Alkali-based pretreatments distinctively extract lignin and pectin for enhancing biomass saccharification by altering cellulose features in sugar-rich Jerusalem artichoke stem

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Abstract

Jerusalem artichoke (JA) has been known as a potential nonfood feedstock for biofuels. Based on systems analysis of total 59 accessions, both soluble sugar and ash could positively affect biomass digestibility after dilute sodium hydroxide pretreatment (A). In this study, one representative accession (HEN-3) was used to illustrate its enzymatic digestibility with pretreatments of ultrasonic-assisted dilute sodium hydroxide (B), alkaline peroxide (C), and ultrasonic-assisted alkaline peroxide (D). Pretreatment D exhibited the highest hexose release rate (79.4%) and total sugar yield (10.4 g/L), which were 2.4 and 2.6 times higher, respectively, than those of the control. The analysis of cellulose crystalline index (CrI), cellulose degree of polymerization (DP), thermal behavior and SEM suggested that alkali-based pretreatments could distinctively extract lignin and pectin polymers, leading to significant alterations of cellulose CrI and DP for high biomass saccharification. Additionally, hydrogen peroxide (H₂O₂) could significant reduce the generation of fermentation inhibitors during alkali-based pretreatments.

Key words: *Helianthus tuberosus* L., alkali-based pretreatments, cellulose CrI, cellulose DP, fermentation inhibitor.

1. Introduction

Lignocellulosic biomass, such as herbaceous energy plants, has been well known as a promising feedstock for biofuel production due to its unlimited geographic distribution, low CO₂ emission, and low cost (Ragauskas et al., 2006; Himmel et al., 2007). The herbaceous plant Jerusalem artichoke (*Helianthus tuberosus* L.) can grow well on non-fertile land and possesses high tolerance to frost and most pests and diseases that usually attack traditional crops (Gunnarsson et al., 2014). As an important commercial crop, it can be cultivated to harvest tubers for inulin and biofuel production. A large amount of stem can be harvested as a biomass feedstock for biofuel as well. Although the utilization of its tuber in biorefinery has been reported briefly, few applications of stems for biofuel are published nowadays (Caserta and Cervigni, 1991; Kim et al., 2013). The main components of JA stem are soluble sugar, degradable lignocellulose and ash (Caserta and Cervigni, 1991). Despite many studies have been conducted to evaluate the chemical components of different accessions (Kim and Kim, 2014; Gunnarsson et al., 2014; Liu et al., 2015), little is known about the effects of chemical components on biomass saccharification, in particular on JA. (Jia et al., 2014; Li et al., 2014; Wu et al., 2014; Huang et al., 2015).

As a kind of dicotyledonous herb, the cell wall of JA stem is mainly composed of cellulose, hemicelluloses, lignin and pectin (Gunnarsson et al., 2014). Cellulose is an unbranched b-1,4-glucans, and cellulose CrI and cellulose DP have been characterized as the key factors negatively affecting biomass enzymatic digestibility in many plants

(Zhang et al., 2013; Jia et al., 2014; Li et al., 2014; Huang et al., 2015). Hemicellulose is a class of heterogeneous polysaccharides with various monosaccharides, and can be effectively extracted using different concentrations of alkali that dissociates the hydrogen bonds of wall polymers (Jia et al., 2014; Li et al., 2014; Si et al., 2015). Lignin is a hydrophobic polymer that has been reported as the negative factor in biomass enzymatic digestions, and the effective lignin extraction could lead to a significant alteration on cellulose CrI for enhancing biomass saccharification (Xu et al., 2012; Li et al., 2014; Si et al., 2015). Pectin is a kind of fermentable polysaccharide (mainly composed of galacturonic acid) accounting for approximately 10% in JA stem which could enhance the bioethanol potential of this type of biomass (Gunnarsson et al., 2014). It has been reported that ammonium oxalate-extractable pectin could significantly enhance biomass enzymatic digestibility by reducing lignocellulose CrI in *Miscanthus* sp. (Wang et al., 2015), but much remains unknown in different plants. As a promising solution, genetic modification of plant cell walls has been proposed for reducing recalcitrance in bioenergy plants (Li et al., 2014; Wang et al., 2015). Hence, it becomes essential to identify the effects of lignin and pectin on biomass digestibility under various pretreatments in JA stems.

Lignocellulosic recalcitrance in nature fundamentally requires a costly pretreatment process involving numerous compounds that inhibit bioethanol conversion. Over the last two decades, various pretreatment methods have been developed and alkali-based pretreatments, e.g. those employing sodium hydroxide (NaOH), are

extensively used in biomass saccharification process. NaOH could effectively reduce lignin level in most crop residue and show less sugar degradation and furan derivatives formation than acid pretreatments, but is less satisfactory for processing lignin-rich biomass (Chandra et al., 2007; Kim et al., 2013; Jia et al., 2014; Li et al., 2014; Wu et al., 2014). Recent studies have shown that alkaline peroxide was more effective by generating radicals, which leads to high delignification (Kang et al., 2011). Ultrasonic irradiation has been increasingly considered as an effectively method to assist in the pretreatment of lignocellulosic biomass with different chemical solutions by producing cavitation (Suslick et al., 1999; Velmurugan and Muthukumar, 2012; Yang and Fang, 2014). And the decomposition of water molecules into free radicals by cavitation aids in increasing the accessible surface area and cleaving the linkages between cell wall polysaccharides and lignin (Velmurugan and Muthukumar, 2012). Thus, is it possible to achieve higher pretreatment efficiency by adding the sonication of the alkaline peroxide pretreatment? Although those physical and chemical pretreatments have been used in many plants (Kang et al., 2011; Velmurugan and Muthukumar, 2012; Kim et al., 2013; Li et al., 2014; Wu et al., 2014 ; Yang and Fang, 2014), much remains unknown about their applications in JA stem. Moreover, the numerous inhibitory compounds (e.g. acetic acid, furfural, and HMF) that generated from pretreatment process still need to be investigated when JA stem is concerned (Palmqvist and Hahn-Hägerdal, 2000).

The objective of the present study was to investigate the relationship between chemical components and biomass enzymatic digestibility, and to compare the effects of

four employed pretreatments on JA biomass saccharification. Specifically, the alteration of lignocellulose structure after each pretreatment was identified by cellulose CrI and DP detection, thermal behavior distribution and surface morphology observation. In addition, the inhibitors released from four pretreatment processes were also investigated. The present study provides useful information for energy crop breeding and potential applications in JA stems and beyond.

2. Materials and Methods

2.1. Jerusalem artichoke accessions and sample preparation

A total of 59 JA accessions collected from 24 provinces in China (Liu et al., 2011) were grown at the China Agricultural University Experimental Station in Zhuozhou, Hebei in 2014. The stem sample of each accession was harvested at its physiological maturity date after a growth period of 120 to 150 days. Each sample was ground in a crusher mill into particles of size 1-2 cm. All of the particles were dried at 45 °C for 48 h after inactivation at 105 °C for 20 min, and ground into powders through a combined -40/+80 mesh screen according to Sluiter et al. (2008). The meshed samples (as dry matter) were stored in a dry container until use.

2.2. Soluble sugar and ash measurements

To determine soluble sugars in the stems of the 59 accessions, 2 g dry matter of each accession was added to 40 mL distilled water and shaken at 150 rpm for 2 h at 50 °C.

After centrifugation at 1800 g for 10 min, the residue was washed five times with 20 mL distilled water. As a result, the soluble sugars in the stem sample were extracted into the supernatant. The residue without soluble sugar (RWSS) was dried at 80 °C in an oven. Soluble sugar was determined with the supernatants by the anthrone/H₂SO₄ method and assayed in a UV–VIS spectrometer (TU-1901, Beijing PURKINJE Instruments Co., Ltd. Beijing, China) according to Li et al. (2014). The standard curve was plotted using D-glucose as a standard (purchased from Xilong Chemical Co., Ltd.). Dry matter (2 g of each) was added to 30 mL ceramic crucibles to determine ash content in a muffle furnace (VULCAN 3-550, Densply international Inc. York, USA).

2.3. Pretreatments and inhibitors extraction

RWSS from all of the 59 JA stems were pretreated with dilute sodium hydroxide (pretreatment A). A 2 g quantity of RWSS from each accession was mixed with 40 mL 2% (w/v) NaOH and shaken at 150 rpm for 2 h at 50 °C.

According to our earlier work (Liu et al., 2011; Liu et al., 2012; Liu et al., 2015), HEN-3, which considered as a most optimal JA accession for biofuels in the north of China, displayed relatively good growth and yield performance from 2008 to 2014 in China Agricultural University Experimental Station. For this reason, the stem of HEN-3 was used as an ideal accession for further study. Three different 2 g quantities of RWSS from HEN-3 stem were pretreated with ultrasonic-assisted dilute sodium hydroxide (Pretreatment B), alkaline peroxide (Pretreatment C) and ultrasonic-assisted alkaline

peroxide (Pretreatment D). For pretreatment B, RWSS was mixed with 40 mL 2% (w/v) NaOH solution and treated by ultrasonic bath (KQ500DE, Kun Shan Ultrasonic Instruments Co., Ltd, China) for 2 h at 50 °C. The operating frequency and power of the apparatus were 40 kHz and 500 W, respectively. For pretreatment C, the RWSS was mixed with 40 mL alkaline peroxide in a 50 mL centrifuge tube and shaken at 150 rpm for 2 h at 50 °C. The final concentration of NaOH and H₂O₂ in the alkaline peroxide solution was 2% (w/v) and 5% (w/v), respectively. For pretreatment D, the RWSS was mixed with 40 mL alkaline peroxide and treated by ultrasonic bath for 2 h at 50 °C. The operating frequency and power of the apparatus were 40 kHz and 500 W, respectively.

Following pretreatments A, B, C and D, the tubes were centrifuged at 3000 g for 10 min. The pretreatment supernatant was collected for inhibitor detection with a high pressure liquid chromatograph (HPLC, Shimadzu LC-20AD). The pretreated RWSS was washed five times with 20 mL distilled water and dried at 80 °C for further analysis.

2.4. Morphology observation

The pretreated and control RWSS were washed with distilled water until pH 7.0 was reached. The color and particle size observation of the RWSS were detected by digital camera (Nikon D9000, Japan). For surface morphology observation, the pretreated and control RWSS were sputter-coated with gold and observed by SEM (JSM-6390/LV, Hitachi, Tokyo, Japan). Each sample was observed for 5-10 times and

the representative images were photographed in the study.

2.5. Cellulose crystalline index

The cellulose CrI of pretreated and control RWSS were determined by X-ray diffraction (XRD) using an X-ray diffractometer (XD-2, Beijing Purkinje General Instrument Co., Ltd., China) as described by Zhang et al. (2013) with minor modifications. The mixed-well powders of RWSS were laid on the glass sample holder (35 × 50 × 5 mm) and analyzed under plateau conditions. Ni-filtered Cu Ka radiation ($\lambda = 0.15406$ nm) was generated at a voltage of 36 kV and a current of 20 mA and scanned at speed of 0.02°/s from 10° to 40°. The CrI was estimated using the intensity of the 200 peak (I_{200} , $h = 22.5^\circ$) and the intensity at the minimum between the 200 and 110 peaks (I_{am} , $h = 18.5^\circ$) as follows: $CrI = 100^\circ (I_{200} - I_{am}) / I_{200}$. I_{200} represents both crystalline and amorphous materials while I_{am} represents amorphous material. The standard error of the CrI method was detected at ± 0.05 -0.15 using five representative samples in triplicate.

2.6. Degree of polymerization

The DP of pretreated and control RWSS was meshed using the viscosity method subjected to the equation: $DP^{0.905} = 0.75 [\eta]$ (Zhang et al., 2013). All experiments were performed using an Ubbelohde viscosity meter and cupriethylenediamine hydroxide (Cuen) as the solvent at 25 ± 0.5 °C. The intrinsic viscosity was calculated by

interpolation using the USP table (USP, 2002) that lists the predetermined values of the product of intrinsic viscosity and concentration, ($[\eta] C$), for cellulose samples exhibiting relative viscosity (η_{re}) values between 1.1 and 9.9. η_{re} was calculated using the relationship: $\eta_{re} = t/t_0$, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent, respectively.

2.7. Thermal behavior measurement

Thermogravimetric analysis and differential thermogravimetry were conducted by a METTLER TOLEDO TGA/DSC 1 thermogravimetric balance. About 8 mg of pretreated RWSS and control were placed, respectively, in a ceramic sample holder and heated at 10 °C/min under an argon atmosphere (50 mL/min) in the temperature range of 35 - 600 °C.

2.8. Enzymatic hydrolysis and structural carbohydrate fractionation

A 1g quantity of pretreated RWSS and the untreated control RWSS were washed 5 times with 20 mL distilled water, and once with 20 mL mixed-cellulase reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8), respectively. The washed residue was added to 0.2% (w/w) mixed cellulases (containing β -glucanase $\geq 3.6 \times 10^4$ U, cellulase ≥ 360 U, and xylanase $\geq 6 \times 10^4$ U from Imperial Jade Bio-technology Co., Ltd) in a final volume of 40 mL in a 50 mL centrifuge tube according to Li et al. (2014). The tubes were shaken under 150 rpm at 50 °C for 48 h. Supernatants (2 mL) were withdrawn at 12, 24,

36 and 48 h and centrifuged at 3000 g for 10 min for structural carbohydrate detection.

According to the NREL LAP, cellulose, hemicelluloses, lignin and pectin were

extracted by a two-step sulfuric acid hydrolysis process (Sluiter et al., 2008).

Sugars (i.e., glucose, xylose, arabinose and galacturonic acid) and inhibitors (i.e., acetic acid, HMF and furfural) released from structural carbohydrates were analyzed using a HPLC equipped with a refractive index detector (Shimadzu RID 10A) and an Aminex HPX-87H chromatography column (Bio-Rad, 300 × 7.8 mm, particle size 9 μm). The mobile phase was 5 mM H₂SO₄ in degassed HPLC-grade water at a flow rate of 0.6 mL/min and the column temperature was 65 °C. The acid-soluble lignin and acid-insoluble lignin were measured by UV–VIS spectrometer (TU-1901, Beijing PURKINJE Instruments Co., Ltd. Beijing, China) and muffle furnace (VULCAN 3-550, Densply international Inc. York, USA), respectively.

2.9. Statistical analysis

All chemical assays were conducted in triplicate and the average values are presented as a percentage of dry weight. The cellulose was determined by the amount of glucose. Hemicellulose was calculated by the sum of xylose and arabinose contents. Lignin was calculated by the sum of acid-soluble lignin and acid-insoluble lignin contents. Pectin was determined by the galacturonic acid content. Correlation coefficients were calculated by performing Pearson rank correlation analysis using IBM SPSS Statistics 20. This analysis used mean values and coefficient of variation (CV)

were calculated from all original determinations and defined as the ratio of the standard deviation to the mean value. The removal rates were calculated as the ratios of the levels of components removed in pretreated RWSS (i.e., soluble sugar, ash, cellulose, hemicellulose, lignin and pectin) to levels of initial components in RWSS. Biomass digestibility, hexose release rate or biomass saccharification is defined by the yield (% cellulose) of hexoses released from the enzymatic hydrolysis of pretreated RWSS (Li et al., 2014).

3. Results and discussion

3.1. Diversity of soluble sugar, ash and cell wall polymers in JA stem

Total of 59 JA accessions that collected nation-wide (Liu et al., 2011), exhibited a large diverse soluble sugar level in the stem (Fig. 1I). The soluble sugar level varied from 1.4% to 32.1% with a relatively high CV of 36.6%, which approximated that in sweet sorghum stems reported in a previous study (Li et al., 2014). Meanwhile, we measured a diverse ash level ranged from 1.8% to 5.2% with a CV of 20.6%, much lower than that in corn stover (Wang et al., 2014). By contrast, JA exhibited relatively lower diversity in cell wall polymers with a CV of 8.5% for cellulose, 11.6% for hemicellulose, 9.9% for lignin and 12.5% for pectin. These results indicate that JA stem could be a quality feedstock for bioethanol production due to its higher carbohydrates and lower ash levels compared with corn stover (Wang et al., 2014).

As the total 59 JA accessions exhibited various ecological types and genetic

germplasms, we further performed a correlation analysis among the soluble sugar, ash and four major wall polymers. Firstly, we detected that soluble sugar was not significantly correlated with ash (Fig. 1II) ($p < 0.01$), indicating that soluble sugar production didn't affect ash accumulation in JA stem. Furthermore, cellulose and pectin, two major wall polysaccharides, showed significantly positive correlation with the soluble sugar at $p < 0.05$ and $p < 0.01$ (Fig. 1 II), respectively. By comparison, both hemicellulose ($p < 0.05$) and lignin ($p < 0.01$) were displayed significantly negative correlations with ash (Fig. 1III), respectively. It suggested that both soluble sugar and ash level could be major factors that affect carbon partitioning on wall polysaccharides biosynthesis.

3.2. Effects of soluble sugar and cell wall polymers on biomass digestibility

In previous studies, biomass digestibility has been widely defined in different energy plants by calculating hexose yield (% cellulose) released from hydrolysis using a crude cellulase mixture of lignocellulose after various pretreatments (Xu et al., 2012; Li et al., 2014; Jia et al., 2014; Wu et al., 2014). In the present study, the stems of 59 JA accessions exhibited diverse biomass digestibility, with hexose yield averaging 70.2% (58.6% - 84.6%) after pretreatment A (Fig. 2I). This value is close to that of maize (Jia et al., 2014), and much higher than that of wheat residue (Wu et al., 2014) and *Miscanthus* sp (Xu et al., 2012). Therefore, this result indicates that JA stem had a considerably high enzymatic digestibility and the mount of 59 accessions was qualified

to investigate the effects of chemical components on biomass saccharification.

As correlation analysis has been extensively conducted to determine the effects of cell wall components on biomass saccharification in many plants (Xu et al., 2012; Li et al., 2014; Jia et al., 2014; Wu et al., 2014; Wang et al., 2015), a further correlation analysis was performed in this study (Fig. 2 II, Fig. 2III and Fig. 2IV). Soluble sugar and ash have been considered as important traits for industrial purposes in energy plants (Gunnarsson et al., 2014; Li et al., 2014). However, little is known about their effects on biomass digestibility, especially in JA (Gunnarsson et al., 2014; Kim et al., 2014; Liu et al., 2015). As a result, soluble sugar and ash exhibited much higher correlation coefficient values at 0.653 and 0.454 at $p < 0.01$, respectively (Fig. 2 II and Fig. 2III). It's indicating that both of these two chemical components shown positive effects on biomass saccharification. By comparison, cellulose displayed a significantly negative correlation with hexose yield ($p < 0.05$), whereas neither hemicellulose nor lignin content was significantly correlated with hexose yield in the 59 JA accessions (Fig. 2IV). Hence, it was revealing that cellulose was a main cell wall polymer hindering enzymatic hydrolysis of biomass in JA stems, consistent with previous findings in other energy plants (Xu et al., 2012; Li et al., 2014; Jia et al., 2014; Wu et al., 2014). As a perennial dicotyledonous herb, JA stem contains large amounts of pectin in its cell wall. However, little is known about the effects of pectin on lignocellulose enzymatic hydrolysis (Gunnarsson et al., 2014; Wang et al., 2015). This study demonstrated that pectin displayed a significant positive correlation with hexose yield ($p < 0.01$) (Fig. 2IV). This

is consistent with the previous findings in hemicellulose-rich *Miscanthus* sp. (Wang et al., 2015), indicating that pectin positively affects lignocellulose saccharification under dilute alkaline pretreatment.

3.3 Effects of four alkali-based pretreatments on cell wall components and cellulose features

Biomass pretreatments have been developing to remove the non-cellulosic polymers and to alter lignocellulose structure for better digestibility (Ravindran and Jaiswal, 2015). In general, the pretreated RWSS was found to have a relatively lower removal rate, ranging from 14.5% to 28.0% (Table 1). The cellulose removal rate ranged between 8.4% and 15.4%, indicating that the four alkali-based pretreatments have a relatively lower effect on cellulose. By contrast, the non-cellulosic polymers (hemicellulose, lignin, and pectin) were largely co-extracted during these processes, leading to increases in porosity and accessibility of biomass particles and/or an decrease in lignocellulose structure (Chandra et al., 2007; Alvira et al., 2010; Ravindran & Jaiswal, 2015; Si et al., 2015). In particular, the RWSS after pretreatment D achieved the highest cellulose level and most non-cellulosic polymers removal, suggesting a dramatic increased cellulase accessible surface area for sequential enzymatic hydrolysis. Overall, the results indicated that alkali-based pretreatments have significant effects on non-cellulosic polymers extraction.

Cellulose has been confirmed as the main wall polymer hindering RWSS enzymatic

hydrolysis in the 59 JA accessions (Fig. 2). To understand the effects of the employed pretreatments on cellulose structure, the cellulose CrI and DP were detected in this study (Fig. 3). The cellulose CrI is used to determine the ratio of crystal region to amorphous region in cellulose, which is believed to be an important cellulose feature affecting biomass saccharification (Himmel et al., 2007; Alvira et al., 2010; Zhang et al., 2013). In this work, the cellulose CrI of RWSS increased significantly after four alkali-based pretreatments and RWSS after pretreatment D achieved the highest CrI at 62.5% (Fig. 3I). This indicated that amorphous substances (non-crystalline cellulose and non-cellulosic polymers) were largely removed by the four alkali-based pretreatments employed, leading to an increase in cellulose CrI (Fig. 3 I and Table 1). With respect to cellulose DP, agricultural residues were reported had cellulose DP values ranging from 1800 to 4000 (Zhang et al., 2013). In the present study, the untreated RWSS of JA stem exhibited a relatively lower value at 793.9 (Fig. 3II). On one hand, the untreated RWSS could not be fully dissolved in ionic liquid for DP measurements without chemical pretreatment (Zhang et al., 2013). On the other hand, a high level of amorphous substances was existed in the untreated RWSS, which could result in relatively lower cellulose DP value (Fig. 3II). After pretreatment A, the amorphous substances were largely extracted and the DP of pretreated RWSS rose rapidly to 2199.2. With ultrasound or H₂O₂ supplementation, the DP of pretreated RWSS after pretreatment B and pretreatment C was reduced to 1532.9 and 1292.5, respectively. When both ultrasound and H₂O₂ were supplemented during dilute sodium hydroxide pretreatment, a

dramatic DP decline from 2199.2 to 785.3 could be observed from the pretreated RWSS.

Thus it can be inferred that the four alkali-based pretreatment have great efficiency on lignocellulose depolymerisation by amorphous substances removal (Fig. 3 II and Table 1). In summary, alkali-based pretreatment shows outstanding ability in amorphous substances co-extraction, leading to significant alterations of cellulose CrI and DP.

To confirm the internal structure alteration of JA lignocellulose, the thermal behavior of control and pretreated RWSS were also investigated by thermal measurements (Fig. 3III and Fig. 3IV). As shown in thermogravimetric analysis distribution, the initial temperature of thermal degradation among the four pretreated RWSS were much lower than that of the control (Fig. 3III), which was mainly caused by removal of amorphous substances during pretreatments, consistent with the above findings (Table 1, Fig. 3 I and Fig. 3 II). In the differential thermogravimetric distribution, each kind of RWSS displays a unique pyrolysis decomposition curve which is basically dependent on its structure and components (Fig. 3IV). The peaks of amorphous substances (mainly non-crystalline cellulose, hemicellulose and pectin) and crystal substances (mainly crystalline cellulose) can be identified at about 363 °C and 321 °C, respectively (Chen et al., 2011). The peak of lignin was distributed over a wider range of temperatures, overlapping other components (Peng et al., 2014; Jiang et al., 2015). After the four alkali-based pretreatments, the peak at 363 °C almost could not be observed, revealing that amorphous substances were significant reduced, consistent with a previous study on sugarcane bagasse (Chen et al., 2011). In addition, the order of

cellulose peak intensity of each pretreated RWSS was $D > C > B > A > \text{Control}$, in agreement with above findings on cellulose CrI (Fig. 3I). Therefore, the results of thermal behavior analysis were strongly in supporting of findings of amorphous substances co-extraction effect on cellulose CrI and DP alterations upon various alkali-based pretreatments in JA stem.

3.4 Observation of pretreated RWSS after four alkali-based pretreatments

To intuitive understand the effects of four alkali-based pretreatments on JA RWSS, a series of observation were conducted step by step in this study. After exposure to dilute sodium hydroxide (A), the color of pretreated RWSS became deeper than the control, whereas the pretreated RWSS by pretreatment B becomes deeper than that of pretreated RWSS by pretreatment A. It may be due to the large extraction of amorphous substances of JA RWSS (Table 1). By H_2O_2 supplementation, the pretreated RWSS was found to become transparent to some extent in the pretreatment C and pretreatment D.

Previously, particle size reduction has been shown to significantly improve the enzymatic hydrolysis of biomass by extending the accessible surface area (Sun & Tomkinson, 2002; Alvira et al., 2010). In this study, the particle size of the control was between 40 and 80 mesh (i.e., 0.18 - 0.38 mm). The average particle size was obviously decreased by all four dilute alkali-based pretreatments, which caused by lignocellulose degradation (Table 1). In particular, a dramatic particle size reduction has been observed on the image of ultrasonic-assisted alkaline peroxide (D) pretreated RWSS, which

indicated a high potential for enzymatic hydrolysis.

We further applied scanning electron microscopy to observe the pretreated RWSS surfaces obtained from four alkali-based pretreatments. The surface morphology of JA particles shown that control (CK) had a smooth surface, with highly ordered fibrils. After pretreatment A, the pretreated RWSS surface became rough to a certain extent so that some cracks were formation. With ultrasound or H₂O₂ supplementation, the disruption of the pretreated RWSS structure becomes more pronounced. In addition, the RWSS after pretreatment C displayed more disorder fibrils than that after pretreatment B, and some tiny holes were exhibited on the surface. When pretreatment D applied, the lignocellulosic structure of pretreated RWSS was serious collapse and a lot of debris was obtained, indicating significantly enhancement on accessible surface area and depolymerization of lignocellulose.

3.5 Biomass digestibility under four alkali-based pretreatments

The aim of pretreatment is to enhance the hexose release rate and total sugar yield, and four alkali-based pretreatments show significant effects on JA RWSS saccharification in this study (Fig. 4). The hexose release rates of both the control and pretreated RWSS experienced rapid increases in first 12 h (Fig. 4 I), which may have been caused by sharp degradation of tiny fibers. Without any pretreatment, only 28.0% of hexoses were released from the RWSS after 12 h of saccharification. In addition, the hexose release rate of pretreated RWSS increased by 48.4% after pretreatment A, and

the RWSS pretreated by pretreatment B, C or D achieved almost same hexose release rate at about 54.0%. After 48 h sequential enzymatic hydrolysis, the order of hexose release rates of JA with different pretreatments is $D > C > B > A > \text{control}$. The RWSS pretreated with ultrasonic-assisted alkaline peroxide (D) achieves the highest hexose release rate at approximately 80.0%, which is 2.4 times higher than that of the control (33.5%). Meanwhile, the maximum hexose released rates were 66.9%, 71.9% and 74.2% for pretreatments A, B, and C, respectively.

The concentrations of hexoses, pentoses, and total sugars yield in the hydrolysate of the control and pretreated RWSS was exhibited the order $D > C > B > A > \text{control}$ (Fig. 4 II, Fig. 4III, Fig. 4IV, Fig. 4 V and Fig. 4VI). This order is exactly the same as that of hexose release rates of control or pretreated RWSS after 48 h saccharification. The total sugar yield after pretreatment D was 10.4 g/L, which was about 2.6 times higher than that of the control. Moreover, the total sugar yield after pretreatments A, B, and C exceeded 8.4 g/L, much higher than that of the control (4.1 g/L). In summary, four alkali-based pretreatments could significant enhance the JA biomass digestibility by amorphous substances extraction and pretreatment D was the most optimal method for JA biomass saccharification (Fig. 4 and Table 1). However, it remains unclear about the mechanism of four alkali-based pretreatment enhancements on biomass digestibility.

3.6 Mechanism of four alkali-based pretreatment enhancements on biomass saccharification

As the alkali-based pretreatment could significant affect cellulose CrI and DP, we performed a correlation analysis between the altering cellulose fearures and biomass digestibility in four pretreated RWSS (Fig. 5). As a result, the cellulose CrI of the pretreated RWSS exhibited a significant positive correlation with hexose release rates at $p < 0.01$ levels with high coefficient value at 0.954, indicating the altering CrI could significantly enhance the biomass saccharification (Fig. 5 I). By contrast, a significant negative correlation was observed between cellulose DP and biomass digestibility, which suggested that cellulose DP played a negative role in biomass digestion and the depolymerization of lignocellulose could increase the biomass digestibility (Fig. 5 II). As the cellulose DP is biologically determined by cellulose biosynthesis (Zhang et al., 2013), it could fundamentally affect cellulose crystallinity for high biomass enzymatic digestibility (Li et al., 2014). Besides, the cellulose DP values were negatively correlated with the cellulose CrI values in the present study (Fig. 5 III). Hence, it could be inferred that the depolymerization of lignocellulose, which caused by amorphous substances extraction, should predominately affect the cellulose CrI, leading to dramatic enhancements in biomass saccharification in JA (Fig. 4).

Alkali-based pretreatment had exhibited efficient effects on amorphous substances removal, leading to altering cellulose features for enhancing biomass digestibility. However, much remains unknown about the mechanism of each non-cellulosic polymers distinct effect on biomass enzymatic digestibility in plants (Huang et al., 2015; Si et al., 2015). As alkali-based agents could distinctively co-extract hemicellulose,

lignin and pectin, we further preformed a correlation analysis between the extracted non-cellulosic polymers and the hexoses yield after four alkali-based pretreatments.

Despite the hemicellulose was reported as a positive factor in biomass saccharification under various pretreatments in many plants (Xu et al., 2012; Li et al., 2014; Jia et al., 2014), such a relationship was not observed between the hemicellulose removal rates and biomass saccharification in JA (Fig. 6 I). By contrast, both lignin and pectin removal rates displayed a significant positive correlation with hexoses yield at $p < 0.01$ levels. Therefore, the results indicated that the extraction of lignin and pectin, other than hemicellulose removal, should predominately affect biomass digestibility in JA under alkali-based pretreatments. Besides, it was reported that hemicellulose exhibits a tight association with lignin and pectin, respectively (Si et al., 2015; Wang et al., 2015). Hence, these results could be inferred that the positive effects of hemicellulose on biomass saccharification should be caused by an effective co-extraction with lignin and pectin under the four alkali-based pretreatments.

Furthermore, the correlation analysis between non-cellulosic polymers extraction and two cellulose features suggested that both lignin and pectin could significant alter JA cellulose features under alkali-based pretreatments (Fig. 6 II and Fig. 6III). Si et al. (2015) reported that lignin extraction could increase cellulose CrI and biomass enzymatic digestibility in *Miscanthus* sp. under various one- and two-step pretreatments with alkali and acid. Wang et al. (2015) also reported that the extractable pectin could reduce cellulose crystallinity and play a predominant role in biomass enzymatic

saccharification upon various chemical pretreatments. In conclusion, the results indicated that lignin and pectin extraction, other than hemicellulose removal, predominately affect biomass enzymatic digestibility by altering cellulose features in JA under four alkali-based pretreatments. Hence, this study also suggests the potential of genetic modification and pretreatment approaches for enhancing lignocellulose digestibility in bioenergy plants.

3.7 Analysis of fermentation inhibitors from alkali-based pretreatments

As the final step in bioethanol production, fermentation has been reported to be significantly prevented by the byproducts of pretreatment, such as acetic acid, HMF, and furfural (Palmqvist & Hahn-Hägerdal, 2000). In the present study, acetic acid, HMF and furfural in the pretreatment supernatant were also quantified (Table 2). As a result, it can be seen that no HMF and furfural can be detected in any of the four alkali-based pretreatment supernatants, consist with previous findings in other alkali-based pretreatments (Cao et al. 2012). Therefore, alkali-based pretreatments can prevent the generation of HMF and furfural during the pretreatment process.

By comparison, a certain amount of acetic acid is produced by the four alkali-based pretreatments. As shown in table 2, the acetic acid concentration of pretreatment A supernatant is 1.87 g/L. With ultrasound supplementation (B), the concentration of acetic acid significantly increased by 19.3% to 2.23 g/L. By contrast, the concentration of acetic acid dramatically decreased by 38.5% to 1.15 g/L in pretreatment C. In

addition, when both ultrasound and H₂O₂ was employed, the supernatant obtained from pretreatment D achieved almost the same concentration of acetic acid as pretreatment A. Hence, the results suggested that H₂O₂, contrary to ultrasound, displays a great effect on hydrolysate detoxification during alkali-based pretreatment.

4. Conclusion

JA stem is a quality feedstock for bioethanol production due to rich soluble sugar and low ash. Correlation analysis of 59 diverse JA accessions has indicated that both sugar and ash significantly affect biomass saccharification. Comparative analyses of four alkali-based pretreatments indicate that pretreatment D is the most suitable method for biomass digestion. Notably, Lignin and pectin removal could distinctively affect the cellulose CrI and DP for high biomass saccharification. Furthermore, H₂O₂, contrary to ultrasound, displays a great effect on hydrolysate detoxification by largely reducing the generation of acetic acid during alkali-based pretreatments.

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Figure captions

Fig.1 Variations in soluble sugar, cellulose, hemicellulose, lignin, pectin and ash, and their correlations for 59 Jerusalem artichoke accessions: Chemical components (I); correlations between soluble sugar and cell wall polymers (II), correlations between ash and cell wall polymers (III).

Fig.2 Effects of soluble sugar, ash and cell wall polymers on hexoses yield (% cellulose) from enzymatic hydrolysis after 2% dilute sodium hydroxide (Pretreatment A) in 59 Jerusalem artichoke accessions: sampling distribution on hexoses yield (I); correlation between soluble sugar and hexoses yield (II); correlation between ash and hexoses yield (III); correlation between four major wall polymers and hexoses yield (IV). R^2 indicates correlation coefficients. ** indicates significant correlation at $p < 0.01$.

Fig. 3 Cellulose crystalline index (I), degree of polymerization (II), thermogravimetric analysis (III) and differential thermogravimetric distribution (IV) of residue without soluble sugar (RWSS) pretreated with dilute sodium hydroxide (A), ultrasonic-assisted dilute sodium hydroxide (B), alkaline peroxide (C), and ultrasonic-assisted alkaline peroxide (D) and the untreated control RWSS (CK) for stem of the Jerusalem artichoke accession HEN-3.

Fig. 4 Time courses of hexose release rates (I) and total sugar yield during saccharification of stem residue without soluble sugar pretreated with dilute sodium hydroxide (III), ultrasonic-assisted dilute sodium hydroxide (IV), alkaline peroxide (V),

and ultrasonic-assisted alkaline peroxide (VI) and the control of untreated RWSS (II) for Jerusalem artichoke accession HEN-3. Data are plotted as means $\pm$ SD (n=3).

Fig. 5 Effects of cellulose crystalline index and degree of polymerization on hexoses yield (% cellulose) under four alkali-based pretreatments: correlation between cellulose crystalline index and hexoses yield (I); correlation between cellulose degree of polymerization and hexoses yield (II); correlation between cellulose crystalline index and degree of polymerization (III). R^2 indicates correlation coefficients. ** indicates significant correlation at $p < 0.01$.

Fig.6 Effects of non-cellulosic polymers (hemicellulose, lignin and pectin) extraction on biomass digestibility and cellulose crystalline index and degree of polymerization: correlation between non-cellulosic polymers removal rates and hexoses yield (I); correlation between non-cellulosic polymers removal rates and cellulose crystalline index (II); correlation between non-cellulosic polymers removal rates and cellulose degree of polymerization (III). R^2 indicates correlation coefficients. ** indicates significant correlation at $p < 0.01$.

Table 1. Mean $\pm$ standard deviation of main components of residue without soluble sugar (RWSS) pretreated by dilute sodium hydroxide (A), ultrasonic-assisted dilute sodium hydroxide (B), alkaline peroxide (C), and ultrasonic-assisted alkaline peroxide (D) and the untreated control RWSS (CK) and their removal rates for stem of Jerusalem artichoke accession HEN-3.

Parameter		CK	A	B	C	D
Component (%)	Cellulose	37.7 $\pm$ 0.6	40.3 $\pm$ 1.1	42.8 $\pm$ 0.2	43.8 $\pm$ 0.8	44.3 $\pm$ 0.5
	Hemicellulose	19.1 $\pm$ 0.1	13.9 $\pm$ 0.7	14.7 $\pm$ 0.3	16.7 $\pm$ 0.8	16.6 $\pm$ 0.1
	Lignin	26.0 $\pm$ 1.1	23.9 $\pm$ 0.6	23.1 $\pm$ 0.1	21.5 $\pm$ 0.1	21.6 $\pm$ 0.2
	Pectin	10.4 $\pm$ 0.2	6.0 $\pm$ 0.1	6.4 $\pm$ 0.8	6.2 $\pm$ 0.3	5.9 $\pm$ 0.1
Removal rate (%)	RWSS	0.0	14.5	21.6	23.8	28.0
	Cellulose	0.0	8.4	11.0	12.4	15.4
	Hemicellulose	0.0	38.0	40.0	33.9	37.6
	Lignin	0.0	21.5	30.4	37.5	40.3
	Pectin	0.0	50.4	52.1	55.2	58.9

Table 2. Mean $\pm$ standard deviation of inhibitory compounds in the supernatant of residue without soluble sugar (RWSS) pretreated with dilute sodium hydroxide (A), dilute sodium hydroxide (B), alkaline peroxide (C), and ultrasonic-assisted alkaline peroxide (D) for stem of Jerusalem artichoke accession HEN-3.

Inhibitor	A	B	C	D
Acetic acid (g/L)	1.87 $\pm$ 0.03	2.23 $\pm$ 0.01	1.15 $\pm$ 0.01	1.89 $\pm$ 0.00
HMF (g/L)	ND	ND	ND	ND
Furfural (g/L)	ND	ND	ND	ND

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6