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Steam explosion distinctively enhances biomass enzymatic saccharification of cotton stalks by largely reducing cellulose polymerization degree in *G. barbadense* and *G. hirsutum*

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HIGHLIGHTS

- Steam explosion has large enhancements on biomass digestibility in cotton stalks.
- Steam explosion largely reduces cellulose DP for enhancing biomass digestibility.
- Steam explosion with dilute acids causes the highest sugar–ethanol conversion rates.
- Extreme alkali has the highest sugar yield and lowest sugar–ethanol conversion rate.
- CrI and Xyl/Ara as major factors on hexoses yields distinctive in two cotton species.

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ABSTRACT

In this study, steam explosion pretreatment was performed in cotton stalks, leading to 5–6 folds enhancements on biomass enzymatic saccharification distinctive in *Gossypium barbadense* and *Gossypium hirsutum* species. Sequential 1% H_2SO_4 pretreatment could further increase biomass digestibility of the steam-exploded stalks, and also cause the highest sugar–ethanol conversion rates probably by releasing less inhibitor to yeast fermentation. By comparison, extremely high concentration alkali (16% NaOH) pretreatment with raw stalks resulted in the highest hexoses yields, but it had the lowest sugar–ethanol conversion rates. Characterization of wall polymer features indicated that biomass saccharification was enhanced with steam explosion by largely reducing cellulose DP and extracting hemicelluloses. It also showed that cellulose crystallinity and arabinose substitution degree of xylans were the major factors on biomass digestibility in cotton stalks. Hence, this study has provided the insights into cell wall modification and biomass process technology in cotton stalks and beyond.

1. Introduction

Cotton is an important fiber crop with enormous masses of stalk residues suitable for conversion into biofuels and biomaterials. As the world's largest cotton-producing country, China can harvest 20–40 million tons (dry weight) of cotton stalks each year (Akdeniz et al., 2004; Kang et al., 2012), but a high proportion of cotton stalks are burned in the field, causing a serious environmental pollution and biomass waste (Kaur et al., 2012).

Cotton stalks have been applied to produce cellulose fibers for biomaterials (Troedec et al., 2011). For instance, cotton stalk fibers reinforced polypropylene (PP) composites have mechanical properties similar to those of jute fibers reinforced PP composites (Reddy and Yang, 2009). In addition, cotton stalks could be used to manufacture pulp (Zheng et al., 2012), thermal insulation

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fiberboard (Zhou et al., 2012), and activated carbons (El-Hendawy et al., 2008; Ozdemir et al., 2011). Despite cotton stalk is lignocellulose-rich biomass, little is yet reported about its applications in biofuel production.

Lignocellulose has been considered as a major renewable biomass source for biofuels (Sun et al., 2013). Principally, biomass conversion involves three major steps: physical and chemical pretreatments for cell wall destruction, enzymatic hydrolysis for soluble sugar release, and yeast fermentation for ethanol production (Wu et al., 2013). However, plant cell wall composition and wall polymer features crucially determine biomass recalcitrance, resulting in a costly lignocellulosic digestion and numerous inhibitory compounds released that inhibit the yeast fermentation (Alvira et al., 2010; Li et al., 2014a). As pretreatment is the initial step, it is important to find out an optimal approach that not only enhances biomass enzymatic saccharification but also releases less yeast fermentation inhibitors.

Acid and alkali chemicals such as H_2SO_4 and NaOH are extensively used in biomass pretreatments (Alvira et al., 2010). Principally, the two chemicals have distinct mechanisms for biomass depolymerization. Alkali pretreatment can mostly cause the dissociation of entire wall polymers whereas acid pretreatment induces the partial release of monosaccharides, oligosaccharides, and lignin monomers (Xu et al., 2012; Li et al., 2014a). However, steam explosion has been increasingly considered as an economical and environment-friendly pretreatment by largely reducing biomass particle size, and partially removing hemicelluloses and lignin (Alvira et al., 2010; Kumar et al., 2009). Although those physical and chemical pretreatments have been used in many plants (Moiser et al., 2005; Li et al., 2013b,2014b; Jia et al., 2014), much remains unknown about their applications in cotton stalks.

Over 98% of the commercially cultivated cottons in the world are produced from the two cotton species: *Gossypium barbadense* (Sea island cotton, Gb) and *Gossypium hirsutum* (Upland cotton, Gh). Gb species has the longest, finest and strongest fibers, whereas Gh species has higher yield of cotton fibers (Al-Ghazi et al., 2009; Li et al., 2013a). In this study, the stalks of two cotton species were pretreated with steam explosion followed by acid (H_2SO_4) or alkali (NaOH) at various concentrations, and the sequential biomass enzymatic saccharification was compared in both species. In addition, the sugar–ethanol conversion rate by yeast fermentation after various pretreatments was also evaluated in order to find out an optimal biomass conversion process for cotton stalks.

2. Methods

2.1. Plant samples

Gossypium hirsutum (Huamian 99) and *Gossypium barbadense* (Junhai 1) were grown in experimental fields of Huazhong Agricultural University, Wuhan, China. The mature cotton stalks of 5–10 plants were harvested, dried at 50 °C, ground into powder through 40 mesh screen and stored in sealed dry container until in use.

2.2. Plant wall polymer extraction

The plant cell wall fraction method was used to extract hemicelluloses and cellulose as described by Peng et al. (2000) and Wu et al. (2013). The soluble sugars, lipid, starch and pectin of the biomass samples were consecutively extracted by using potassium phosphate buffer (pH 7.0), chloroform–methanol (1:1, v/v), DMSO–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate. Extracted the remaining pellets with 4 M KOH and 1.0 mg/mL sodium borohydride at 25 °C, 150 rpm for 1 h, then washed with 5 mL distilled water twice, and collected all supernatant with two parallels; one parallel was neutralized and dialyzed for KOH-extractable hemicelluloses monosaccharides analysis, and another parallel was collected for the determination of free pentoses and hexose as the KOH-extractable hemicelluloses. For the remaining two parallel non-KOH-extractable residues, one parallel was sequentially treated with trifluoroacetic acid (TFA) for 1 h at 120 °C for non-KOH-extractable hemicelluloses monosaccharides analysis; and other one was further extracted with H_2SO_4 (67%, v/v) at 25 °C, 150 rpm for 1 h, and the supernatants were collected for determination of free hexoses and pentoses as total cellulose and non-KOH-extractable hemicelluloses, respectively. In a parallel experiment, the residues of the 4 M KOH extraction were treated with acetic–nitric acids (acetic acid/nitric acid/water: 8:1:2, v/v) for 1 h at 100 °C, the remaining residues were defined as crystalline cellulose. The experiments were conducted in technological triplicate.

2.3. Colorimetric assay of hexoses and pentoses

The hexoses and pentoses of biomass residues were determined using an UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China). Hexoses were detected by the anthrone/H_2SO_4 method (Fry, 1988), and the pentoses was measured by the orcinol/HCl method (Dische, 1962). The standard curves for hexoses and pentoses assay were drawn by using D-glucose and D-xylose (purchased from Sinopharm Chemical Reagent Co., Ltd.) as standards, respectively. Regarding the high pentose levels that affect the absorbance reading at 620 nm for hexoses assay, the deduction from pentoses reading at 660 nm was carried out for a final hexoses calculation. A series of xylose concentrations were used for plotting the standard curve referred for the deduction, verified by GC–MS analysis. All experiments were carried out in technological triplicate.

2.4. Lignin and monolignol assay

Total lignin content was measured by the two-step acid hydrolysis method according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (Sluiter et al., 2008) with minor modification as described by Wu et al. (2013). All experiments were carried out in technological triplicate.

Monolignols were detected by HPLC as described by Li et al. (2014a). Standard chemicals: *p*-Hydroxybenzaldehyde (H), vanillin (G) and syringaldehyde (S) were purchased from Sinopharm Chemical Reagent Co., Ltd, and the samples were run by HPLC (Waters 1525) with Kromat Universil C18 column (4.6 mm × 250 mm, 5 μm) operating at 28 °C with CH_3OH:H_2O:HAc (25:74:1, v/v/v) carrier liquid (flow rate: 1.1 mL/min). Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better, and the detection of the compounds was carried out with a UV-detector at 280 nm.

2.5. Hemicellulose monosaccharides determination by GC–MS

Hemicellulose monosaccharides were determined by GC–MS as described by Xu et al. (2012) and Li et al. (2013b). TFA and *myo*-inositol were purchased from Aladdin Reagent Inc. Acetic anhydride, acetic acid and monosaccharide standards components: L-rhamnos (Rha), L-arabinose (Ara), L-fucose (Fuc), D-xylose (Xyl), D-galactose (Gal), D-glucose (Glu) and D-mannose (Man) were all obtained from Sinopharm Chemical Reagent Co., Ltd. 1-methylimidazole was purchased from Sigma–Aldrich Co. LLC. The samples were run by GC–MS (SHIMADZU GCMSQP2010) at Analytical conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25um df column. Carrier gas: He. injection method: split. Injection port: 250 °C, interface: 250 °C. Injection volume: 1.0 μL. The temperature

program: from 170 °C (held for 12 min) to 220 °C (held for 8 min) at 3 °C/min. Ion source temperature: 200 °C, ACQ Mode: SIM. The mass spectrometer was operated in the EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 m/z in 0.45 s. Calibration curves of all analytes routinely yielded correlation coefficients in 0.999 or better.

2.6. Cellulose crystallinity detections

Cellulose crystallinity was defined by detecting biomass crystalline index (CrI) using X-ray diffraction method as described by Zhang et al. (2013). The Rigaku-D/MAX instrument (Uitima III, Japan) was used, and the well-mixed powders of biomass samples were analyzed under plateau conditions. Ni-filtered Cu Kα radiation ($\lambda = 0.154056$ nm) was generated at a voltage of 40 kV and a current of 40 mA, and scanned at a speed of 10°/min from 5° to 45°. The CrI was estimated using the intensity of the 200 peak (I_{200}, $\theta = 22.5°$) and the intensity at the minimum between the 200 and 110 peaks (I_{am}, $\theta = 18.0°$) as follow: $CrI = 100 \times (I_{200} - I_{am})/I_{200}$ (Segal et al., 1959); I_{200} represents both crystalline and amorphous materials and I_{am} represents amorphous material. The CrI method was detected with ± SD from 0.05 to 0.15 using five representative samples in triplicate.

2.7. Measurement of degree of polymerization (DP) of cellulose

Degree of polymerization (DP) of cellulose samples was measured using the viscosity method (Puri, 1984) with minor modification described by Zhang et al. (2013). The method bases on the relationship: $DP^{0.905} = 0.75\,[\eta]$, and the $[\eta]$ is the intrinsic viscosity of the solution. All experiments were conducted at 25 ± 0.5 °C using an Ubbelohde viscosity meter and cupriethylenediamine hydroxide (Cuen) as solvent. The intrinsic viscosity was calculated by interpolation using the USP table (USP, 2002) showing the predetermined values of the product of intrinsic viscosity and concentration. The $[\eta]C$, for cellulose samples exhibiting relative viscosity (η_{rel}) values between 1.1 and 9.9. η_{rel}, was calculated using the equation: $\eta_{rel} = t/t_0$, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent, respectively. All experiments were performed in technological triplicate.

2.8. Biomass pretreatments and enzymatic hydrolysis

2.8.1. Steam explosion

Cotton stalk samples were cut into the 5–8 cm size, sprayed with deionized water to the moisture at 50% and then stored at room temperature for steam explosion. The chopped cotton stalk samples (200 g, dry matter) were loaded into 5-L steam explosion reactor (QBS-200, Hebi Zhengdao Machine Factory, Hebi, China), and treated at 225 °C (2.5 MPa) for 3 min (severity factor: $R_0 = 4.22$). Then, the steam exploded samples were dried in air and stored in dry container until in use.

2.8.2. H_2SO_4 pretreatment

The well-mixed biomass powder samples (0.3 g) were treated with 6 mL H_2SO_4 at various concentrations: 0.5%, 1.0%, 2.0%, 4.0%, 8.0%, 12.0%, 16.0% H_2SO_4 (v/v) used for raw stalk samples, and 0.25%, 0.5%, 1.0%, 1.5% H_2SO_4 (v/v) for the samples after steam explosion. The sealed sample tubes were heated at 121 °C for 20 min in an autoclave (15 psi), and shaken under 150 rpm for 2 h at 50 °C. The samples were then centrifuged at 3000g for 5 min, and the pellets were washed with 10 mL distilled water for 3–5 times until no sugar detected, and stored at −20 °C until in use for enzymatic hydrolysis. All supernatants were collected for determining released of total sugars (pentoses and hexoses) from pretreatments. As a control, the biomass sample was only added with 6 mL distilled water under shaking for 2 h at 50 °C. All samples were carried out in technological triplicate.

2.8.3. NaOH pretreatment

The well-mixed biomass samples (0.3 g) were treated with 6 mL NaOH at various concentrations: 0.5%, 1.0%, 2.0%, 4.0%, 8.0%, 12.0%, 16.0%, 20.0% NaOH (w/v) used in raw stalk samples, and 0.25%, 0.5%, 1.0%, 1.5% NaOH (w/v) for the biomass samples after steam explosion. The sealed sample tubes were shaken under 150 rpm for 2 h at 50 °C, and then centrifuged at 3000g for 5 min. The pellets were washed with 10 mL distilled water for 3–5 times until no sugar detected, and stored at −20 °C until in use for enzymatic hydrolysis. All supernatants were collected for determining total sugars (pentoses and hexoses) released from pretreatments. As a control, the biomass sample was only added with 6 mL distilled water under shaking for 2 h at 50 °C. All samples were carried out in technological triplicate.

2.8.4. Enzymatic hydrolysis

The remaining biomass residues from steam explosion and chemical pretreatments were washed 2–3 times with 10 mL distilled water until the supernatants at pH 7.0, and once more with 10 mL of mixed-cellulase reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8). The washed residues were incubated with 6 mL (1.6 g/L) of mixed cellulases containing β-glucanase ($\geqslant 2.98 \times 10^4$ U), cellulase ($\geqslant 298$ U) and xylanase ($\geqslant 4.8 \times 10^4$ U) purchased from Imperial Jade Bio-technology Co., Ltd). The sealed sample tubes were shaken under 150 rpm at 50 °C for 48 h, and then centrifuged at 3000g for 5 min. The supernatants were collected for determining total pentose and hexose yields released from enzymatic hydrolysis. As a control, the sample was only added with 6 mL of reaction buffer, and shaken under 150 rpm for 48 h at 50 °C. All samples were carried out in technological triplicate.

2.9. Yeast fermentation and ethanol determination

Yeast fermentation was performed as described by Li et al. (2014a) with minor modification. *Saccharomyces cerevisiae* was purchased from Angel yeast Co., Ltd., Yichang, China and dissolved in 0.2 M pH 4.8 phosphate buffer for all fermentation experiments. The pretreatments were performed with 16% NaOH (w/v) or 12% H_2SO_4 (v/v) on raw material and 1% NaOH (w/v) or 1% H_2SO_4 (v/v) on steam exploded material of cotton stalks as former described. Collected the supernatants and washed the residues twice with 5 mL distilled water. The combined supernatants were neutralized to pH 4.8 using appropriate amounts of H_2SO_4 or NaOH, and 0.2 M phosphate buffer. The fermentation medium consists of combined supernatants released from chemical pretreatment and sequential enzymatic hydrolysis of cotton stalks, and appropriate amounts of glucose adjusted to final hexoses concentration at 200.00 g/L. The fermentation mediums were transferred to 30-mL glass tubes, sealed with rubber plugs and then sterilized in autoclave under 0.15 Mpa at 25 °C for 20 min. Thereafter, the tubes were inoculated to an initial cell mass concentration of 2.00 g/L (cell dry weight) and incubated at 37 °C for 48 h. The fermentation liquid was distilled after 48 h for determination of ethanol content. All of the experiments were performed in technological triplicate.

Ethanol yield was detected using the dichromate oxidation method (Fletcher and van Staden, 2003) with minor modifications (Li et al., 2014a). The fermentation liquid was distilled at 100 °C for 15 min to produce ethanol liquor. The diluted ethanol sample (final volume, 1 mL) with 2.00 mL of 5.00% potassium dichromate ($K_2Cr_2O_7$) (5.00 g $K_2Cr_2O_7$ dissolved in 90.00 mL distilled water and 10.00 mL 98% sulfuric acid) was heated for 10 min in a boiling

bath. After cooling, distilled water was added to a final volume of 10.00 mL, and the absorbance was read at 600 nm. Absolute ethanol was used as the standard.

The sugar–ethanol conversion rate was calculated by the following equation: $S - E = E/A/H \times 100\%$ [$S - E$: sugar–ethanol conversion rate; E: total ethanol weigh (g) at the end of fermentation; A: the conversion rate at 51.11% (92/180) in the case that glucose is completely conversed to ethanol according to the Embden–Meyerhof–Parnas pathway in *S. cerevisiae*; and H: total hexoses weight (g) at the beginning of fermentation]. All experiments were performed in technological triplicate.

3. Results and discussion

3.1. Steam explosion extractions with wall polymers in Gb and Gh species

In the present work, the mature stalks of two cotton species (Gb and Gh) were initially pretreated with steam explosion (Tables 1 and S1). Without steam explosion pretreatment, the Gb and Gh species showed a significantly different cell wall composition (cellulose, hemicelluloses and lignin) at $p < 0.05$ and $p < 0.01$ (Table 1). In particular, Gb had much lower cellulose and hemicelluloses levels than those of Gh by 25% and 20%. However, after steam explosion, both cotton species had similar cellulose and lignin contents, only that Gb exhibited a significantly lower hemicelluloses level than that of the Gh by 22% at $p < 0.01$ (Table 1). On the other hand, the steam explosion largely reduced hemicelluloses levels of Gb and Gh by 132% and 129%, respectively, but it not much on lignin contents (Table S1). Notably, the Gb exhibited much increased cellulose content by 27% from the steam explosion, whereas the Gh had the raised cellulose by 9% at insignificant level ($p > 0.05$). Hence, the data indicated that the steam explosion could predominately extract hemicelluloses in raw cotton stalks, which relatively enhanced the cellulose compositions distinctive in Gb and Gh species.

3.2. Steam explosion enhancements on biomass saccharification

Biomass enzymatic saccharification (or digestibility) has been measured by calculating the hexoses yield (% cellulose) released from cellulases hydrolysis after pretreatment (Li et al., 2013b; Wu et al., 2013). Under the first-step, steam explosion pretreatment, Gb and Gh stalks exhibited the hexoses yields released from enzymatic hydrolysis at 34.5% and 29.6%, respectively (Fig. 1; Table S2), which were 5–6 folds higher than that of the raw stalks (control, without steam explosion). While the steam-exploded stalks were sequentially pretreated with 0.25% H_2SO_4, Gb and Gh showed the increased hexoses yields at 38% and 36.7%, but had slightly enhanced hexoses yields under relatively higher concentrations of H_2SO_4 (0.5%, 1%, 1.5%; Fig. 1A). Thus, the second-step pretreatment of the steam-exploded residues with 1% H_2SO_4 could reach to the highest hexoses yields at 39.6% and 37.4% in the Gb and Gh species (Table S2). As a contrast, pretreated with 0.25% NaOH, the steam-exploded stalks of Gb had a reduced hexoses yield at 30.5% at $p < 0.05$, whereas the Gh did not show any significant change (Fig. 1B; Table S2). Furthermore, pretreated with relatively higher NaOH concentrations (0.5%, 1%, 1.5%), the steam-exploded stalks did not exhibit any enhanced hexoses yields compared with the 0.25% NaOH pretreatment. It has been reported that steam explosion is somehow similar to the diluted acid pretreatment that could predominately remove hemicelluloses

Table 1
Cell wall compositions in the raw cotton stalks and steam-exploded stalk residues.

Biomass sample	Species	Cell wall compositions (% dry matter)					
		Cellulose		Hemicelluloses		Lignin	
Raw stalks	Gb	32.0 ± 0.3**		14.4 ± 0.5*		24.5 ± 0.1**	
	Gh	39.9 ± 0.8	24.6%#	17.2 ± 0.1	19.6%	25.6 ± 0.1	4.45%
Steam-exploded residues	Gb	40.6 ± 0.2		6.19 ± 0.16**		24.5 ± 1.6	
	Gh	43.5 ± 1.5	7.09%	7.52 ± 0.22	21.5%	24.8 ± 0.8	1.50%

* or **Significant difference between the raw stalks and steam-exploded residues in *G. barbadense* (Gb) and *G. hirsutum* (Gh) species by *t*-test at $p < 0.05$ or $p < 0.01$ ($n = 3$).
\# Indicated the increased percentage between Gb and Gh species: subtraction of two values divided by low value. Data indicated as mean ± SD ($n = 3$).

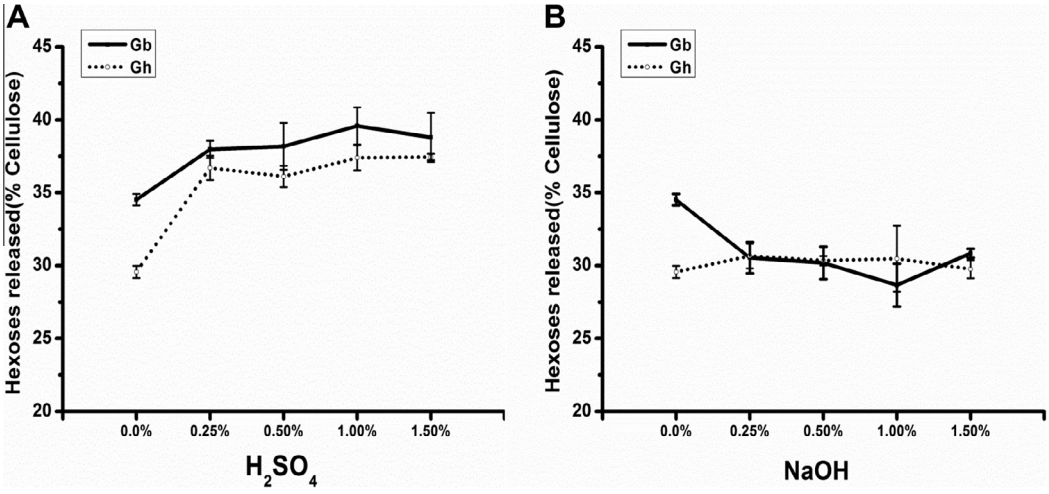

Fig. 1. Hexoses yields (% cellulose) released from enzymatic hydrolysis of the steam-exploded stalk residues. The steam-exploded stalk residues in Gb and Gh species were sequentially pretreated with/without (0.0%) H_2SO_4 (A) and NaOH (B) at four concentrations; the bar indicated SD ($n = 3$).

(Chen and Qiu, 2007; Alvira et al., 2010). However, the steam explosion condition (R_0 = 4.22, 2.5 MPa/3 min) performed in this study might not be enough for more hemicelluloses extraction, and thus the sequential dilute acid pretreatment could have the further enhancement on biomass saccharification. On the other hand, as only high concentration of alkali (4 M KOH) is favor for hemicelluloses disassociation with cellulose by breading hydrogen bonds (Xu et al., 2012), it could interpret that the sequential dilute alkali pretreatment had little effect on the steam-exploded residues in this work.

In sum, the first-step pretreatment of steam explosion could largely enhance biomass enzymatic saccharification of the cotton stalks distinctive in Gb and Gh species, and the second-step pretreatment with dilute acid (H_2SO_4), other than alkali (NaOH), could further significantly increase biomass digestibility in both cotton species.

3.3. Biomass digestibility under various H_2SO_4 and NaOH pretreatments

To compare with the various steam-explosion pretreatments, classic chemical pretreatments were also performed in the raw cotton stalks (without steam explosion) using various concentrations of H_2SO_4 and NaOH (Fig. 2; Table S3). Pretreated with relatively low concentrations of H_2SO_4 (0.5%, 1%, 2%, 4%), the raw stalks remained the slightly enhanced hexoses yields released from enzymatic hydrolysis, but both Gb and Gh species respectively showed the highest hexoses yields at 22.6% and 18.1% under pretreatment of 12% H_2SO_4 (Fig. 2A), which were much lower than that of the steam explosion pretreatment. As a comparison, the NaOH pretreatments could cause the enzymatic saccharification of raw stalks by 3-folds higher than those of H_2SO_4 pretreatments. Notably, the alkali pretreatments in the other examined grass plants only exhibited 1–2 folds higher biomass saccharification than those of their acid pretreatments (Xu et al., 2012; Wu et al., 2013; Li et al., 2014b; Jia et al., 2014), which may be due to the cotton stalks having the relatively lower hemicelluloses levels (Table 1). In addition, Gb and Gh species respectively exhibited the maximum hexoses yields at 78% and 59% under 16% NaOH pretreatments (Fig. 2B; Table S3), which were 2–3 folds higher than that of 12% H_2SO_4 pretreatments (Fig. 2A). In addition, the Gb species had the hexoses yields higher than those of Gh under both H_2SO_4 and NaOH pretreatments, consistent with the previous observations under various steam explosion pretreatments.

3.4. Yeast fermentation inhibition from various pretreatments

It has been reported that physical and chemical pretreatments could produce various toxin compounds that inhibit yeast fermentation for ethanol production (Li et al., 2014a). In this study, the supernatants obtained from pretreatments and sequential enzymatic hydrolysis were supplied into yeast fermentation course, and the sugar–ethanol conversion rates were then detected as an adverse parameter of accounting for yeast fermentation inhibition (Table 2). Using the supernatants obtained from pretreatments of the steam explosion followed with 1% H_2SO_4, the Gb and Gh species respectively showed the highest sugar–ethanol conversion rates by 65% and 63% among all pretreatments performed in this study (Table 2). As a comparison, using the supernatants from 1% H_2SO_4 pretreatment of raw stalks (other than the steam-exploded residues), both cotton species exhibited sugar–ethanol conversion rates by 50% and 49%, indicating that the steam explosion pretreatment could produce the relatively less toxin compounds that inhibit yeast fermentation. Notably, despite that 16% NaOH pretreatment of raw stalks could result in the highest hexoses yields among all pretreatments performed (Fig. 2B), it caused the lowest sugar–ethanol conversion rates by 22% in Gb and Gh species, which was almost 3-folds lower than that of pretreatments of the steam explosion followed with 1% H_2SO_4 (Table 2). Taken together, the results indicated that pretreatments of the steam explosion

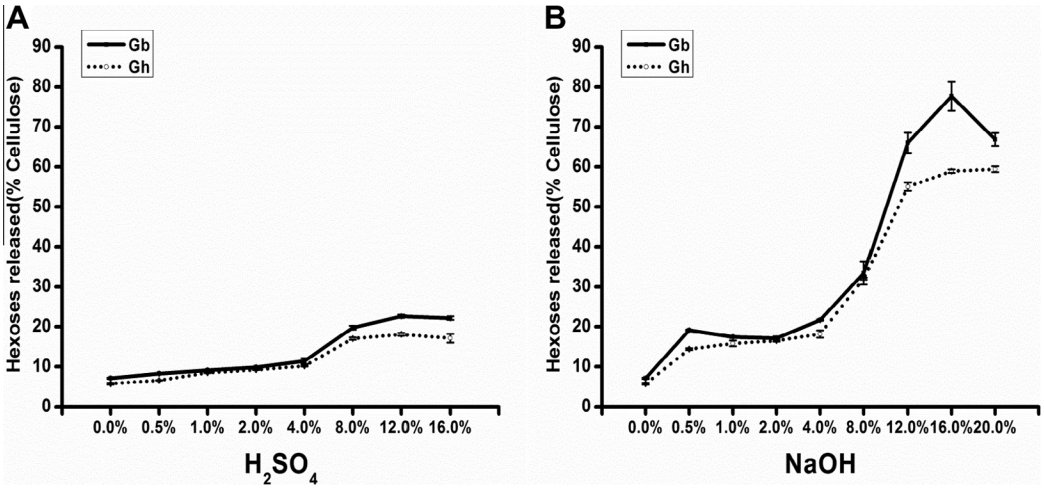

Fig. 2. Hexoses yields (% cellulose) released from enzymatic hydrolysis of the raw stalks. The raw stalks were pretreated with/without (0.0%) H_2SO_4 (A) and NaOH (B) at series concentrations; the bar indicated SD (n = 3).

Table 2
Sugar–ethanol conversion rate (%) by yeast fermentation supplied with supernatants from pretreatment and enzymatic hydrolysis in cotton stalks.

Pretreatment	Raw stalks				Steam-exploded residues	
	1% H_2SO_4	1% NaOH	12% H_2SO_4	16% NaOH	1% H_2SO_4	1% NaOH
Gb	50.4 ± 1.1	44.6 ± 1.4	28.0 ± 0.3	21.8 ± 1.3	65.1 ± 1.8	45.9 ± 0.7
Gh	48.7 ± 0.4	42.3 ± 1.6	24.5 ± 1.3	22.3 ± 1.1	62.7 ± 0.7	50.1 ± 0.9

Data indicated mean ± SD (n = 3).

Table 3
Lignocellulose crystalline index (CrI) and degree of polymerization (DP) of crystalline cellulose in the raw cotton stalks and steam-exploded stalk residues.

Biomass samples	Species	CrI (%)		DP	
Raw stalks	Gb	44.8		247 ± 3**	
	Gh	50.3	12.3%#	219 ± 6	−12.3%
Steam-exploded residues	Gb	57.7		151 ± 0	
	Gh	56.9	−1.52%	149 ± 3	−1.43%

** Significant different between Gb and Gh species *by t*-test at $p < 0.01$ ($n = 3$).
# Percentage of the increased or decreased level between two species: subtraction of two values divided by low value. CrI method was detected with SD from 0.05 to 0.15 using five representative samples in triplicate. DP data indicated mean ± SD ($n = 3$).

followed with dilute acid could not only largely enhance biomass enzymatic saccharification, but also mostly reduce the inhibition to yeast fermentation for ethanol production in cotton stalks.

3.5. Mechanism of steam explosion enhancements on biomass saccharification

To understand the steam explosion enhancement on biomass enzymatic digestibility, two major cellulose features in cotton stalks were detected including lignocellulose crystalline index (CrI) and crystalline cellulose DP (Tables 3 and S4). Lignocellulose CrI has been characterized as the negative factor on biomass enzymatic digestibility under various pretreatments in many plants examined (Chen and Li, 2000; Zhang et al., 2013; Wu et al., 2013; Li et al., 2014b,c). In this work, Gb species showed a significantly lower CrI value than that of Gh by 12.3% in the raw stalks, consistent with the finding about the Gb with a relatively higher biomass saccharification under physical (steam explosion) and chemical (acid and alkali) pretreatments (Figs. 1 and 2). However, both Gb and Gh species were detected with a similar CrI value in the steam-exploded stalk residues, suggesting that the steam-exploded residue CrI should no longer be the major factor affecting the biomass digestibility under the second-step dilute acid and alkali pretreatments (Table 3). Meanwhile, Gb exhibited a higher DP value than that of Gh in the raw stalks, but both species also had a close DP in the steam-exploded residues. On the other hand, despite that the steam explosion pretreatment could increase lignocellulose CrI by 13% and 29%, it could strongly reduce cellulose DP by 48% and 64% in the Gb and Gh species, respectively (Table S4), suggesting that the steam-explosion may mainly affect cellulose DP.

It has been reported that hemicelluloses negatively affect cellulose CrI for the enhanced biomass digestibility in the plants examined (Xu et al., 2012; Wang et al., 2014), whereas the crystalline cellulose DP positively affects CrI for the reduced biomass saccharification in *Miscanthus* (Zhang et al., 2013). Hence, the steam explosion extraction with large proportion of hemicelluloses should be the major factor on raising lignocellulose CrI in the steam-exploded stalk residues.

Although the steam explosion could reduce biomass residue sizes for enhancing lignocellulose saccharification (Qiu and Chen, 2012; Alvira et al., 2010), to our knowledge, little is yet reported about its drastically decreasing crystalline cellulose DP, in particular on cotton stalks. Since both cellulose DP and CrI are the negative factors affecting biomass enzymatic digestibility in *Miscanthus* (Zhang et al., 2013), the large reduction of crystalline cellulose DP by the steam explosion pretreatment, rather than increase of cellulose CrI, should be the main factor enhancing biomass enzymatic saccharification in cotton stalks. However, as discussed above, the cellulose CrI in the raw stalks, other than DP, should be the main factor on biomass enzymatic digestibility under acid and alkali pretreatments (Table 3).

Furthermore, hemicellulosic monosaccharides and lignin monomers (monolignols) were determined in Gb and Gh cotton stalks (Tables 4 and 5). In the raw stalks, Gb showed a much lower Xyl/Ara ratio value (11.0) than that of Gh (26.0; Table 4), consistent with the previous findings about the Xyl/Ara being the negative factor on biomass enzymatic digestibility in the grass plants examined (Li et al., 2013b,2014b,c; Wu et al., 2013; Jia et al., 2014). Despite that Gb and Gh showed a similar lignocellulose CrI and crystalline cellulose DP in the steam-exploded stalks (Table 3), Gb remained a much lower Xyl/Ara value (42.6) than that of Gh (73.2) in the steam-exploded residues (Table 4), which could interpret that Gb maintained a consistently higher biomass enzymatic digestibility than that of Gh under the second-step dilute acid pretreatments (Fig. 2).

Lignin has dual roles in biomass enzymatic saccharification, due to the proportions of three monolignols (*S*, *G*, *H*) distinctive in different grass plants examined (Xu et al., 2012; Wu et al., 2013; Li et al., 2014b; Jia et al., 2014). In this study, three monolignol proportions (*S*/*G*, *H*/*C*, *S*/*H* ratios) exhibited a large variation in both raw stalks and steam-exploded residues between Gb and Gh species. However, the cotton stalks had an extremely low *H* monomer level, greatly different from the other grass plants examined (Xu et al., 2012; Wu et al., 2013; Li et al., 2014b; Jia et al., 2014). Notably, despite that total lignin was not significantly extracted with the steam explosion (Table S1), both Gb and Gh respectively showed much lower *S*/*H* values (8.46, 3.21) in the steam-exploded residues than ones (42.0, 76.1) in the raw stalks (Table 5), due to a much higher *H* level in the steam-exploded residues. Hence, it suggests that the *H* level may mainly affect the lignin–hemicelluloses co-extraction with the steam explosion for enhancing biomass saccharification in cotton stalks.

Table 4
Monosaccharide composition (% of total) of hemicelluloses in the raw stalks and steam-exploded stalk residues.

Biomass	Species	Rha (%)	Fuc (%)	Ara (%)	Xyl (%)	Man (%)	Glu (%)	Gal (%)	Xyl/Ara
Raw stalks	Gb	3.5	0.2	7.1	78.1	3.2	1.4	6.5	11.0
	Gh	2.8	0.2	3.4	88.0	1.4	0.6	3.5	26.0
Steam-exploded residues	Gb	0.7	0.4	2.0	84.3	4.0	6.6	2.0	42.6
	Gh	0.4	0.4	1.1	80.3	2.9	13.7	1.2	73.2

Table 5
Three monolignol composition (% of total) in the raw stalks and steam-exploded stalk residues.

Biomass	Species	*S* (%)	*G* (%)	*H* (%)	*S*/*G*	*H*/*G*	*S*/*H*
Raw stalks	Gb	45.50	53.40	1.10	0.852	0.02	42.0
	Gh	45.80	53.60	0.60	0.854	0.011	76.1
Steam-exploded residues	Gb	54.10	39.60	6.40	1.37	0.162	8.46
	Gh	33.30	56.40	10.30	0.59	0.183	3.21

4. Conclusion

Steam explosion pretreatments of cotton stalks have caused 5–6 folds enhancements on biomass enzymatic saccharification distinctive in Gb and Gh species by largely reducing crystalline cellulose DP and extracting hemicelluloses. Sequential dilute-acid (1% H_2SO_4) pretreatment of the steam-exploded stalks could not only further increase biomass digestibility, but also lead to the highest sugar–ethanol conversion rate among all pretreatments performed in this study, probably by reducing the toxin compounds that inhibit yeast fermentation. As a contrast, pretreatments of raw stalks with extremely high concentration alkali (16% NaOH) have caused the highest biomass saccharification, but remain the lowest sugar–ethanol conversion rate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.01.020.

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