Bioresource Technology 167 (2014) 14–23

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Sugar-rich sweet sorghum is distinctively affected by wall polymer features for biomass digestibility and ethanol fermentation in bagasse

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HIGHLIGHTS

- Rich soluble sugar did not affect biomass digestibility in sweet sorghum bagasse.
- Two features (DP and Xyl/Ara) affected lignocellulose CrI for high biomass digestion.
- G-monomer determined lignin effects on biomass digestibility and yeast fermentation.
- Hemicelluloses and lignin affected biomass enzymatic digestions by distinct ways.
- Suggested potential genetic modification in sweet sorghum for high biofuels.

ARTICLE INFO

Article history:
Received 25 January 2014
Received in revised form 23 April 2014
Accepted 26 April 2014
Available online 10 May 2014

Keywords:
Sweet sorghum
Soluble sugar
Dry bagasse
Plant cell walls
Biomass digestibility

ABSTRACT

Sweet sorghum has been regarded as a typical species for rich soluble-sugar and high lignocellulose residues, but their effects on biomass digestibility remain unclear. In this study, we examined total 63 representative sweet sorghum accessions that displayed a varied sugar level at stalk and diverse cell wall composition at bagasse. Correlative analysis showed that both soluble-sugar and dry-bagasse could not significantly affect lignocellulose saccharification under chemical pretreatments. Comparative analyses of five typical pairs of samples indicated that DP of crystalline cellulose and arabinose substitution degree of non-KOH-extractable hemicelluloses distinctively affected lignocellulose crystallinity for high biomass digestibility. By comparison, lignin could not alter lignocellulose crystallinity, but the KOH-extractable G-monomer predominately determined lignin negative impacts on biomass digestions, and the G-levels released from pretreatments significantly inhibited yeast fermentation. The results also suggested potential genetic approaches for enhancing soluble-sugar level and lignocellulose digestibility and reducing ethanol conversion inhibition in sweet sorghum.

1. Introduction

Global energy crisis and greenhouse gas emissions have drawn the attention on renewable and sustainable fuels, such as the production of energy plants as biofuel feedstocks (Cotton et al., 2013). Among annual herbaceous crops, sweet sorghum (*Sorghum bicolor* L.) has emerged as one of leading bioenergy crops, because it contains large amounts of fermentable soluble sugar at stalk and degradable lignocellulose at bagasse (Zegada-Lizarazu and Monti, 2012; Zegada-Lizarazu et al., 2012). Despite sweet sorghum has been briefly reported about its soluble sugar and bagasse residue applications on biofuels, little is known about the effects of soluble sugar on bagasse yield and biomass saccharification (Mallikarjun et al., 1998; Kumar and Kothari, 1999).

Plant cell wall represents a major biomass resource for biofuels and chemical products. Principally, biomass conversion into bioethanol involves three major steps: initial physical and chemical pretreatments towards cell wall destruction, consequential

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http://dx.doi.org/10.1016/j.biortech.2014.04.086
0960-8524/

enzymatic hydrolysis leading to soluble sugar release, and final yeast fermentation for ethanol production. However, plant cell wall recalcitrance largely determines an unacceptably costly biomass process with numerous compounds released that inhibit yeast fermentation. As a promising solution, genetic modification of plant cell walls has been proposed for reducing biomass recalcitrance in bioenergy crops (Xie and Peng, 2011). Hence, it becomes essential to identify the key factors of plant cell walls that affect both biomass enzymatic digestion and ethanol conversion upon various pretreatments in bioenergy crops. In terms of sweet sorghum, it is also important to find out the soluble sugar impact on lignocellulosic saccharification.

Plant cell walls are composed primarily of cellulose, hemicelluloses and lignin. Cellulose is a linear polymer composed of β-1,4-glucans (Arioli et al., 1998), and its crystalline index (CrI) and degree of polymerization (DP) have been characterized as the major features that affect biomass enzymatic hydrolyses in plants (Zhang et al., 2013). Hemicelluloses are a heterogeneous class of polysaccharides, and xylans are major components of hemicelluloses in grasses (Scheller and Ulvskov, 2010). It has been reported that either total hemicelluloses level or arabinose substitution degree of xylans can negatively affect cellulose crystallinity for high biomass enzymatic digestibility in *Miscanthus* (Xu et al., 2012; Li et al., 2013). However, hemicelluloses levels are not the major factor on biomass saccharification upon various chemical pretreatments in wheat (Wu et al., 2013). Lignin is a complex composed of three major phenolic components: *p*-coumaryl (H), coniferyl (G), and sinapyl (S) alcohols (Ralph et al., 2004). Due to its structural diversity and heterogeneity, lignin has been recently characterized with dual roles in biomass enzymatic digestions, but much remains unknown in different plants (Boudet et al., 2003; Ziebell et al., 2010; Xu et al., 2012; Wu et al., 2013).

Initial acid and alkali chemical pretreatments have been broadly used for biomass process (MacDonald et al., 1983; Hendriks and Zeeman, 2009; Garlock et al., 2011). As a consequence, a wide range of compounds can be released or formed from chemical pretreatments as inhibitors on yeast fermentation. Among the inhibitors, the phenolic compounds released from lignin are considered with a major inhibiting role in yeast fermentation, but little remains known about lignin influence on ethanol product in plants, in particular on sweet sorghum (Heredia-Olea et al., 2012,2013).

Over the past years, various physical and chemical pretreatments have been applied for bagasse digestions in sweet sorghum (Himmel et al., 2007; McIntosh and Vancov, 2010; Zhang et al., 2011; Chen et al., 2012; Matsakas and Christakopoulos, 2012), it has little been reported about the main factors of plant cell walls on biomass enzymatic digestibility and yeast fermentation (Billa et al., 1997). In this study, we initially performed a large-scale analysis of total 63 representative sweet sorghum accessions that displayed a diverse cell wall composition and varied bagasse biomass digestibility. Then we examined five typical pairs of sweet sorghum samples in order to rule out the effects of wall polymer features on biomass enzymatic digestibility and yeast fermentation under various chemical pretreatments.

2. Methods

2.1. Collection of sweet sorghum samples

Total 63 sweet sorghum germplasm accessions collected worldwide were grown in Hubei experimental field in 2011, and the mature stalks were harvested from 90 to 140 days after sowing. The stalks tissues were dried at 50 °C for 36 h after inactivation at 105 °C for 20 min, ground into powders through a 40 mesh screen as biomass samples, and stored in a dry container until use. All samples were prepared in biological triplicates.

2.2. Measurements of soluble sugars and dry bagasse

To determine total soluble sugars, the biomass sample (0.3 g) was added with 6 mL distilled water, and shaken at 150 rpm for 2 h at 50 °C. After centrifugation at 3000*g* for 10 min, the biomass residues were washed five times with distilled water, and all supernatants were collected as total soluble sugars (pentoses and hexoses) released from stalks. Soluble sugars were detected by colorimetric assay as described below, and dry bagasse was calculated according to subtraction between dry biomass and soluble sugars. All experiments were performed in biological triplicates.

2.3. Plant cell wall fractionation

Cellulose and hemicelluloses were extracted using the plant cell wall fractionation method described by Peng et al. (2000) with minor modifications suggested by Wu et al. (2013). The soluble sugar, lipids, starch and pectin of the biomass samples were consecutively removed by 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. The remaining pellet was extracted with 4 M KOH with 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and washed with distilled water until soluble sugars were not detectable. The combined supernatant was then neutralized, dialyzed and lyophilized as KOH-extractable hemicelluloses. The remaining pellet was extracted with H_2SO_4 (67%, v/v) for 1 h at 25 °C and the supernatants were collected for determination of free hexoses and pentoses as total cellulose and non-KOH-extractable hemicelluloses. All experiments were conducted in biological triplicates.

2.4. Colorimetric assay of hexoses and pentoses

Hexoses and pentoses were assayed using an UV–VIS spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) according to Xu et al. (2012). Hexoses were detected by the anthrone/H_2SO_4 method (Fry, 1988), and pentoses were assayed using the orcinol/HCl method (Dische, 1962). The standard curves for hexoses and pentoses were plotted using D-glucose and D-xylose as standards (purchased from Sinopharm Chemical Reagent Co., Ltd.). Considering the high pentoses level can affect the absorbance at 620 nm for hexoses content, the deduction from pentoses at 660 nm was carried out for final hexoses calculation. A series of xylose concentrations were analyzed for plotting the standard curve referred for the deduction, verified by GC–MS analysis. All experiments were carried out in biological triplicates.

2.5. Determination of hemicelluloses level and monosaccharide composition

Hemicelluloses level was calculated based on determinations of total hexoses and pentoses as described above. Monosaccharide composition of hemicelluloses was determined by GC–MS as described by Xu et al. (2012) and Li et al. (2013). Acid hydrolysis: The hemicelluloses sample was dialyzed for 36 h after neutralization with acetic acid. The dialyzed sample dissolved in 2.5 mL TFA (2 M) was heated in a sealed tube at 121 °C in an autoclave (15 psi) for 1 h. *Myo*-inositol (200 μg) was added as the internal standard. Derivatisation of monosaccharides to alditol acetates: Distilled water (800 μL) and a freshly prepared solution of $NaBH_4$ (400 μL, 100 mg/mL in 6.5 M aqueous NH_3) were added to each sample. Sample was capped, mixed well and incubated at 40 °C for 30 min. Excess $NaBH_4$ was decomposed by adding acetic acid (800 μL). 400 μL sample was then moved into a 25 mL glass tube.

Acetic anhydride (4 mL) was added and the solution was shaken again. Then 1-methylimidazole (600 μL) was added. After mixing, the sample was allowed to stand for 10 min, and excess acetic anhydride was decomposed by adding distilled water (10 mL). Then dichloromethane (3 mL) was added, mixed gently, and centrifuged (2000*g*, 10 s) for phase separation. After removing the upper phase, the lower phase was washed with distilled water (3 × 20.0 mL). The collected lower phase was dehydrated by adding anhydrous sodium sulfate and stored at −20 °C until analysis by GC–MS (SHIMADZU GCMS-QP2010 Plus).

GC–MS Analytical Conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25 μm 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 0.999 or better.

2.6. Detection of total lignin and monolignin composition

Total lignin level of biomass samples was detected by two-step acid hydrolysis method according to analytical procedure of the National Renewable Energy Laboratory with minor modification by Wu et al. (2013). The acid-insoluble lignin was calculated gravimetrically after correction for ash, and the acid-soluble lignin was measured using UV spectroscopy. All experiments were carried out in biological triplicates.

Monolignin determination was described by Li et al. (2013) and Wu et al. (2013) with minor modification. Standard chemicals: *p*-Hydroxybenzaldehyde (H), vanillin (G) and syringaldehyde (S) were purchased from Sinopharm Chemical Reagent Co., Ltd. After removal of soluble sugars from biomass sample, the bagasse was extracted with benzene-ethanol (2:1, v/v) in a Soxhlet for 4 h, and the remaining pellet was collected as cell wall residue (CWR). The supernatants from 1% NaOH and 1% H_2SO_4 pretreatments as described below, were neutralized with HCl and NaOH respectively, and then mixed with 10 mL 4 M NaOH as wall pretreatment supernatant (WPS). About 0.05 g CWR was added with 5 mL 2 M NaOH and 0.5 mL nitrobenzene, and a stir bar was put into a 25 mL Teflon gasket in a stainless steel bomb for monolignin detection of CWR. 10 mL WPS was added with 1 mL nitrobenzene, and a stir bar was put into a 25 mL Teflon gasket in a stainless steel bomb for WPS detection.

The bomb was tightly sealed and heated at 170 °C (oil bath) for 3.5 h and stirred at 20 rpm. The bomb was then cooled with cold water. The chromatographic internal standard (ethyl vanillin) was added to the oxidation mixture. The alkaline oxidation mixture was washed 3 times with 30 mL CH_2Cl_2/ethyl acetate mixture (1/1, v/v) to remove nitrobenzene and its reduction by-products. The alkaline solution was acidified to pH 3.0–4.0 with 6 M HCl, and then extracted with CH_2Cl_2/ethyl acetate (3 × 30 mL) to obtain the lignin oxidation products which were in the organic phase. The organic extracts were evaporated to dryness under reduced pressure (20 mm Hg) at 40 °C. The oxidation products were dissolved in 10 mL chromatographic-grade pure methanol.

HPLC analysis: The solution was filtered with membrane filter (0.22 μm). 20 μL solution was injected into HPLC (Waters 1525 HPLC) column Kromat Universil C18 (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.

2.7. Detection of cellulose level and features

Celluloses sample was dissolved in 67% H_2SO_4 (v/v) for 1 h at 25 °C, and the hexoses were detected by the anthrone/H_2SO_4 method as cellulose level.

Degree of polymerization (DP) of cellulose was described by Zhang et al. (2013) using the viscosity method subjective to the equation: $DP^{0.905} = 0.75\ [\eta]$ (Puri, 1984; Kumar and Kothari, 1999). All experiments were performed at 25 ± 0.5 °C using an Ubbelohde viscosity meter and cupriethylenediamine hydroxide (Cuen) as the solvent. 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_{rel} was calculated using the relationship: $\eta_{rel} = t/t_0$, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent, respectively. Each sample was measured in triplicate.

Crystallinity index (CrI) of lignocellulose was detected by X-ray diffraction (XRD) method using Rigaku-D/MAX instrument (Uitima III, Japan) as described by Zhang et al. (2013) with minor modification. The mixed-well powders of dry bagasse were laid on the glass sample holder (35 × 50 × 5 mm) and were analyzed under plateau conditions. Ni-filtered Cu Kα radiation ($\lambda = 0.154056$ nm) generated at voltage of 40 kV and current of 18 mA, and scanned at speed of 0.0197°/s from 10° to 45°. The crystallinity index (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}, $\theta = 18.5°$) as the follow: $CrI = 100 \times (I_{200} - I_{am})/I_{200}$. 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.

2.8. Observation of biomass residue surface

Bagasse samples were pretreated with 1% NaOH or 1% H_2SO_4, and hydrolyzed with the mixed-cellulases. The remaining residues were washed with distilled water until pH 7.0. The surface morphology of the sample was sputter-coated with gold and observed by scanning electron microscope (SEM JSM-6390/LV, Hitachi, Tokyo, Japan) as described by Xu et al. (2012).

2.9. Analysis of biomass enzymatic digestibility

Chemical pretreatment methods were described by Huang et al. (2012) with minor modifications. H_2SO_4 pretreatment: the bagasse sample was washed 5 times with 10 mL distilled water, and added with 6 mL H_2SO_4 at three concentrations (0.25%, 1%, 4%, v/v), respectively. The sample tube was sealed and heated at 121 °C for 20 min in autoclave (15 psi) after mixing well, and then shaken at 150 rpm for 2 h at 50 °C. After centrifugation at 3000*g* for 5 min, the pellet was washed three times with 10 mL distilled water and stored for enzyme hydrolysis. NaOH pretreatment: The bagasse sample was washed 5 times with 10 mL distilled water, and added with 6 mL NaOH at three concentrations (0.5%, 1%, 4%, w/v). The sample tube was shaken at 150 rpm for 2 h at 50 °C. After centrifugation at 3000*g* for 5 min, the pellet was washed three times with 10 mL distilled water and stored for enzyme hydrolysis. All experiments were conducted in biological triplicates.

The bagasse samples from above pretreatments were washed 5 times with 6 mL distilled water, and once with 10 mL mixed-cellulases reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8). The washed residues were added with 0.16% (w/v) 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 from Imperial Jade

Bio-technology Co., Ltd) to 6 mL. During the enzymatic hydrolysis, the samples were shaken under 150 rpm at 50 °C for 48 h. After centrifugation at 3000*g* for 10 min, the supernatants were collected for total sugar (pentoses and hexoses) assay. The samples with 6 mL reaction buffer were shaken for 48 h at 50 °C as the control. All samples were carried out in biological triplicates.

2.10. Ethanol fermentation

The supernatants from 1% NaOH and 1% H_2SO_4 pretreatments of bagasse were respectively neutralized with HCl and NaOH, mixed with cellulases hydrolysates (10.00 mL), and added to the fermentation tube along with appropriate amount of glucose to 200.00 g/L hexose in all fermentation vessels. All samples were sterilized in an autoclave at 0.15 Mpa, 121 °C for 20 min.

The fermentations were conducted using *Saccharomyces cerevisiae* for ethanol production (Angel yeast Co., Ltd., Yichang, 443000, China). The yeast powder was suspended in an appropriate amount of pH 4.8 phosphate buffer to achieve an inoculum consisting of 2.00 g/L (cell dry weight) in all fermentation vessels. The fermentation was performed in 30 mL glass test tube sealed with rubber plugs to allow the liberation of CO_2. The glass test tubes were incubated at 37 °C for 48 h.

Ethanol was measured using $K_2Cr_2O_7$ method. The fermentation liquid was distilled at 100 °C for 15 min to achieve ethanol liquor. Appropriate amount of ethanol sample in 2.00 mL 5.00% $K_2Cr_2O_7$ (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 water bath. After cooling, distilled water was added to 10.00 mL, and the absorbance was read at 600 nm. Absolute ethanol was taken as the standard.

The sugar-bioethanol conversion rate at the end of fermentation was calculated according to the formula: E-C = $E/A/H \times 100\%$ [E-C: 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 converted to ethanol according to Embden-Meyerhof-Parnas (EMP) pathways in *S. cerevisiae*; *H*: total hexose weigh (g) at the beginning of fermentation]. All experiments were carried out in biological triplicates.

2.11. Statistical calculation of correlation coefficients

Correlation coefficients were calculated by performing Spearman rank correlation analysis for all pairs of measured traits across the whole population. This analysis used average values calculated from all original determinations for a given traits pair.

3. Results and discussion

3.1. Diversity of soluble sugar and wall polymers in sweet sorghum samples

Among the C4 grasses, sweet sorghum is typically rich in soluble sugar at the stalk. In this study, we initially detected a large varied soluble sugar level in the total 63 representative sweet sorghum samples that world-wide collected (Fig. 1A). The soluble sugar levels were ranged from 0.5% to 40.82% (% dry matter) with the coefficient variation (CV) at 63.87%. Meanwhile, we measured a diverse dry bagasse level ranged from 5.48 g to 183.3 g with relatively high CV at 74.78% (Fig. 1B). As the total 63 sweet sorghum accessions exhibited various ecological types and genetic germplasms, we also determined a diverse cell wall composition (cellulose, hemicelluloses, and lignin) in the bagasse (Fig. 1C). But, the three major wall polymers had much lower CV values at 7.63%, 8.02% and 10.52%, compared with the sugar and bagasse levels (Details can be found in Electronic Annex Table A.1).

As the 63 sweet sorghum displayed a large population and a wide sampling distribution, we further performed a correlation among the soluble sugar, dry bagasse and three major wall polymers. Notably, a significantly positive correlation was observed between soluble sugar levels and dry bagasse contents at $p < 0.01$ (Fig. 1D), indicating that biomass production could not affect sugar accumulation in sweet sorghum. Furthermore, we detected that cellulose and hemicelluloses, two major wall polysaccharides, were not correlated with either soluble sugar or dry bagasse levels (Fig. 1D and E), suggesting that the soluble sugar level was not the major factor that affects carbon partitioning on wall polysaccharides biosyntheses in sweet sorghum. By comparison, lignin level showed a positive correlation at $p < 0.01$ with the soluble sugar and dry bagasse levels, indicating that lignin should be an important factor for soluble sugar accumulation and biomass production in sweet sorghum (Details can be found in Electronic Annex Table A.2). Hence, the results concluded that sweet sorghum is a typical species of rich soluble sugar and high biomass product, and it could be considered as one of the leading bioenergy crops.

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

In our previous studies, biomass enzymatic digestibility (saccharification) has been defined by calculating the hexoses yield (% cellulose) released from hydrolysis by a crude cellulase mixture of lignocellulose after pretreatment (Xu et al., 2012; Li et al., 2013; Wu et al., 2013). In the present study, we determined the hexoses yields (% cellulose) released from enzymatic hydrolysis after 1% NaOH pretreatments of the bagasses in total 63 sweet sorghum accessions. As a result, the sweet sorghum samples displayed large variations of hexoses yields from 37% to 100% (Fig. 2A). Notably, the three quarters of sweet sorghum samples showed their hexoses yields at more than 60%, whereas at least one quarter of samples had the hexoses yields at above 80%. Hence, the results indicated that the sweet sorghum bagasse had a relatively high biomass enzymatic digestibility, compared with *Miscanthus* and wheat species (Xu et al., 2012; Wu et al., 2013).

Soluble sugar (% per dry stalk) and dry bagasse (g per dry stalk) have been considered as major agronomic traits for bioenergy purposes in sweet sorghum (Mallikarjun et al., 1998; Zegada-Lizarazu et al., 2012). However, little is known about their effects on lignocellulose enzymatic digestibility. As the 63 sweet sorghum samples displayed a normal distribution on hexoses yields (Fig. 2A), we performed a correlation among soluble sugar, dry bagasse and biomass enzymatic digestibility (Fig. 2). As a result, the soluble sugar and dry bagasse levels were not significantly correlated with the hexoses yields released by enzymatic hydrolysis after 1% NaOH pretreatments (Fig. 2B and C), indicating that both agronomic traits were not the factors affecting biomass digestibility in sweet sorghum bagasse. By comparison, cellulose and lignin displayed a negative correlation with hexoses yields at $p < 0.01$, whereas hemicelluloses were not significantly correlated (Fig. 2D), different from *Miscanthus* observation on hemicelluloses positive effect (Xu et al., 2012). Hence, the results suggested that increasing soluble sugar and dry bagasse levels could not affect lignocellulose enzymatic digestibility in sweet sorghum.

3.3. Analysis of five typical pairs of sweet sorghum samples

To test three major wall polymers impacts on biomass digestibility, we selected five typical pairs of sweet sorghum samples (Table 1) and compared their hexoses yields released from enzymatic hydrolysis under various chemical pretreatments (Fig. 3).

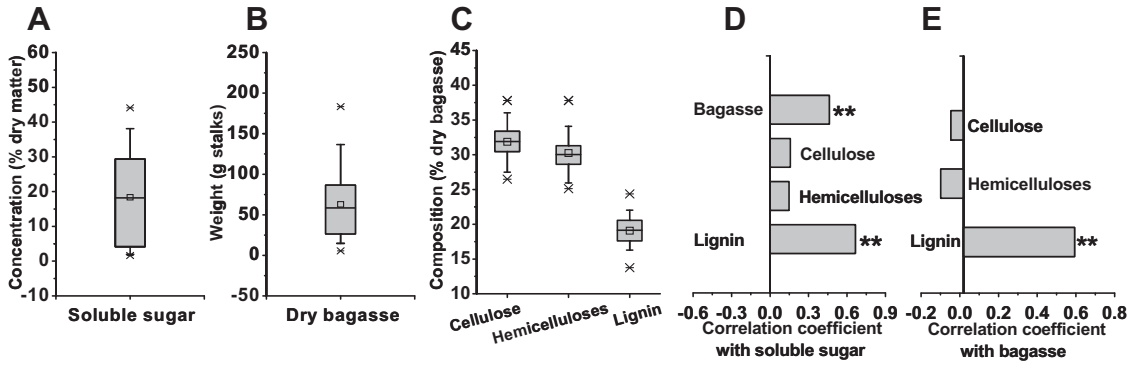

Fig. 1. Variations of soluble sugar, dry bagasse and cell wall composition, and their correlations in total 63 representative sweet sorghum accessions: (A) soluble sugar; (B) dry bagasse; (C) cell wall composition; (D) and (E) correlations among soluble sugar, dry bagasse and three major wall polymers (cellulose, hemicelluloses, and lignin).

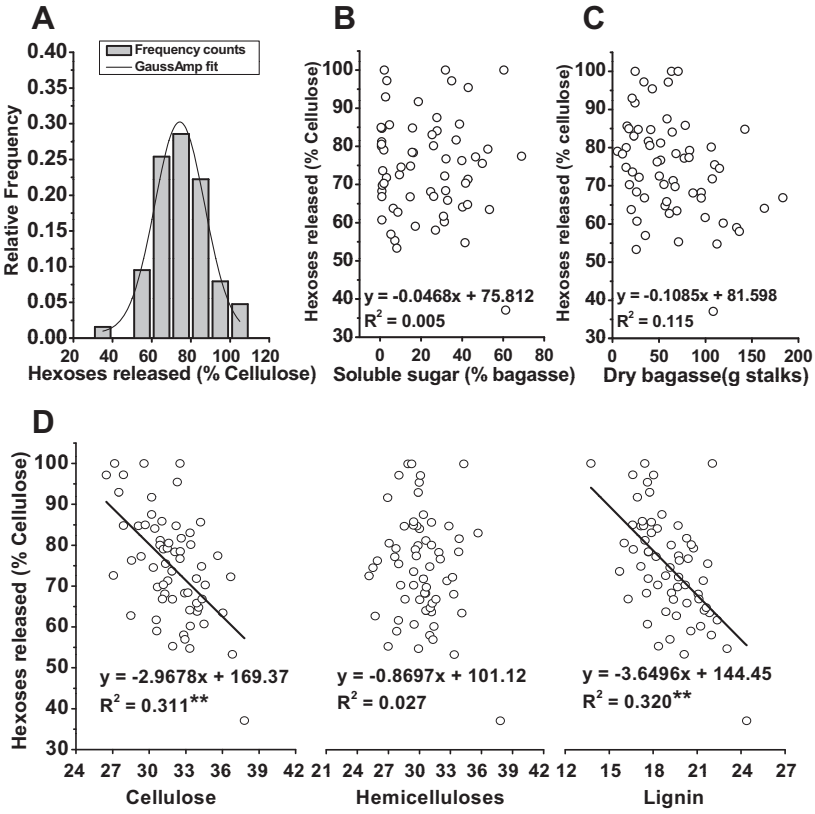

Fig. 2. Effects of soluble sugar, dry bagasse and wall polymers on hexoses yields (% cellulose) released from enzymatic hydrolysis after 1% NaOH pretreatment in the 63 representative sweet sorghum accessions ($n = 63$): (A) sampling distribution on hexoses yields; (B) correlation between soluble sugar and hexoses yields; (C) correlation between dry bagasse and hexoses yields; (D) correlation between three major wall polymers and hexoses yields. ** Indicated significant correlation at $p < 0.01$ level.

In the first three pairs (I-1, I-2, I-3), each displayed a significant difference ($p < 0.01$, $n = 3$) of single wall polymer (cellulose, hemicelluloses, and lignin) by 13.13%, 16.41% and 32.27%, respectively (Table 1). By comparison, other two wall polymers of each pair were only altered by less than 6% at insignificant levels ($p > 0.05$, $n = 3$). Hence, the three pairs of sample can be applied to test effect of single wall polymer level on biomass enzymatic digestibility in sweet sorghum.

The NS113 and NS10 samples with relatively lower cellulose and lignin levels at Pairs I-1 and I-3, respectively displayed much higher hexoses yields up to 1.4-folds at $p < 0.01$, compared to the other paired samples (NS65, NS199) after pretreatments with NaOH and H_2SO_4 at three concentrations (Fig. 3). Notably, both NS113 and NS10 samples could even have a complete lignocellulose enzymatic hydrolysis (100% hexoses yields) when treated with 4% NaOH. As a comparison, NS199 sample at Pair II with lower hemicelluloses level, did not show much higher hexoses yields (1.1-folds) than that of its paired sample (NS25), in particular when treated with H_2SO_4 (Fig. 3). Therefore, the results were consistent with previous reports about three major wall polymers impacts on biomass enzymatic saccharification in sweet sorghum bagasse (Fig. 2D).

Furthermore, we detected the Pairs II samples (NS171, NS76) that displayed both cellulose and lignin reductions by 21.2% and 29.7% at $p < 0.01$ (Table 1). Despite that cellulose and lignin levels were much decreased at Pair II, such reductions could not cause much more hexoses yields increased (only up to 1.4 and 1.5-folds), compared with the Pairs I-1 and I-3 (Fig. 3). The data suggested

Table 1
Cell wall compositions in five typical pairs of sweet sorghum samples. The bold values in it represented the comparative cell wall compounds.

Pair	Samples	Cell wall composition (% dry bagasse)					
		Cellulose		Hemicelluloses		Lignin	
I-1	**NS113(H)**[&]	**32.53 ± 0.64**[**]	**−13.13%**[@]	32.01 ± 0.40	−4.22%	20.38 ± 0.32	1.30%
	NS65(L)	**36.80 ± 0.12**		33.36 ± 0.80		20.12 ± 0.21	
I-2	**NS199**	30.58 ± 0.84	2.03%	**27.97 ± 0.40**[**]	**−16.41%**	22.38 ± 0.68	−5.14%
	NS25	31.20 ± 0.68		**32.56 ± 0.67**		21.23 ± 1.01	
I-3	**NS10(H)**	30.20 ± 0.25	−1.26%	26.93 ± 0.27	−3.86%	**16.92 ± 0.18**[**]	**−32.27%**
	NS199(L)	30.58 ± 0.84		27.97 ± 0.40		**22.38 ± 0.68**	
II	**NS171(H)**	**27.50 ± 0.18**[**]	**−21.20%**	29.89 ± 0.47	−0.10%	**17.76 ± 0.32**[**]	**−29.73%**
	NS76(L)	**33.33 ± 0.59**		29.92 ± 0.47		**23.04 ± 0.25**	
III	**NS148(H)**	33.41 ± 0.27	−1.44%	31.16 ± 0.07	−0.26%	20.59 ± 0.68	1.41%
	NS151(L)	33.89 ± 0.32		31.24 ± 0.02		20.30 ± 0.27	

[&] (H) or (L) indicated the sample in the pair with relatively high (H) or low (L) biomass digestibility.
[**] A significant difference at pair by *t*-test at $p < 0.01$ ($n = 3$).
[@] Percentage of the increased or decreased level at pair: subtraction of two samples divided by low value at pair.

that both cellulose and lignin should not have the synergistic effects on biomass enzymatic saccharification in sweet sorghum bagasse. In addition, we determined the Pair III samples that exhibited a close cell wall composition with three major wall polymer alterations by less than 2% at insignificant levels ($p > 0.05$; Table 1). However, the NS148 sample at Pair III also had the higher hexoses yields than that of its paired sample (NS151) up to 1.2-folds at $p < 0.01$ levels (Fig. 3). Hence, the results indicated that the wall polymer features should also play an important role in biomass enzymatic digestions in sweet sorghum (Details can be found in Electronic Annex Table A.3).

3.4. Observation of biomass residue surfaces

Using scanning electron microscopy, we further observed the biomass residues in the five typical pairs of sweet sorghum samples after pretreatments with 1% NaOH and 1% H_2SO_4 and sequential enzymatic hydrolysis. SEM images of the bagasse residue surfaces can be found in Electronic Annex Fig. A.1 and A.2. The samples (NS113, NS10, NS171, and NS148) with relatively higher hexoses yields at four pairs (I-1, I-3, II, III), displayed a coarse biomass residue surface, whereas their paired samples (NS65, NS199, NS76, and NS151) exhibited a relatively smooth surfaces when treated with 1% NaOH or 1% H_2SO_4 pretreatment. Notably, the four pairs of sweet sorghum samples could even show much more profound coarse faces of biomass residues from the sequential enzymatic hydrolysis after pretreatment. Hence, the sweet sorghum showing a rough biomass residue face is attributed to a relatively effective enzymatic hydrolysis, similar to the observations in *Miscanthus*, rice and wheat (Xu et al., 2012; Li et al., 2013; Wu et al., 2013). By comparison, the two samples (NS199, NS25) of Pair I-2 did not show any visible differences on the biomass residue surfaces either from 1% NaOH and 1% H_2SO_4 pretreatment, or from sequential enzymatic digestions, consistent with

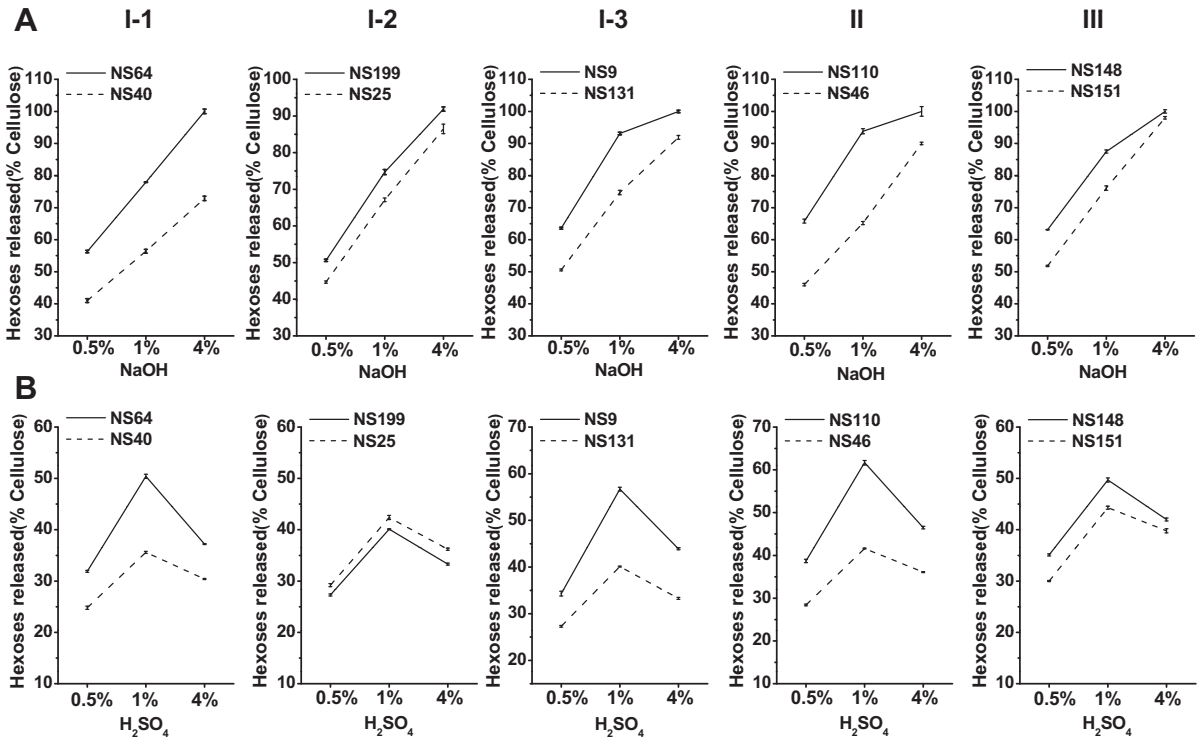

Fig. 3. Biomass enzymatic digestibility in the five typical pairs of sweet sorghum samples: (A) hexoses yields (% cellulose) under NaOH pretreatments with three concentrations; (B) hexoses yields under H_2SO_4 pretreatments with three concentrations. Bar indicated as SD ($n = 3$).

their close biomass enzymatic saccharification. In addition, as the Pair III samples had a similar cell wall composition (Table 1), it suggested that the distinct residue surfaces should be due to their wall polymer feature impact.

3.5. Effects of wall polymer features on biomass saccharification

Cell wall polymer features have been characterized with distinct effects on biomass enzymatic digestibility in *Miscanthus*, rice and wheat (Li et al., 2013; Wu et al., 2013; Zhang et al., 2013). In this study, we detected three major wall polymer features in the five pairs of sweet sorghum samples (Fig. 4). With respect to the cellulose features, we examined crystalline index (CrI) of raw material and degree of polymerization (DP) of crystalline cellulose. In general, the sweet sorghum samples with relatively higher hexoses yields at four pairs (I-1, I-3, II, III), showed much lower CrI and DP values than that of their paired samples, but the Pair I-2 samples only displayed a small alteration, consistent with their close hexoses yields (Figs. 3 and 4A). Furthermore, we conducted a correlation between the two features and hexoses yields from NaOH and H_2SO_4 pretreatments at three concentrations (Fig. 4B). A negative correlation was observed at $p < 0.01$ or 0.05 among the five pairs of sweet sorghum samples, except on CrI with 4% H_2SO_4 pretreatment. Hence, the results indicated that both CrI and DP could be applied in accounting for cellulose negative impact on biomass enzymatic digestibility in sweet sorghum (Fig. 2D), as reported in *Miscanthus* and other plants (Zhang et al., 2013; Wu et al., 2013). Lignocellulose crystallinity has been well characterized as the key factor that negatively affects biomass enzymatic digestibility in plants (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005; Xu et al., 2012; Wu et al., 2013). Notably, the crystalline cellulose DP even showed much higher correlative coefficient values than that of the raw material CrI, suggesting that the DP should be another important factor on lignocellulose enzymatic digestions in sweet sorghum.

With regard to hemicelluloses features, we determined monosaccharide compositions of KOH-extractable and non-KOH-extractable hemicelluloses in the five pairs of sweet sorghum samples. Correlative analysis indicated that all six detectable monosaccharides in the two types of hemicelluloses did not show any significant correlation with the hexoses yields from NaOH and H_2SO_4 pretreatments at three concentrations, except mannose with 0.25% and 1% H_2SO_4 pretreatments. As Xyl/Ara ratio has been applied as a negative indicator on Ara substitution degree of xylans in *Miscanthus*, rice and wheat (Xu et al., 2012; Li et al., 2013; Wu et al., 2013), we also calculated the Xyl/Ara values of both

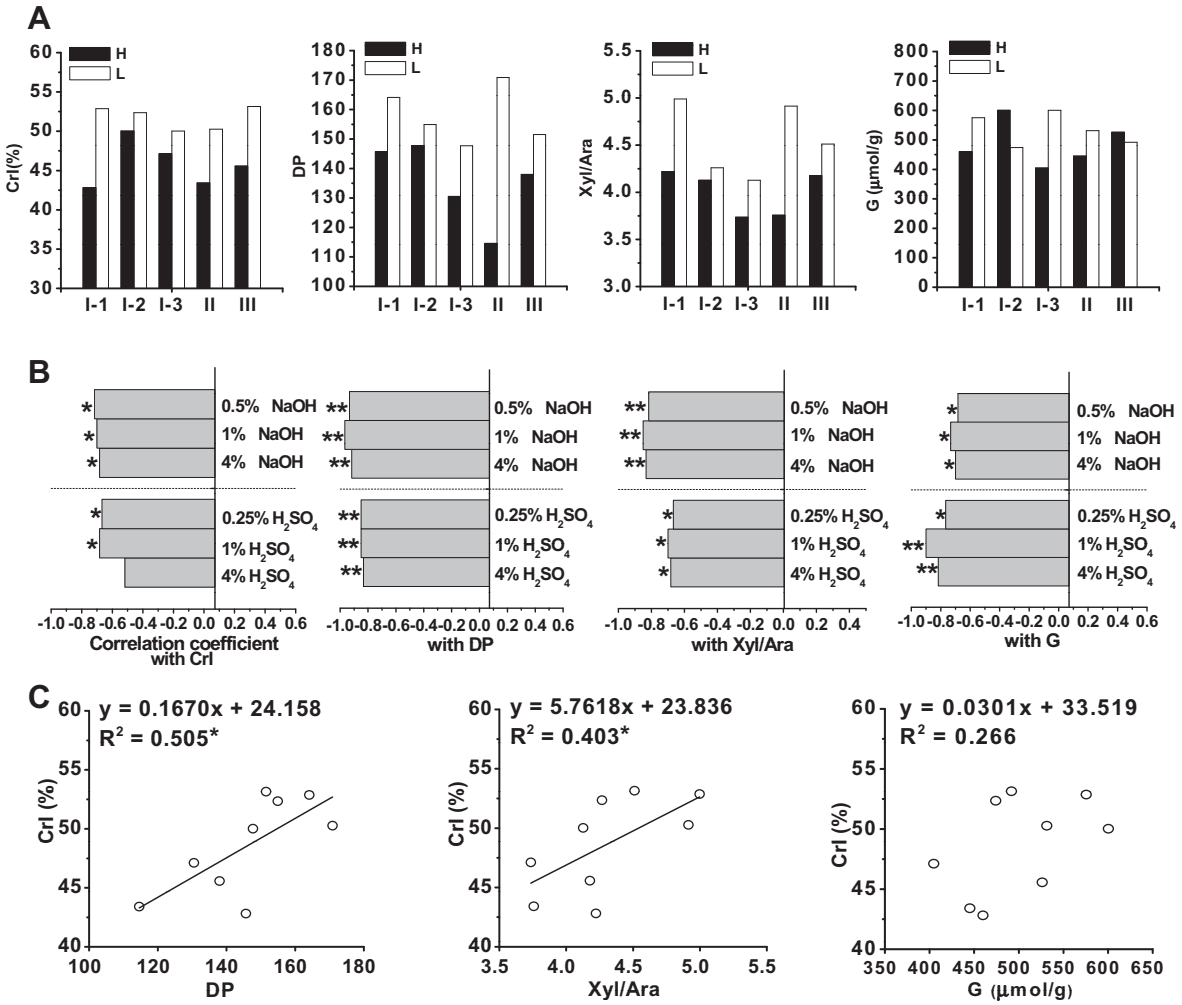

Fig. 4. Effects of wall polymer features on biomass enzymatic digestibility in the five typical pairs of sweet sorghum samples: (A) raw material crystalline index (CrI), degree of polymerization (DP) of crystalline cellulose, Xyl/Ara of non-KOH-extractable hemicelluloses and G-monomer level of KOH-extractable lignin; (B) correlations between four wall polymer features (CrI, DP, Xyl/Ara, G-monomer) and hexoses yields released from enzymatic hydrolyses after NaOH and H_2SO_4 pretreatments with three concentrations; (C) correlations among raw material CrI, crystalline cellulose DP, non-KOH-extractable Xyl/Ara and KOH-extractable G-monomer. * and ** Indicated significant correlations at $p < 0.05$ and 0.01, respectively ($n = 9$); (H) and (L) indicated samples with relatively high (H) and low (L) biomass enzymatic digestibility each pairs of sweet sorghum samples.

KOH-extractable and non-KOH-extractable hemicelluloses in sweet sorghum. As a result, the Xyl/Ara in the non-KOH-extractable hemicelluloses, rather than the KOH-extractable, displayed a negative correlation at $p < 0.01$ or 0.05 with hexoses yields after various chemical pretreatments (Fig. 4B). As a comparison, the samples at four pairs (I-1, I-3, II and III) with relatively higher hexoses yields, displayed much lower Xyl/Ara values than that of their paired samples in the non-KOH-extractable hemicelluloses (Fig. 4A). Similar to the cellulose CrI and DP, the Pair I-2 samples only displayed a small alteration of Xyl/Ara. Hence, it indicated that Ara substitution degree of the non-KOH-extractable hemicelluloses positively affected biomass enzymatic digestibility in sweet sorghum, similar to Xyl/Ara role in *Miscanthus*, rice and wheat (Xu et al., 2012; Li et al., 2013; Wu et al., 2013).

Lignin has been recently reported with the dual impacts on biomass enzymatic digestibility, due to the three monolignin (S, G, H) compositions and ratios (S/G, H/G, S/H) distinctive in various plant species (Boudet et al., 2003; Ziebell et al., 2010; Wu et al., 2013). In this study, we detected three monolignins levels in the KOH-extractable and non-KOH extractable lignin. With regard to the lignin negative impact to biomass enzymatic digestibility (Fig. 2D), only G monomer levels in the KOH-extractable lignin displayed a negative correlation with hexoses yields at $p < 0.01$ or 0.05 from NaOH and H_2SO_4 pretreatments (Fig. 4B). However, Pairs I-2 samples showed a contrast G content and Pair III displayed a close G level (Fig. 4A), consistent with the previous findings that Pair I-2 samples had a similar biomass enzymatic saccharification, and Pair III samples were less altered on hexoses yields than that of Pairs I-1, I-3 and II from various pretreatments (Fig. 3). Despite that three monolignin ratios have been reported as the important features affecting biomass digestibility in *Miscanthus* and other plants, this study did not show any significant correlation between three monolignin ratios (S/G, H/G, S/H) and hexoses yields in the two types of lignin, suggesting that the lignin affected biomass digestibility by different way in sweet sorghum.

3.6. Mechanism on biomass enzymatic digestion in sweet sorghum

Lignocellulose crystallinity has been well characterized as the key factor that negatively affects biomass enzymatic digestibility in plants (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005; Zhang et al., 2013). To understand biomass enzymatic saccharification in sweet sorghum, we further performed correlation analyses between raw material CrI and three major wall polymers features (DP, Xrl/Ara, G-monomer) in the five typical pairs of samples (Fig. 4C). As a result, the raw material CrI exhibited a positive correlation with either the crystalline cellulose DP or the non-KOH-extractable Xyl/Ara at $p < 0.05$ levels, similar to the observations in *Miscanthus*, rice and wheat (Zhang et al., 2013; Li et al., 2013; Wu et al., 2013). 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 in sweet sorghum. In addition, despite that the non-KOH-extractable hemicelluloses cover less than 30% of total, they should be tightly associated with the cellulose microfibrils by hydrogen bonds that could reduce lignocellulose crystallinity. It also suggested that the Ara branched with xylan may have a direct interlinking with β-1,4-glucans for decreasing lignocellulose crystallinity, as discussed in other plants (Li et al., 2013; Wu et al., 2013). As a comparison, the G-monomer did not show any significant correlation with lignocellulose CrI (Fig. 4C), indicating that the lignin should play a different role in biomass enzymatic digestions in sweet sorghum. Hence, the results suggested that lignin may independently form a penetration barrier against the enzyme accessible to cellulose surface, and G-monomer should play a dominant role in monolignin network construction, leading to a low biomass enzymatic digestibility in sweet sorghum.

3.7. Effect of monolignin composition on ethanol fermentation

Ethanol fermentation is the final step for biofuel production after biomass pretreatment and sequential enzymatic hydrolysis (Chen et al., 2012; Cotton et al., 2013). However, various chemical compounds can be released or formed from pretreatments as yeast inhibitors (Ban et al., 2008; Heredia-Olea et al., 2012). In particular, phenolic compounds have been considered as the major inhibitors, but little is known about three monolignin moieties in sweet sorghum (Heredia-Olea et al., 2012,2013). In this study, we conducted an ethanol fermentation using *S. cerevisiae*, and detected monolignin inhibitions by adding 1% NaOH and 1% H_2SO_4 extracts into yeast culture media. Under 1% NaOH pretreatment, the three monolignins released from five pairs of sweet sorghum samples, displayed a significantly negative effect on ethanol conversion rates at $p < 0.01$ or 0.05 levels (Fig. 5A), but G monomer remained a relatively higher coefficient value with R^2 at 0.406 than that of H- and S-monomers with R^2 at 0.315 and 0.319 (Fig. 5B). By comparison, only G monomer released from 1% H_2SO_4 pretreatment showed a negative impact on ethanol conversion rate at $p < 0.05$, whereas the released H- and S-monomers did not display any significant effects. To our knowledge, therefore, it was at first time to report that the G-monomer, other than H- and S-monomers, was the dominant factor not only affecting biomass enzymatic digestions, but also inhibiting yeast fermentation in sweet sorghum (Details can be found in Electronic Annex Table A.4).

3.8. Potential cell wall modification for high biofuel production

Genetic modification of plant cell walls has been considered as a promising solution for reducing lignocellulosic recalcitrance towards high biofuel production in bioenergy crops. Hence, it becomes important to identify the key factors of plant cell walls that affect biomass digestibility and ethanol fermentation rate. As sweet sorghum can provide large amount of soluble sugars for a direct and efficient use in biofuel production, we at first have to rule out the soluble sugar effects on biomass production and lignocellulose process. In the present study, it has been demonstrated that the soluble sugar level could not significantly affect biomass yields and lignocellulose enzymatic digestibility, indicating that the sugar level should not be the main factor on cell wall modification in sweet sorghum. It has also suggested that the sweet sorghum could be attractive as bioenergy crop for both remaining sugars associated to the stalk and the enzymatic saccharification of the bagasse.

As lignocellulose crystallinity (CrI) is a crucially negative factor on lignocellulosic recalcitrance in plants, this study has indicated two major factors of cell walls that could reduce CrI by either increasing Ara substitution degree of non-KOH-extractable hemicelluloses, or decreasing DP of crystalline cellulose in sweet sorghum. Since the non-KOH-extractable hemicelluloses cover less than 30% of total, modification of Ara substitution degree should be a relatively simply work in sweet sorghum, as discussed in *Miscanthus*, rice and wheat (Xu et al., 2012; Li et al., 2013; Wu et al., 2013). In addition, lignin could not affect lignocellulose crystallinity, but its G-monomer has been detected with dominant effects on biomass enzymatic digestibility and ethanol fermentation in sweet sorghum. Hence, modification of G-monomer level should be another genetic approach for both enhancing biomass enzymatic saccharification and reducing inhibition to yeast fermentation in sweet sorghum.

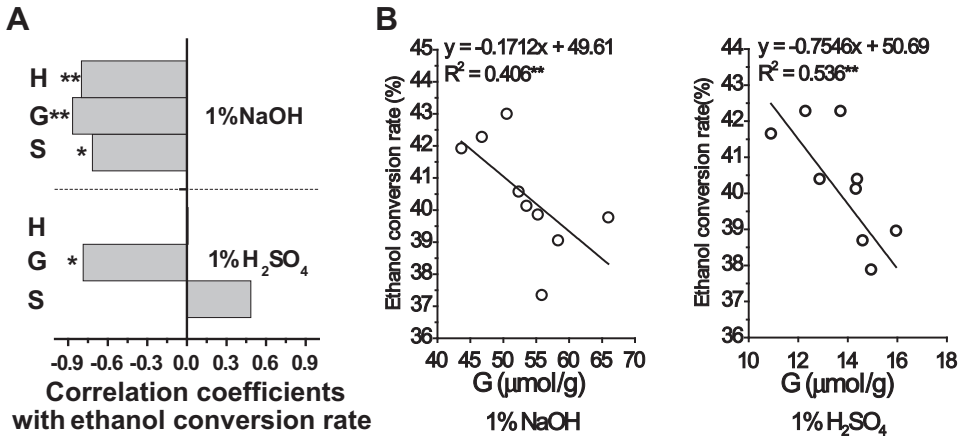

Fig. 5. Effects of three monolignin on yeast fermentation in the five typical pairs of sweet sorghum samples: (A) correlation between ethanol conversion rates and three monolignins (H-, G-, S-monomers) released from 1% NaOH and 1% H_2SO_4 pretreatments; (B) correlative distribution between ethanol conversion rates and G-monomer released from 1% NaOH and 1% H_2SO_4 pretreatments. * and ** Indicated significant correlations at $p < 0.05$ and 0.01, respectively ($n = 9$).

4. Conclusions

Total 63 sweet sorghum accessions are detected with diverse soluble sugar and dry bagasse levels, and both sugar and bagasse products are also examined without significant impact on lignocellulose digestibility. However, either DP of cellulose or Ara substitution degree of non-KOH-extractable hemicelluloses largely affects lignocellulose crystallinity (CrI) for high biomass saccharification upon various NaOH and H_2SO_4 pretreatments. In addition, G-monomer could determine lignin negative effect on biomass enzymatic saccharification and has significant inhibition to yeast fermentation towards ethanol production. Hence, this study can suggest the potential genetic modification of plant cell walls for high biofuels in sweet sorghum.

Acknowledgements

This work was supported in part by grants from the 111 Project of Ministry of Education of China (B08032), the Transgenic Plant and Animal Project of Ministry of Agriculture of China (2009ZX08009-119B), the 973 Pre-project of Ministry of Science and Technology of China (2010CB134401), HZAU Changjiang Scholar Promoting Project (52204-07022), and Fundamental Research Funds for the Central Universities Project (2013QC042).

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.2014.04.086.

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