine dioxide formed from SC under acidic condition to achieve depolymerization of lignin, ring-opening and oxidation of ring-conjugated structures (Gierer 1986). By these oxidative actions, lignin becomes depolymerized and hydrophilic, and thus is removed from the cell wall.

Enzymatic hydrolysis of pretreated substrates for release of sugars

Comparison of different commercial cellulase cocktails

The enzymatic hydrolysis of the pretreated substrates was analyzed and compared by using different commercial cellulase cocktails with the same cellulase loading (15 FPU/g solid). As shown in Fig. 1 for the time courses of glucan and xylan conversion, the pretreated substrates showed greatly enhanced enzymatic digestibility than the raw (untreated) bagasse. Although the xylan content in the pretreated substrate was high (24–30%), relatively high glucan conversion (76–95%) was still obtained for all of the samples. However, there were still some differences for glucan conversion depending on cellulases cocktails used and pretreatment approach. For example, AP, APAA and SC pretreatment obtained glucan conversion of 91.4%, 94.3% and 87.5%, respectively, when CTec 2 was used, while corresponding glucan conversion were 83.9%, 80.3% and 76.2%, respectively, when Habio cellulases cocktail was used. The glucan conversion for other cellulase cocktails was in the above range. This difference might be ascribed to the different contents of the cellulase components which affected their synergism for cellulose hydrolysis. Moreover, other components such as xylanase and protein stabilizer might be also different in these enzyme formulations, and therefore the glucan conversion definitely might be different. However, the above results indicated that the negative effects of hemicelluloses on glucan conversion seems to be limited, because the glucan conversion of AP-pretreated sample was similar to or just somewhat higher than that of SC-pretreated sample, though the xylan content of the former substrates was lower by 5%. Chen et al. (2016) prepared samples with different xylan contents by sulfuric acid hydrolysis plus SC oxidative delignification. They similarly found that removing hemicellulose could improve the initial enzymatic hydrolysis rate of cellulose, but the final glucan conversion for a relatively long time incubation was not significantly affected by xylan content when a considerable part of lignin had been removed.

In terms of the xylan conversion during enzymatic hydrolysis, the results indicated that these pretreatments showed significant difference on both the rate of xylan hydrolysis and the final xylan conversion. However, the same trend of xylan digestibility was observed no matter what cellulase cocktail was used, namely APAA > AP > SC. The final xylan conversion was in the range of 50–100% depending on the pretreatment and cellulase cocktail

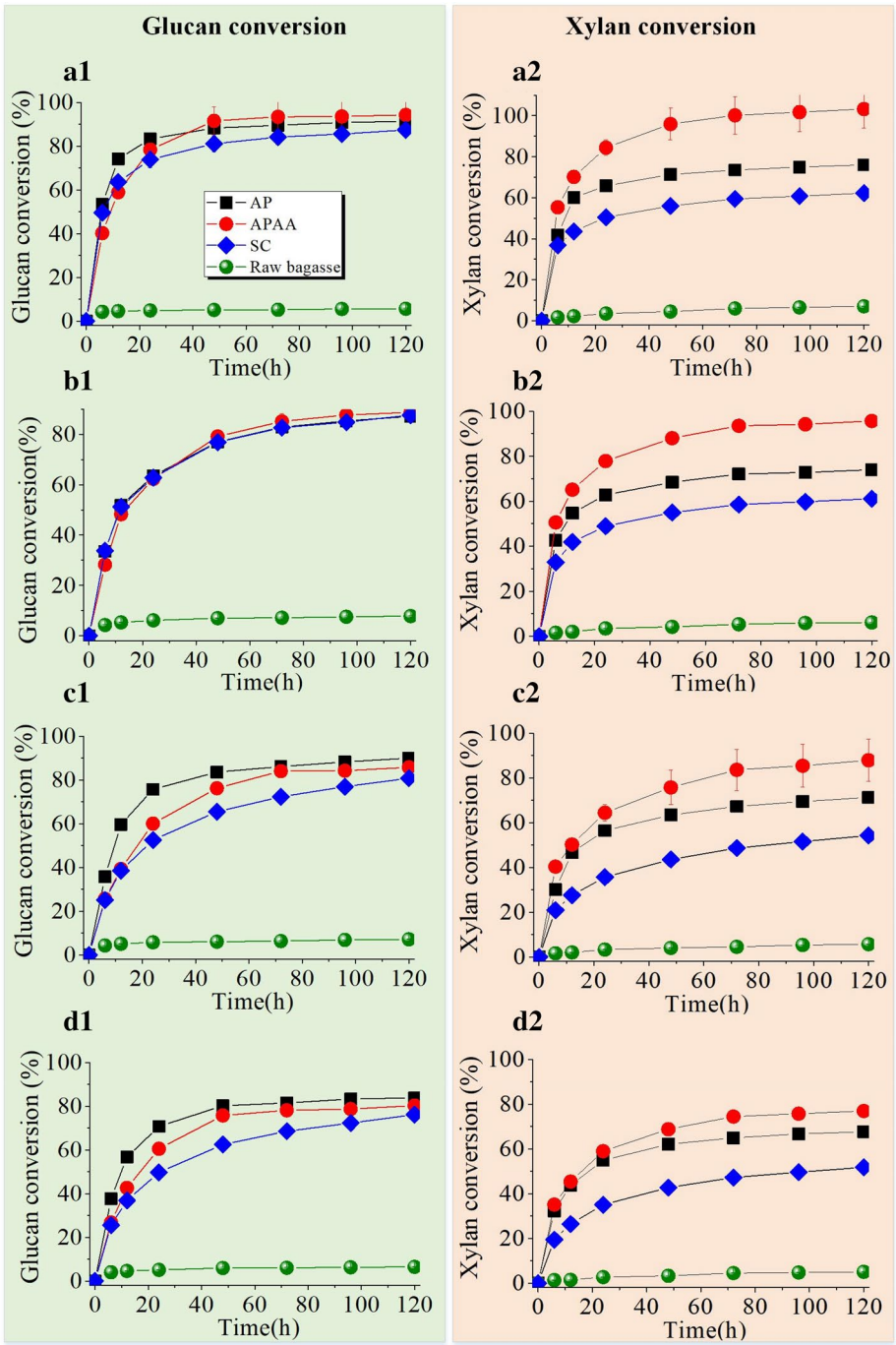

Fig. 1 Enzymatic hydrolysis of several oxidative pretreatment of sugarcane bagasse for cellulose and xylan conversion to glucose and xylose. a1 and a2: CTec 2 cellulase cocktail; b1 and b2: Celluclast 1.5L cellulase cocktail; c1 and c2: Vland cellulase cocktail; d1 and d2: Habio cellulase cocktails. Cellulase loading was 15FPU/g solid; solid loading was 2% (w/v, mg/ml)

used. APAA pretreatment achieved the highest xylan conversion, which might be due to the lowest lignin content in the pretreated substrate. For APAA-pretreated substrates, the highest xylan conversion (~100%) was achieved by using CTec 2 cellulase, while the lowest xylan conversion (75%) was obtained by using Habio cellulase. Similarly, for SC-pretreated substrates, CTec 2

obtained the highest xylan conversion, while Habio cellulase obtained the lowest, but the final (120 h) xylan conversion decreased to 62% and 52%, respectively. The above results suggested that cellulase cocktails showed great influence on the xylan conversion. This was primarily because the xylanase activity in the cellulase cocktails was different, and thus the degree of xylan hydrolysis was different. However, the above results also revealed that once a great part of lignin had been removed, high xylan conversion could be obtained by using cellulase cocktails containing xylanase activity, even if the xylan content in the pretreated substrate was higher than that in raw bagasse.

Effects of cellulase loading on polysaccharide conversion

The pretreated substrates were further estimated for their enzymatic digestibility with different cellulase loading of CTec 2 cellulase cocktail. As shown in Fig. 2, for the kinetic curves of glucan and xylan conversion, increasing cellulase loading from 5 FPU/g solid to 10% FPU/g solid significantly increased the rate of glucan conversion. However, further increasing cellulase loading to 15 FPU/g solid somewhat increased the initial hydrolysis rate, but the final glucan conversion did not change significantly for AP and APAA-pretreated substrates. The results corroborated again the superior cellulose digestibility of AP and APAA-pretreated substrates. The glucan conversion could reach about 80% at cellulase loading of 5 FPU/g solid, and higher than 90% at cellulase loading of 10 FPU/g solid. However, cellulase loading showed

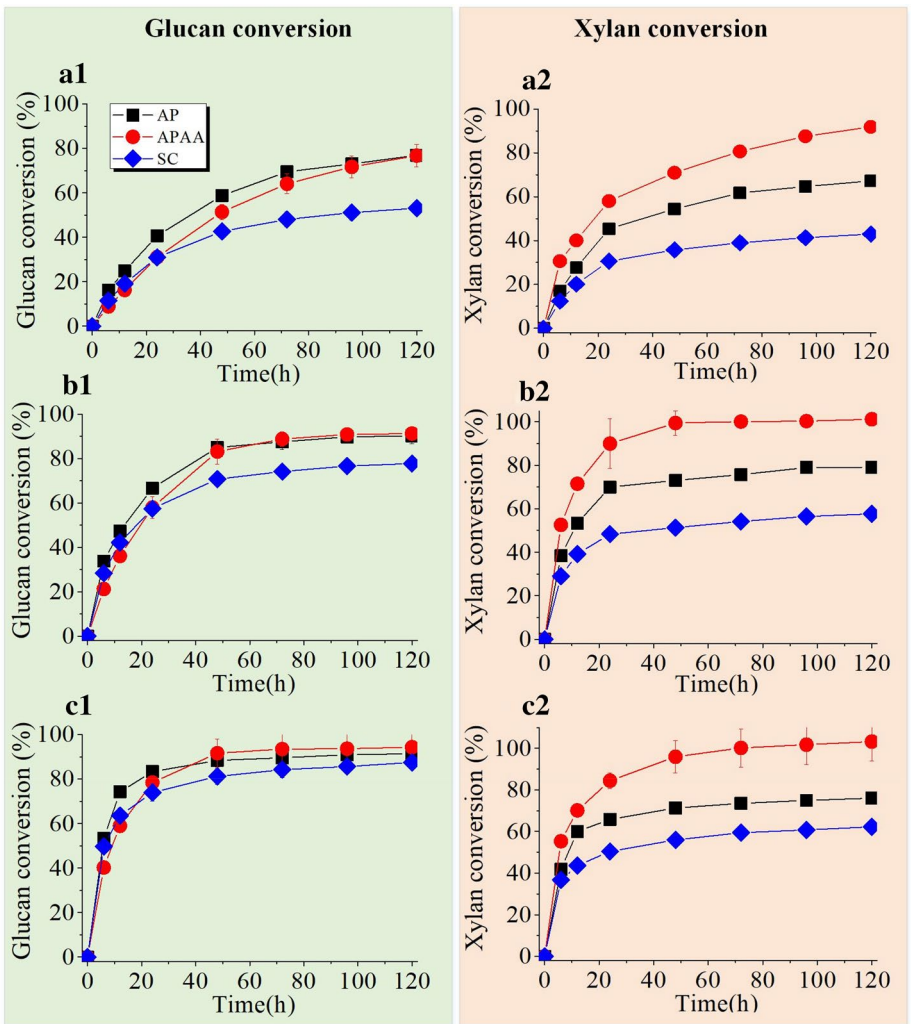

Fig. 2 Effects of cellulase loading on glucan and xylan conversion of substrates obtained by different oxidative pretreatments. a1 and a2: 5 FPU/g solid; b1 and b2: 10 FPU/g solid; c1 and c2: 15 FPU/g solid. The cellulase used was CTec 2 cellulase cocktail

significant effects on both the hydrolysis rate and final extent of glucan conversion for SC-pretreated bagasse. For example, the 120 h glucan conversion at 5, 10 and 15 FPU/g solid was 53.1 ± 2.8%, 77.8 ± 0.3% and 87.5 ± 2.3%, respectively.

Similar trend of the effect of cellulase loading on xylan conversion was also observed. The rate of xylan hydrolysis for AP and APAA-pretreated substrates increased apparently when cellulase loading increased from 5 FPU/g solid to 10 FPU/g solid. However, the final xylan conversion was not improved when CTec 2 cellulase loading increased to 15 FPU/g solid. APAA-pretreated substrates obtained the highest xylan conversion at any cellulase loading, while SC-pretreated solid had the lowest one. Cellulase loading also showed the most significant influence on the hydrolysis of xylan in SC-pretreated substrates. The 120-h xylan conversion increased from 43.0 ± 1.1% at cellulase loading of 4 FPU/g solid to 57.7 ± 0.4% at 10 FPU/g and 62.3 ± 1.1% at cellulase loading of 15 FPU/g, respectively.

Although a high *DD* was obtained by these oxidative pretreatments, there still was some lignin retained in the pretreated substrates, which probably might cause non-productive adsorption of cellulase, leading to the decrease in the observed glucan and xylan conversion. Therefore, 5 g/L non-ionic surfactant Tween 20 was added to the system to see whether the glucan and xylan conversion could be promoted. As shown in Additional file 1: Figure S1, addition of Tween 20 could well increase the glucan and xylan conversion for AP and SC-pretreated bagasse. However, for APAA-pretreated substrate, addition of Tween 20 oppositely decreased the glucan and xylan conversion. This was probably because the AP and SC-pretreated sample had relatively higher residual lignin content, while APAA pretreatment retained only a little lignin in the pretreated solid, and the obtained solid was nearly pure polysaccharide. As found by Chen et al. (2018), the improving action of Tween-20 on the enzymatic hydrolysis of pretreated lignocellulose was greatly dependent on the pretreatment process and enzymatic hydrolysis conditions. However, for hydrolysis of pure cellulose, addition of 5 g/L non-ionic surfactant could lead to decrease in glucose yield primarily due to the interaction between surfactant and cellulase proteins that reduced the productive adsorption of cellulase on cellulose substrates (Zhou et al. 2015).

It should be noted that all of these oxidative pretreatments removed considerable part of lignin, and the content of the residual lignin was low. However, there were still some differences in the enzymatic digestibility of the pretreated substrates, especially for the xylan conversion. This was because the enzymatic digestibility of cell wall polysaccharides was not only affected by the chemical compositions of the substrates, but also greatly affected by the associated physical structure. Therefore, the structure change caused by these oxidative pretreatments should be further interpreted and compared.

Structural features of the pretreated substrates

The pretreated substrates were first characterized by FTIR to study the change of functional group. As shown in Fig. 3a, all of the samples showed typical FTIR spectra of cellulose, as the bands for O–H stretching at 3409-3450 cm^{-1}, C–H stretching at 2915 cm^{-1}, the HCH and OCH in-plane bending vibrations at 1430 cm^{-1}, C–H deformation vibration at 1375 cm^{-1}, C–O–C asymmetric vibration at 1155 cm^{-1}, glucose ring asymmetric stretching at 1108 cm^{-1}, C–O stretching at 1010 cm^{-1} and C–O–C stretching at β-glycosidic linkages between glucose at 898 cm^{-1} (Oh et al. 2005; Zhao et al. 2010) were clearly observed. The strong peak at 1640 cm^{-1} was primarily ascribed to the O–H bending of the adsorbed water. Compared with the pretreated substrates, the raw bagasse showed clear bands at 1602, 1510 and 835 cm^{-1} which were assigned to the aromatic skeletal vibrations and aromatic C–H out-of-plane bending typically for syringyl moieties of lignin (Ronnols et al. 2015). However, these bands became greatly weakened or disappeared after oxidative pretreatment, indicating the high degree of delignification. Raw bagasse and SC-pretreated samples showed apparent band at 1730–1740 cm^{-1}, which was ascribed to the unconjugated carbonyl stretching of the acetyl group linking with hemicelluloses. However, AP and APAA-pretreated samples showed very weak signal at this band, indicating that deacetylation took place during the pretreatment. This was because of the saponification action of alkalis causing the hydrolytic lavage of the acetyl group. Acetyl group has been considered as another important factor limiting the enzymatic hydrolysis of cellulose, because it can interfere with enzyme recognition thereby reducing the productive adsorption of the cellulases (Zhao et al. 2012a). The high degree of deacetylation for AP and APAA pretreatment also might be a reason for the better enzymatic digestibility of the pretreated substrates than SC-pretreated sample.

To investigate whether the oxidative pretreatment could alter the cellulose crystallinity and polymorph, X-ray diffraction (XRD) diagrams were recorded as shown in Fig. 3b. It is clear that all the samples showed typical XRD diagrams of cellulose I, namely the natural cellulose. It indicated that these oxidative pretreatments did not change the polymorph of cellulose. The crystallinity indexes (*CrI*s) of the samples were determined as 44.7%, 55.2%, 52.7% and 44.6% for raw bagasse, AP, APAA and SC-pretreated substrates, respectively. One of the major reasons for the increase in the *CrI*s of AP and

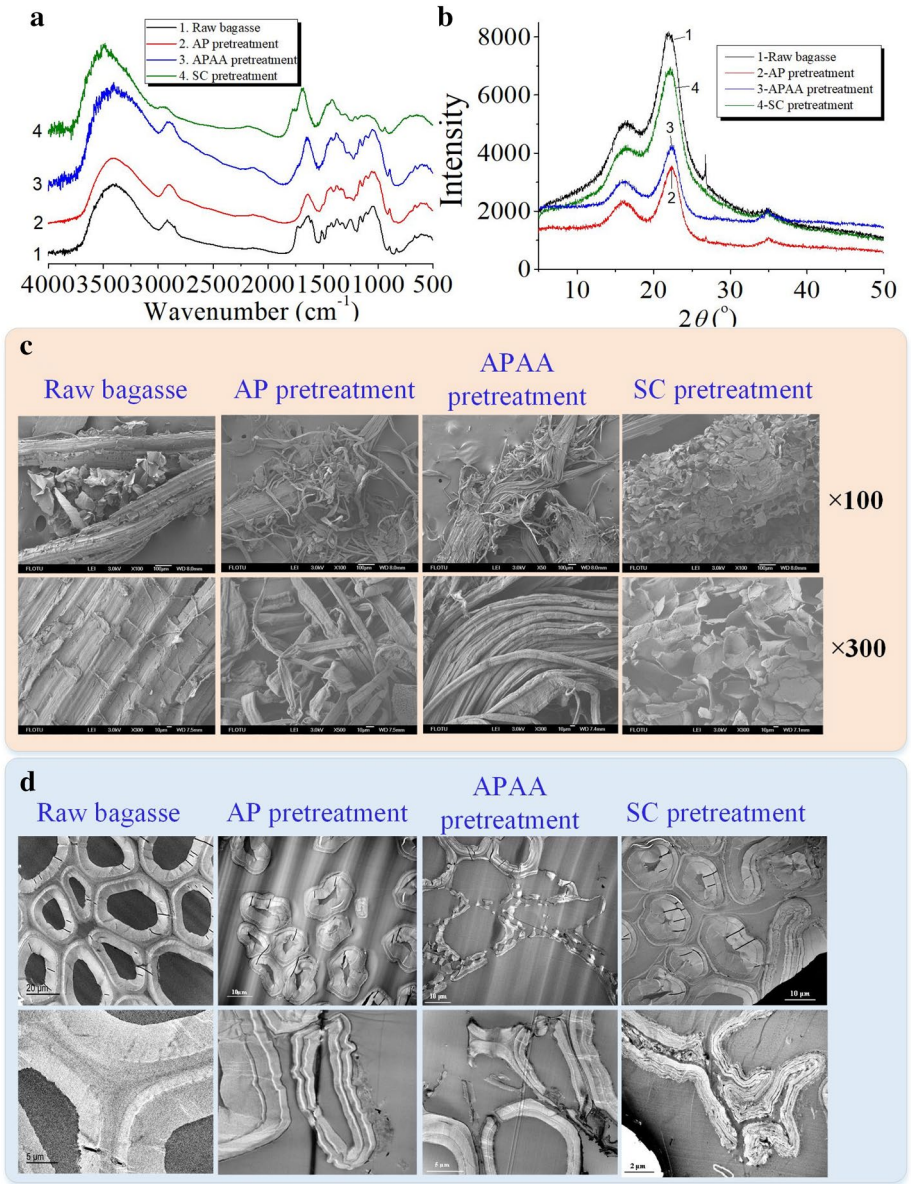

Fig. 3 Structure change of sugarcane bagasse after different oxidative pretreatment. **a** FTIR; **b** XRD; **c** SEM; **d** TEM (The upper line images were about 2000-time magnification; the lower line images were 6000–10,000-time magnification.)

APAA-pretreated sample was the removal of amorphous fraction especially lignin that resulted in the increase in cellulose content. However, the APAA-pretreated samples had lower *CrI* than AP-pretreated one, though it had a higher cellulose content. This was probably because the oxidative action of PAA might convert a part of the disordered region in the cellulose chains to crystalline structure (Zhao et al. 2008c). The SC-pretreated samples showed similar *CrI* with the raw bagasse; however, SC pretreatment indeed removed a considerable part of lignin. Therefore, the oxidative action might also have resulted in the change of cellulose crystallinity. This speculation should be further confirmed.

The surface morphology and cell wall structure of the raw bagasse and pretreated samples were observed by SEM and TEM, respectively. As shown in Fig. 3c, the raw bagasse showed relatively smooth and compact surface with no porous structure. After AP and APAA pretreatment, the sugarcane bagasse was greatly deconstructed with fibers clearly observed. This was attributed to the high degree of delignification that could liberate cellulose fibers from the cell wall. For SC pretreatment, no fibers

were observed, but the substrate surface became much coarser. At a higher magnification, thin petaloid flakes were clearly observed. Definitely, this alteration on the surface morphology could increase the cellulose accessibility for enzymatic hydrolysis. The TEM images shown in Fig. 3d further corroborated the deconstruction of cell wall structure. The raw bagasse showed regular and tightly cohered cell wall. Multi-layered structure with middle lamella (ML), primary wall (P) and second wall (S) could be clearly observed at a higher magnification (the first image in the second line of Fig. 3d). After AP pretreatment, clearly liberation of the cell wall was observed, and the cell wall underwent deformation and fracture. Even more serious etching, deformation and fracture of cell wall was observed for APAA pretreated substrates. SC pretreatment also caused great deformation of cell wall with disappearance of some middle lamella. As observed by Ding et al. (2012), acid chlorite treatment of corn stover effectively removed lignins in the second wall and the warty layer in the second wall, thereby exposing microfibrils to enzyme access. However, in this work, some cell walls were found to still "cohere" together, but more pores were formed in the cell wall which might provide access for cellulase enzymes. These results could provide interpretation on the reason why APAA pretreatment achieved the highest glucan and xylan conversion.

Confirmation of the improvement of cellulose hydrolysis by oxidative delignification with sodium chlorite

The above results demonstrated that oxidative delignification could greatly improve cellulose digestibility. However, AP and APAA pretreatment also remove about 40% hemicelluloses though the *DD* was as high as 90%. SC pretreatment obtained much higher selectivity for delignification. Therefore, to further confirm the contribution of delignification to increase cellulose hydrolyzability, SC pretreatment was performed for different time to obtain different *DD* but remaining more than 90% of hemicellulose un-removed. As shown in Table 2, SC pretreatment for 0.25–2 h indeed could obtain samples with varied lignin content with *DD* of 59.5–90.7%. The solid recovery was higher than 80%. The glucan and xylan solubilization were low, which again confirmed the high selectivity of SC pretreatment for delignification. One of the reasons for the high selectivity of SC delignification relied on the presence of lignin, because lignin has more electron-rich structures and SC may more preferably react with lignin rather than degrade cellulose. The work of Hubbell and Ragauskas (2010) also supported this conclusion as they found that Avicel cellulose samples showed a degree of polymerization (*DP*) reduction of nearly 5% with no addition of lignin during acid–chlorite delignification in contrast to a 1% drop in DP with addition of 30% lignin.

According to the experimental results on the kinetic curves of glucan and xylan conversion during enzymatic hydrolysis, it was apparently observed that both glucan and xylan conversion were greatly improved with the increase in *DD*. The relationship between glucan or xylan conversion and *DD* was further plotted as shown in Fig. 4. The initial hydrolysis rate represented as the conversion at 6 h (@6 h) and final conversion represented as the conversion at 120 h (@120 h) obtained by different cellulase cocktails were compared. The used cellulase cocktails showed clear difference for the glucan and xylan conversion@6 h, while the difference became smaller for conversion@120 h. CTec 2 cellulase was the robustest with the highest conversion@6 h; however, no significant difference was found for CTec 2 and Celluclast for conversion@120 h. The enzymatic conversion of glucan and xylan generally increased with *DD* in a linear relationship, illustrating that removing lignin indeed could dramatically improve both glucan and xylan conversion. The glucan conversions@120 h with CTec 2 cellulase cocktails reached 41.1 ± 2.3%, 49.4 ± 0.8%, 62.4 ± 1.0% and 89.1 ± 1.6% at *DD* of 59.5%, 61.9%, 73.3% and 90.7%, respectively. Corresponding xylan conversions@120 h were 37.1 ± 5.7%, 40.6 ± 1.2%, 52.1 ± 5.7% and 67.3 ± 5.0%, respectively.

The structures of SC-delignified substrates were further characterized as shown in Fig. 5. The FTIR spectra (Fig. 5a) confirmed the removal of lignin as the bands at 1602, 1510 and 835 cm^{-1} became greatly weakened. The intensity of the band at 1735 cm^{-1} decreased for SC pretreatment for 0.25–0.5 h, but increased for pretreatment

Table 2 Chemical compositions of sodium chlorite-pretreated sugarcane bagasse for different time

SC pretreatment time (h)	Solid yield (%)	Glucan (%)	Xylan (%)	Lignin (%)	Acetyl group (%)	Glucan removal (%)	Xylan removal (%)	Lignin removal (%)
0 (raw bagasse)	100	41.3 ± 1.3	25.9 ± 0.9	24.0 ± 1.2	2.40 ± 0.3	0	0	0
0.25	87.6 ± 0.9	44.8 ± 1.7	27.2 ± 0.3	11.1 ± 0.2	1.06 ± 0.03	4.98 ± 0.54	8.00 ± 1.10	59.5 ± 0.8
0.5	87.1 ± 1.2	46.6 ± 2.3	28.5 ± 0.9	10.5 ± 0.8	1.08 ± 0.08	1.72 ± 0.89	4.16 ± 3.12	61.9 ± 1.3
1	83.2 ± 1.5	47.1 ± 0.1	28.6 ± 0.1	7.7 ± 0.4	1.13 ± 0.01	5.12 ± 0.23	8.13 ± 0.27	73.3 ± 7.0
2	80.0 ± 0.8	47.5 ± 1.0	29.5 ± 0.2	2.8 ± 0.1	1.13 ± 0.03	7.99 ± 1.97	8.88 ± 0.62	90.7 ± 5.8

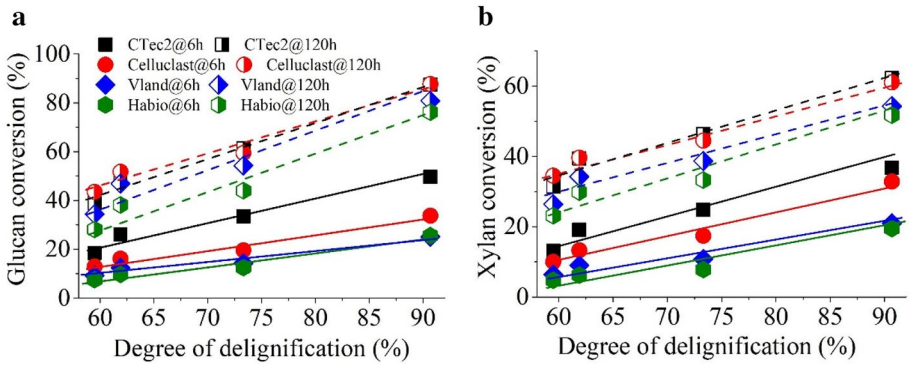

Fig. 4 Relationship between enzymatic conversion of glucan or xylan and degree of delignification when the pretreated substrates were hydrolyzed by different cellulase cocktails. **a** Glucan conversion; **b** xylan conversion

for 1–2 h, suggesting the occurrence of deacetylation and oxidation of the substrates. No other significant difference was observed. XRD diagrams (Fig. 5b) illustrated that cellulose polymorph was not altered by SC delignification. However, the *CrI* was found to somewhat decrease with the increase in SC pretreatment time, being 47.1%, 45.6%, 44.7% and 44.6% for SC pretreatment of 0.25, 0.5, 1.0 and 2.0 h, respectively, but such difference actually was not significant statistically. To further confirm whether cellulose crystallinity could be altered by SC treatment, filter paper as a representative pure cellulose was treated by SC for 0.25–2 h. XRD diagrams shown in Additional file 1: Figures S2A further confirmed that SC treatment did not change the cellulose polymorph. The *CrI* of untreated filter paper was 81.1%, but after SC treatment for 0.25, 0.5, 1 and 2 h, *CrI* changed to 78.6%, 83.2%, 82.4 and 80.4%. This difference actually was not significant, suggesting that SC treatment did not dramatically change cellulose crystallinity. Enzymatic hydrolysis of treated filter paper (Additional file 1: Figure S2B) also showed that SC oxidative treatment of filter paper under the employed condition did not change the rate and final extent of glucan conversion. Similar conclusion was obtained by Park et al. (2015) when *a*-cellulose was subjected to oxidative pretreatment.

In terms of the surface morphology of the SC-delignified substrates, SEM images (Fig. 5c) showed that the bagasse surface become coarser and coarser as *DD* increased. TEM images (Fig. 5d) also revealed that the cell wall underwent etching and fracture, while at a high *DD* some middle lamella disappeared and cell wall was significantly deformed. However, most of the cell walls still looked like being adhering together, probably because the presence of hemicelluloses that played as "iron wires" to bundle the cellulose fibers. Mechanical beating could be used to further liberate the fibers. However, the above results confirmed that delignification could greatly modify the surface morphology and deconstruct cell wall structure to overcome the biomass recalcitrance for efficient polysaccharide hydrolysis. Ding et al. (2012) also concluded that ideal pretreatments should maximize lignin removal and minimize polysaccharide modification, thereby retaining the essentially native microfibrillar structure.

Lignin not only plays as physical barrier to limit cellulose accessibility to cellulose enzymes, but also non-productively adsorb proteins by hydrophobic interactions, electrostatic attractions and hydrogen-bonding interactions (Lou et al. 2013). Particularly, condensed lignin such as klason lignin may have more hydrophobic surface which is apt to adsorb more cellulase enzymes (Sun et al. 2016; Li et al. 2018). To confirm whether oxidative pretreatment could modify lignin surface and affect the non-specific adsorption of cellulases, sugarcane bagasse klason lignin was prepared and treated with SC under the same conditions for pretreatment of bagasse. The treated klason lignin was then added to the filter paper system with a loading of 4% based on cellulose weight for enzymatic hydrolysis. It was clearly observed that addition of klason lignin significantly decreased filter paper hydrolysis with final enzymatic conversion decreasing by about 30% (Additional file 1: Figure S3). However, after SC oxidative pretreatment, the inhibitive action of klason lignin was eliminated. The rates and final extents of glucan conversion were similar to those of control (no addition of klason lignin). This result strongly confirmed that oxidative pretreatment also caused modification of lignin surface which might greatly decrease the hydrophobic interaction thus reducing non-specific adsorption of lignin. Reduced cellulase adsorption was also reported

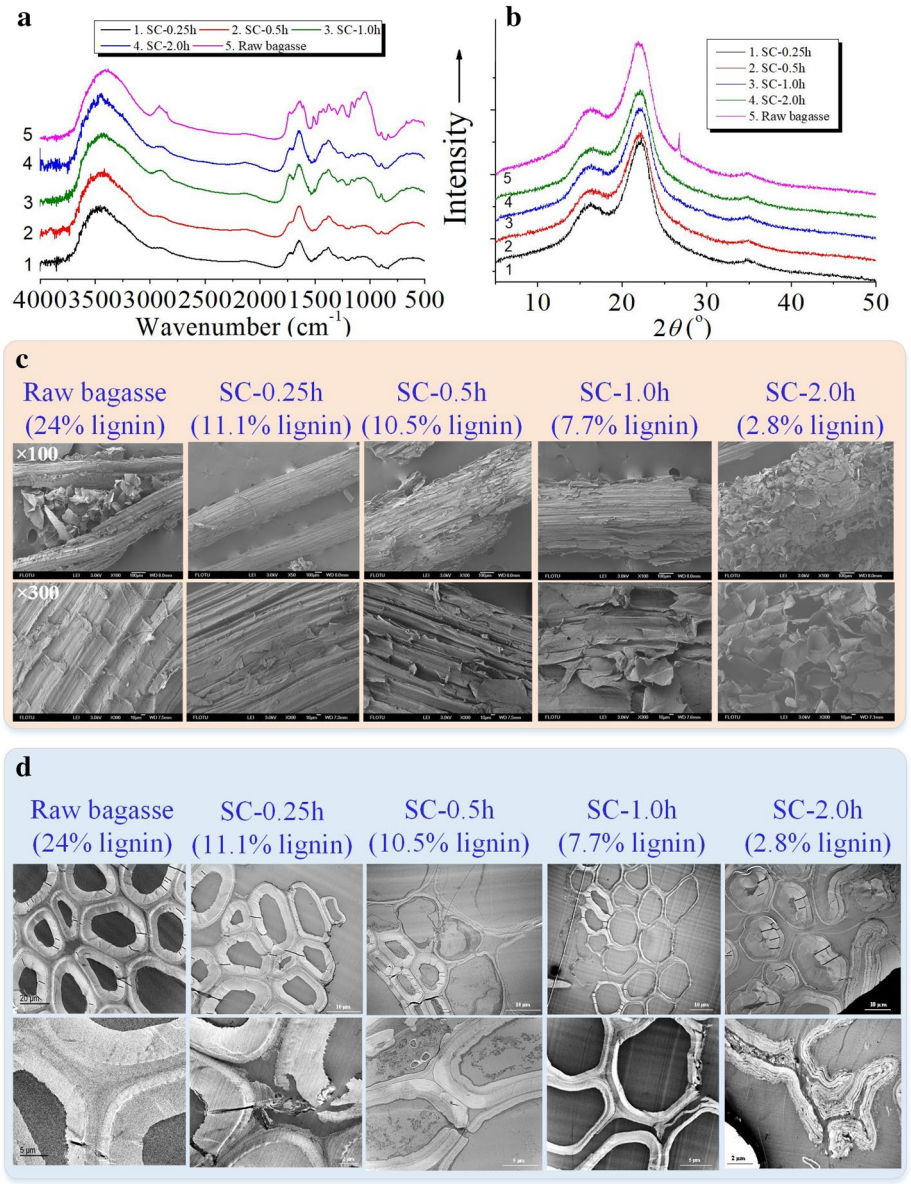

Fig. 5 Structure change of sugarcane bagasse after sodium chlorite (SC) pretreatment for different time. **a** FTIR; **b** XRD; **c** SEM; **d** TEM (The upper line images were about 2000-time magnification; the lower line images were 6000–10,000-time magnification)

when alkaline lignin was treated by Fenton oxidation (Ying et al. 2018).

Various oxidative pretreatments also have corroborated the effectiveness of delignification on improving cellulose digestibility. Bhalla et al. performed alkaline pre-pretreatment followed by AP delignification with addition of $CuSO_4$ as a catalyst for pretreating hardwoods (hybrid poplar and eucalyptus). The glucose yield by enzymatic hydrolysis could be over 80% for a 72-h hydrolysis (Bhalla et al. 2019). Fernandez-Delgado et al. (2019) found that mild alkaline and oxidative pretreatment of brewer's spent grains with hydrogen peroxide efficiently improve the yield of biobutanol during ABE fermentation of the pretreated substrate. Song et al. (2019a) employed a two-stage alkalioxygen assisted liquid hot water pretreatment of lignocellulosic biomass, and found that biomass saccharification was significantly improved by selective removal and oxidative modification of lignin through the alkalioxygen pretreatment. Liu et al. (2019) obtained a high ethanol concentration (109.2 g/L) when simultaneous saccharification and co-fermentation of H_2O_2-pretreated corn stover by *Saccharomyces cerevisiae*

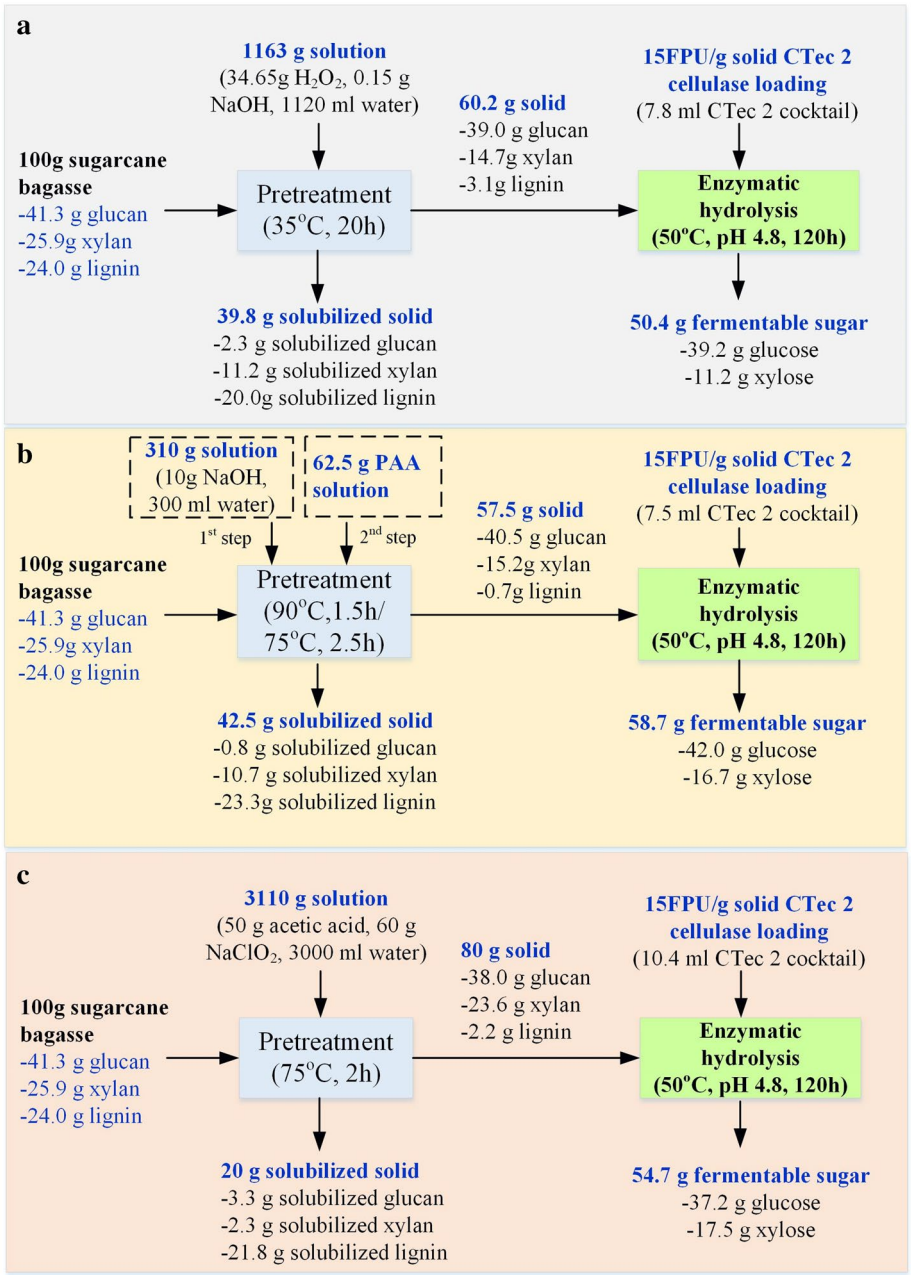

Fig. 6 Mass balance for AP, APAA and SC pretreatments of sugarcane bagasse followed by enzymatic hydrolysis with Novozymes CTec 2 for fermentable sugar production. **a** AP pretreatment; **b** APAA pretreatment; **c** SC pretreatment for 2 h; CTec 2 Cellulase loading was 15FPU/g solid; solid loading was 2% (w/v, mg/ml)

and *Candida tropicalis* due to the greatly increased cellulose digestibility. These results indicated that oxidative delignification could be an efficient approach for improving cellulose accessibility.

Mass balance and comparison of several oxidative pretreatment for fermentable sugar production

Mass balance for sugar production by oxidative pretreatment followed by enzymatic hydrolysis with CTec 2 cellulase cocktail was performed as shown in Fig. 6. For AP pretreatment, 50.4 g fermentable sugars including 39.2 g glucose and 11.2 g xylose could be obtained by enzymatic

hydrolysis from 100 g of raw bagasse. The pretreatment consumed 34.7 g H_2O_2 and 0.15 g NaOH. Usually H_2O_2 was available at 30wt% concentration, thus the consumption of 30% H_2O_2 solution was 115.7 g. APAA pretreatment could obtain 58.7 g fermentable sugar from 100 g of raw bagasse, including 42 g glucose and 16.7 g xylose. APAA pretreatment involves two steps, namely alkaline pre-pretreatment and PAA post-treatment, which consumed 10 g NaOH and 62.5 g PAA solution, respectively. The PAA solution was prepared by reaction of 34.2 g acetic acid with 27.4 g 30wt% H_2O_2 with addition of 0.4 g sulfuric acid as the catalyst. SC pretreatment obtained 54.7 g fermentable sugars/100 g raw bagasse including 37.2 g glucose and 17.5 g xylose. The process consumed 50 g acetic acid and 60 g sodium chlorite. APAA pretreatment obtained the highest fermentable sugar yield, but two-step operation made this process relatively complicated with consumption of a large amount to water to wash the alkali-treated solid prior to PAA delignification. SC pretreatment employed chlorine-containing chemicals thus the negative effects on environment should be considered. AP pretreatment seemed to be the most feasible without significant environment pollution. However, the consumption of H_2O_2 was also high. Therefore, although high sugar yield could be obtained by the above oxidative pretreatments, it seems that these processes are still far away from practical application in large scale. Although the process parameter may be further optimized to reduce the usage of the chemicals, the oxidants are still too expensive to be used for low-cost biomass pretreatment. Finding recyclable oxidants might provide a solution to reduce the pretreatment cost, which is worthy studying. However, the above results demonstrated in academic aspect that delignification under mild pretreatment condition can be very helpful to improve the enzymatic hydrolysis of cellulose by commercial cellulase cocktails even if the substrates has a high hemicellulose content. Moreover, retaining xylan in the pretreated substrates and converting it to xylose by subsequent enzymatic hydrolysis could increase fermentable sugar yield and eliminate the detoxification step. The utilization of the residual lignin or degradation products might also help to reduce the operation cost. Therefore, integrated production of multiple high value-added products in a biorefinery based on oxidative pretreatment might be a promising way to increase the process economic feasibility.

Conclusion

To investigate the "contribution" of delignification to the increase in lignocellulosic cellulose enzymatic digestibility, several oxidative pretreatments under mild conditions that showed relatively high selectivity of delignification were performed and compared in terms of the cellulose and xylan hydrolyzability as well as the substrates structures. Four commercial cellulase cocktails were employed for hydrolysis of the pretreated solids. The results revealed that delignification indeed could greatly improve cellulose hydrolysis and relatively high glucan conversion could be obtained though there was some difference for the hydrolysis rate by different cellulase cocktails. Alkaline peroxide and alkaline/peracetic acid pretreatments resulted in the disappearance of middle lamella and liberation of cellulose fibers with significant etching, deformation and fracture of cell wall structure, thus significantly increasing cellulose accessibility. SC pretreatment greatly modified the sugar bagasse surface morphology. The surface became much coarser as the degree of delignification increased. The cell wall underwent more serious fracture and deformation with some middle lamella getting disappeared at higher degree of delignification. However, SC treatment did not change the polymorph and crystallinity of pure cellulose (filter paper). Nevertheless, SC oxidative pretreatment might cause modification of lignin structure and surface properties and thus reducing nonspecific absorption of enzyme proteins. The above results supported the conclusion that delignification under mild pretreatment condition can be very helpful to improve the enzymatic hydrolysis of cellulose by commercial cellulase cocktails even if the substrates has a high hemicellulose content. However, these oxidative pretreatments consumed a relatively large amount of non-recyclable oxidants, and thus they were too expensive for practical application in large scale.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-020-00312-y.

Additional file 1: Figure S1. Effects of 5 g/L Tween on enzymatic hydrolysis of different oxidative pretreatment of sugarcane bagasse. Cellulase loading was 15 FPU/g solid. **Figure S2.** XRD diagrams (A) and enzymatic hydrolysis (B) of filter paper treated by sodium chlorite (SC) for different time.

Abbreviations

AP: Alkaline-hydrogen peroxide pretreatment; APAA: Alkaline-peracetic acid; *CrI*: Crystallinity index; *DD*: Degree of delignification; DP: Degree of polymerization; FPU: Filter paper unit; FTIR: Fourier transform infrared spectroscopy; HP: Hydrogen peroxide; LCC: Lignin–carbohydrate complex; PAA: Peracetic acid; SC: Sodium chlorite; SEM: Scanning electron microscope; TEM: Transmission electron microscope; XRD: X-ray diffraction.

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Authors' contributions
YH carried out all of the experiments; YC carried out most of the data analysis; JZ carried out a part of data analysis and provided project funds for the work; DL provided supervision and funds for the work; XZ conceived the work, wrote the manuscript and made revision, provided supervision and funds for the work. All authors read and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article and its Additional file 1.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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