Distinct polymer extraction and cellulose DP reduction for complete cellulose hydrolysis under mild chemical pretreatments in sugarcane

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ABSTRACT

In this study, liquid hot water (LHW) and chemical (H_2SO_4, NaOH, CaO) pretreatments were performed in *Saccharum* species including sugarcane bagasse. In comparison, the LHW and CaO pretreatments significantly enhanced biomass enzymatic hydrolysis, leading to much high bioethanol yield obtained at 19% (% dry matter) with an almost complete hexoses-ethanol conversion in the desirable So5 bagasse sample. Despite the LHW and CaO are distinctive for extracting hemicellulose and lignin, both pretreatments largely reduced cellulose degree of polymerization for enhanced lignocellulose enzymatic saccharification. Further chemical analysis indicated that the pretreated So5 sample had much lower cellulose crystalline index, hemicellulosic Xyl/Ara and lignin S/H ratio than those of other biomass samples, which explained that the So5 had the highest bioethanol yield among *Saccharum* species. Therefore, a mechanism model was proposed to elucidate how mild pretreatments could enhance biomass enzymatic saccharification for a high bioethanol production in the desirable sugarcane bagasse.

1. Introduction

Saccharum species are typically high photosynthetic-efficient C4 plants with huge lignocellulose residues for biofuels and chemical production (Cai et al., 2005; D'Hont et al., 1995). There are three major *Saccharum* species including *officenarum*, *spontaneum* and *arundinaceum* (D'Hont et al., 1995). In particular, sugarcane (*officenarum*) has been broadly used for sugar production, but its bagasse remains under development for biofuel purpose (Brienzo, Tyhoda, Benjamin, & Görgens, 2015; Cai et al., 2013). Although both *spontaneum* and *arundinaceum* species contain low sugars in stalks, they are increasingly considered as the potential desirable bioenergy crops, due to their fast growth and well adaptation to various environmental stresses (Piperidis,

Christopher, Carroll, Berding, & D'Hont, 2000; Ram, Sreenivasan, Sahi, & Singh, 2001).

In principle, lignocellulose conversion process is associated with three major steps for bioethanol production: initial physical and chemical pretreatments to disturb wall polymers, sequential enzymatic hydrolysis to release soluble sugars, and final yeast fermentation to produce bioethanol (Caspeta, Caro-Bermúdez, TPonce-Noyola, Alfredo, & Martinez, 2014). However, biomass recalcitrance basically determines an unacceptable costly biomass process with secondary waste release from harsh physical and chemical pretreatments (Kumari & Das, 2015). Therefore, it is important to find out an optimal biomass process technology (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010).

Biomass recalcitrance is fundamentally determined by plant cell

wall compositions, wall polymer features and wall network styles (Das & Sarmah, 2015; Lim et al., 2013). Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin (Xu et al., 2012). Cellulose consists of β-1,4-glucans that form microfibrils by hydrogen bonds. As two major features, cellulose crystalline index (CrI) and degree of polymerization (DP) of β-1,4-glucans have been characterized to negatively affect biomass enzymatic digestions under various pretreatments in biomass residues examined (Alvira et al., 2010; Kumari & Das, 2015). Hemicelluloses are heterogeneous polysaccharides and xylans are the major components in grassy plants. Recent reports have indicated that the arabinose (Ara) substitution degree of xylans (defined as reverse Xyl/Ara ratio) could reduce cellulose crystallinity for positively affecting biomass enzymatic digestibility under chemical pretreatments in *Miscanthus* and other grass plants (Chen, Zhao, Hu, Zhao, & Liu, 2015; De Souza et al., 2015; Souza, Leite, Pattathil, Hahn, & Buckeridge, 2013; Wu et al., 2013). Lignin is a stable non-carbohydrate polymer composed of three major phenolic units: *p*-coumaryl (H), coniferyl (G), and sinapyl (S) alcohols, and its deposition negatively affects biomass saccharification as the barrier against enzyme access and loading onto microfibrills (Jia et al., 2014; Li, Zhao et al., 2014). More recently, it has been reported that three monolignols proportions play dual roles in lignocellulose enzymatic hydrolysis based on distinct pretreatments performed in grass plants including sugarcanes (Brienzo et al., 2016; Carlos, Alves, Da, & Igor, 2013; Li, Feng et al., 2014, 2018a; Li, li, & Yuanyuan, 2011; Souza et al., 2013).

As the initial step, physical and chemical pretreatments have been broadly applied in biomass process (Li, Zhuo et al., 2018; Liu et al., 2013; Singh, Suhag, & Dhaka, 2015). Liquid hot water is an environment-friendly pretreatment, but its effectiveness is limited for the sequential enzymatic hydrolysis in the most biomass residues (Li, Li, Sang, & Xu, 2013; Yu et al., 2013). In principle, chemical pretreatments are effective for largely extracting hemicellulose and lignin using acids and alkalis, but they could not avoid to release wastes and secondary pollution (Li, Si et al., 2014). In particular, alkali pretreatment could largely extract lignin by breaking hydrogen and other covalent bonds, whereas acid pretreatment mainly remove hemicelluloses by splitting strong chemical bonds under high temperature (Si et al., 2015). In addition, Tween has been examined to be the excellent surfactant for enhancing enzymatic hydrolysis of pretreated biomass residues by lessening cellulase absorption with lignin (Jin et al., 2016). Hence, it becomes essential to employ mild physical and chemical pretreatments for largely enhancing biomass saccharification and bioethanol production.

Over the past years, many physical and chemical pretreatments are conducted to enhance biomass saccharification and bioethanol production in sugarcane bagasse with the highest bioethanol yield obtained at 20% (% dry matter), but most pretreatment approaches have used harsh conditions such as high temperature, high pressure and expensive acid and alkali chemicals at high concentrations (Amores et al., 2013; Cao & Aita, 2013; Carvalho, Queiroz, & Colodette, 2016; Chandel, Kapoor, Singh, & Kuhad, 2007, 2014; Hernandez-Salas et al., 2009; Martins, Rabelo, & Costa, 2015; Mesquita, Ferraz, & Aguiar, 2016; ; You et al., 2016; Zhang et al., 2015). Hence, it remains to explore an optimal technology for high saccharification and efficient sugar-ethanol conversion rate. In this study, we initially performed various pretreatments in *Saccharum* biomass samples, and found out that short-time liquid hot water and mild CaO pretreatments are effective enough to enhance biomass saccharification. Notably, we determined the second high bioethanol yield at 19% (% dry matter) and the highest sugar-ethanol conversion rate at 99.6%-100% in the desirable sugarcane bagasse, while 1.6% Tween-80 was co-supplied into enzymatic hydrolysis. Finally, this study explained why the desirable sugarcane bagasse had the high bioethanol production and conversion efficiency by proposing a mechanism model that highlights the major impacts on biomass enzymatic hydrolysis and bioethanol production.

2. Material and methods

2.1. Plant samples

Three *Saccharum* species (*Saccharum officenarum* L., *Saccharum spontaneum* L., *Saccharum arundinaceum*) were collected from Guangdong experimental field, China. The mature straws were dried at 50 °C and ground through a 40 mesh screen. The well-mixed powders were stored in a sealed dry container until use.

2.2. Plant cell wall fractionation

The procedure of plant cell wall fractionation was used to extract pectin, hemicelluloses and cellulose as described by Peng, Hocart, Redmond, and Williamson, (2000) and Jin et al. (2016). All experimental analyses were conducted in biological triplicates.

2.3. Colorimetric assay of hexoses and pentoses

The anthrone/H_2SO_4 method (Fry, 1988) and orcinol/HCl method (Dische, 1962) were respectively used for hexoses and pentoses assay. D-glucose and D-xylose were applied to plot standard curves for hexoses and pentose measurements, and the deduction from pentoses reading at 660 nm was carried out for final hexoses calculation in order to eliminate the interference of pentose on hexose reading at 620 nm, which was verified by GC/MS analysis as described below. All experiments were performed in biological triplicate.

2.4. Total lignin and monolignol detection

Two-step acid hydrolysis method was applied for total lignin assay, according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (Sluiter et al., 2008). Three lignin monomers were measured by HPLC using nitrobenzene oxidation method as previously described by Zahoor et al. (2017).

2.5. Hemicellulose monosaccharide determination

GC-MS (SHIMADZU GCMS-QP2010 Plus) was used for detection of monosaccharide composition of hemicellulose as previously described by Fan et al. (2017). Trifluoroacetic acid (TFA) and *myo*-inositol were obtained from Aladdin Reagent Inc. 1-Methylimidazole was purchased from Sigma-Aldrich Co. LLC. Acetic anhydride and acetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd.

2.6. Detection of degree of polymerization (DP) of cellulose

The dry powders of biomass samples (0.2–1 g) were extracted with 4 M KOH (containing sodium borohydride at 1.0 mg/mL) at 25 °C for 1 h. After centrifugation at 4000 *g* for 5 min, the pellet was re-extracted with 4 M KOH, and washed five times with distilled water until pH at 7.0. The pellet was further extracted with 10 mL 8% $NaClO_2$ at 25 °C for 72 h (change $NaClO_2$ every 12 h). After centrifugation, the residues were washed five times with distilled water until pH at 7.0, and dried with vacuum suction filtration. The DP of crude cellulose sample was measured using the viscosity method (Puri, 1984) with minor modification (Li et al., 2017). The cellulose DP was measured at 25 ± 0.5 °C using cupriethylenediamine hydroxide (Cuen) as the solvent in Ubbelohde viscometer. The relative viscosity (ηrel) values was calculated using the ratio of t/t0, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent. The intrinsic viscosity was calculated by interpolation using the United States Pharmacopeia table (USP, 2002). The intrinsic viscosity values were converted to cellulose DP according to equation: $DP^{0.905} = 0.75[\eta]$, [η] is the
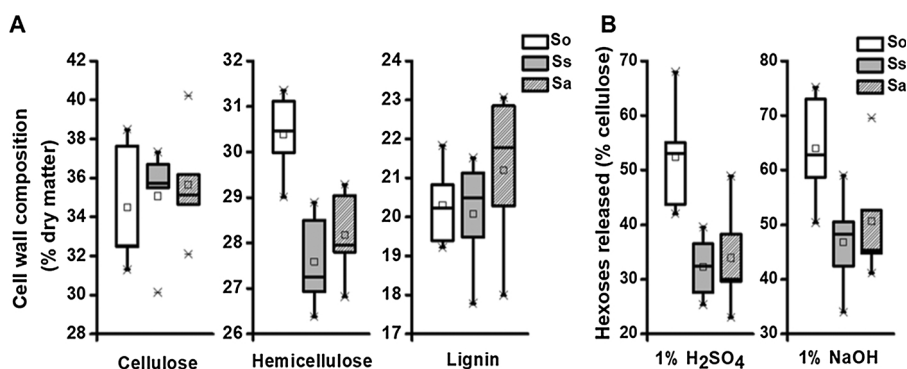
Fig. 1. Variations of cell wall compositions and hexoses yields released from enzymatic hydrolysis under 1% H$_2$SO$_4$ and 1% NaOH pretreatments in three *Saccharum* species (n = 5). So: *Saccharum officenarum* L.; Ss: *Saccharum spontaneum* L.; Sa: *Saccharum arundinaceum*.

intrinsic viscosity of the solution calculated by interpolation using the USP table. All experiments were performed in biological triplicate.

2.7. Detection of cellulose crystalline index

X-ray diffraction method was applied to detect cellulose crystalline index (CrI), as previously described by Li et al. (2017) using the Rigaku-D/MAX instrument (Uitima III, Japan). Technical standard errors of the CrI method were measured at ± 0.05 ~ 0.15 using five representative samples in triplicate.

2.8. Biomass pretreatments

H$_2$SO$_4$ pretreatment: The well-mixed biomass samples were treated with 6 mL H$_2$SO$_4$ at various concentrations (0.5%, 1%, 2%, 4%, 8% v/v). The sample tubes were sealed and heated at 121 °C for 20 min in autoclave (0.15 Mpa). The pellets were washed with 10 mL distilled water for 5-6 times until pH at 7.0, and the samples added with 6 mL distilled water and shaken for 2 h at 50 °C were used as control.

NaOH pretreatment: The well-mixed biomass samples were incubated with 6 mL NaOH at various concentrations (1%, 2%, 4%, 8%, 16%, w/v). The pellets were washed with 10 mL distilled water for 5-6 times until pH 7.0. Samples were added with 6 mL distilled water and shaken for 2 h at 50 °C as control.

CaO pretreatment: The well-mixed biomass samples were treated with 6 mL CaO at various concentrations (5%, 10%, 15%, 20% w/w) at 50 °C for 48 h. The pellets were neutralized with 10% HCl and washed with 10 mL distilled water for 6 times until pH 7.0. Sample was added with 6 mL distilled water and shaken for 48 h at 50 °C as control.

Liquid hot water (LHW) pretreatment: The well-mixed biomass samples were added into well-sealed stainless steel bombs, and heated at 200 °C under 15 rpm shaking for 0, 4, 8, 16, 32, 64 min, respectively. Then, the sealed bombs were cool down immediately. The solid-liquid/water ratio for pretreatment is 1:20. The sealed samples were shaken under 150 rpm for 2 h at 50 °C, and centrifuged at 3,000 *g* for 5 min. All supernatants were combined for pentoses and hexoses assay and the remained pellets were used for enzymatic hydrolysis as described below. All samples were conducted in biological triplicate.

2.9. Enzymatic hydrolysis of pretreated biomass residues

The remaining residues from pretreatments were washed 5 ~ 6 times with 10 mL distilled water until 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 cellulases at 10.60 FPU g-1 biomass and xylanase at 6.72 U g-1 biomass from Imperial Jade Bio-technology Co., Ltd), and shaken under 150 rpm for 48 h at 50 °C. The samples were centrifuged at 3,000 *g* for 5 min, and the supernatants were collected for pentoses

and hexoses assay. The samples only added with 6 mL reaction buffer were shaken for 48 h at 50 °C and as the control. All experiments were carried out in biological triplicate.

2.10. Enzymatic hydrolysis co-supplied with Tween-80

The pretreated biomass residues were incubated with 6 mL (1.6 g/L) of mixed-cellulases co-supplied with Tween-80 at various concentrations (0%, 0.4%, 0.8%, 1.6%, 3.2%, v/v). The sealed samples were shaken under 150 rpm for 48 h at 50 °C (solid-liquid ratio, 1:20). After centrifugation at 3,000 *g* for 5 min, the supernatants were collected for pentoses and hexoses assay. All experiments were performed in biological triplicate.

2.11. Yeast fermentation and ethanol measurement

The yeast fermentation was conducted using *Saccharomyces cerevisiae* strain (purchased from Angel yeast Co., Ltd., Yichang, China) as previously described by Jin et al. (2016) and Zahoor et al. (2017). The experiments were performed with biological triplicate.

2.12. Statistical calculation of correlation coefficients

Correlation coefficients were generated by performing Spearman rank correlation analysis for all measured traits or parameters using average values calculated from all original determinations.

3. Results and discussion

3.1. Diverse cell wall composition and biomass saccharification among three Saccharum species

In this study, we detected cell wall compositions (cellulose, hemi-celluloses, lignin) of total 15 representative biomass samples among three *Saccharum* species (Fig. 1). In comparison, the sugarcane bagasse samples showed cellulose levels varied from 31.31% to 38.48% (% dry matter), whereas other *spontaneum* and *arundinaceum* species had cellulose contents varied from 30.13% to 40.22% (Fig. 1A; Table S1). Notably, three *Saccharum* species displayed a close average level of cellulose around 35%, but sugarcanes samples had higher average hemicellulose level (30.38%) than those of the *spontaneum* and *arundinaceum* species (27.59%, 28.18%). In addition, three species had much low average levels of lignin from 20.30% to 21.19%.

Biomass saccharification (digestibility) has been characterized by calculating the hexoses yields (% cellulose) released from lignocellulose enzymatic hydrolysis after various pretreatments or total hexoses yields (% dry matter) released from both enzymatic hydrolysis and pretreatment. In the present work, we detected hexoses yields in total 15 biomass samples of three *Saccharum* species under pretreatment with 1%
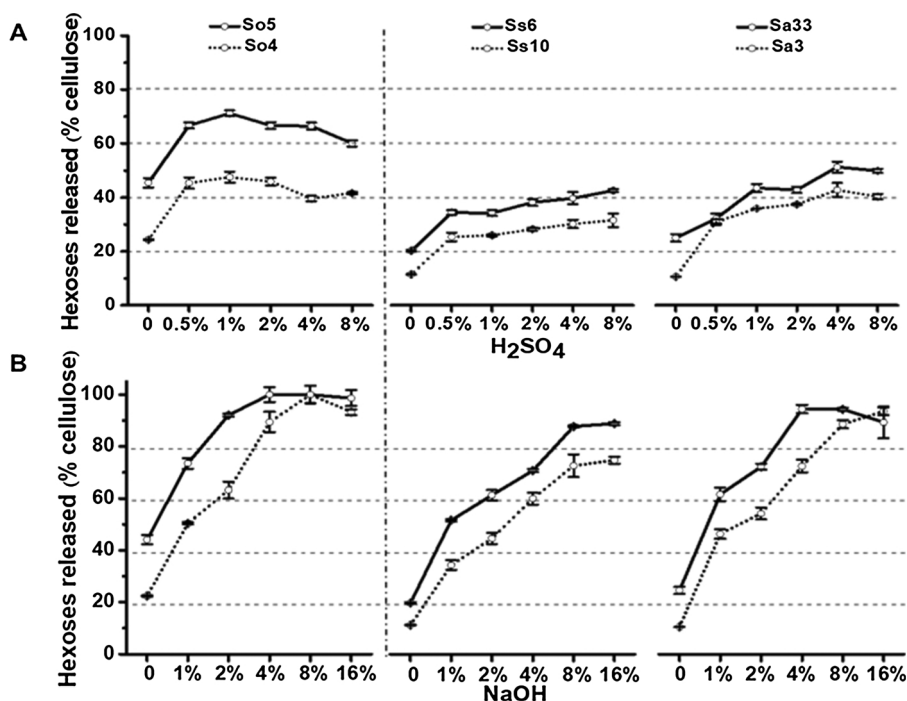
Fig. 2. Hexoses yields (% cellulose) released from enzymatic hydrolysis after H_2SO_4 and NaOH pretreatments under a series concentrations. Bar as mean $\pm$ SD (n = 3).

H_2SO_4 or 1% NaOH (Fig. 1B). In general, the sugarcane bagasse samples exhibited large variations of hexose yields (% cellulose) ranged from 51% to 79%, whereas the *spontaneum* and *arundinaceum* species had varied hexoses yields from 32% to 51% (Table S1), indicating that sugarcane bagasse had much higher biomass enzymatic saccharification than those of the *spontaneum* and *arundinaceum* species. In addition, 1% NaOH pretreatment could lead to much higher hexoses yields than those of 1% H_2SO_4 pretreatment in all three species samples, consistent with the previous reports in other C4 grass plants (Keshwani & Cheng, 2009; Lu et al., 2010; Zhang et al., 2013).

3.2. High biomass enzymatic digestibility under H_2SO_4 and NaOH pretreatments

To find out the highest biomass saccharification from chemical pretreatments, we selected one pair samples from each *Saccharum* species that showed relatively high and low hexoses yields described above (Table S1, S2), and then performed H_2SO_4 and NaOH pretreatments using a series of concentrations (Fig. 2). Under H_2SO_4 pretreatments, two sugarcane bagasse samples showed much higher hexoses yields from enzymatic hydrolysis than those of other two *Saccharum* species (Fig. 2A). However, the highest hexoses yield from sugarcane sample (So5) remained less than 75% (% cellulose) from 1% H_2SO_4 pretreatments, and all other samples had the hexoses yields at less than 50% even though under 8% H_2SO_4 pretreatments. As the H_2SO_4 pretreatments were performed under high temperature (121 °C for 20 min), it should not be cost-effective for biomass process of *Saccharum* species.

By comparison, all *Saccharum* species had much higher hexoses yields from NaOH pretreatments than those from H_2SO_4 pretreatments (Fig. 2B). In particular, two sugarcane bagasse samples showed a complete biomass saccharification with hexoses yields at 100% (%cellulose) from 4% or 8% NaOH pretreatment, whereas other two *Saccharum* species had the hexoses yields at less than 92% even though under 16% NaOH pretreatments. Hence, the results suggest that alkali pretreatment (50 °C for 2 h) was effective for biomass enzymatic saccharification of *Saccharum* species samples, in particular on the sugarcane bagasse.

3.3. Enhanced biomass digestibility upon CaO and liquid hot water pretreatments

Although NaOH pretreatment was preferable for biomass saccharification of *Saccharum* species as described above (Fig. 2B), it is an expensive chemical agent. In this study, we performed CaO pretreatments as an alternative (Fig. 3A). Notably, the desirable sugarcane bagasse sample (So5) showed a complete biomass enzymatic saccharification from 15% CaO pretreatment, whereas another bagasse sample (So4) had the hexoses yield at 80% (% cellulose). By comparison, other *Saccharum* species had relatively lower hexoses yields at less than 75% even though from 20% CaO pretreatments (Fig. 3A). Hence, the results suggest that CaO agent should be the excellent alternative of NaOH for the desirable sugarcane bagasse and other *Saccharum* species.

Meanwhile, this study performed a time course of liquid hot water (LHW) pretreatments in all *Saccharum* species samples (Fig. 3B). Under 4 min LHW pretreatment, the desirable sugarcane bagasse (So5) had the hexoses yield at 82% from enzymatic hydrolysis, whereas other samples had hexoses yields at less than 60% even though under long time LHW pretreatments from 8 min to 64 min. Because LHW is an environment-friendly pretreatment, it should be applicable for biomass process of *Saccharum* species samples, in particular on the sugarcane bagasse sample. Hence, compared to the NaOH and H_2SO_4 pretreatments, the LHW and CaO pretreatments should be of relatively lower cost and less waste release, but they remained high enhancements of biomass enzymatic saccharification in the desirable sugarcane bagasse.

3.4. Increased bioethanol production under co-supplement with Tween-80

As described above, despite the 15% CaO and long-time LHW pretreatments could lead to the highest hexoses yields in the most *Saccharum* biomass samples, they remained slightly increased rates of hexoses yields, compared to 10% CaO and 4 min LHW pretreatments (Fig. 3). Hence, this study focused to use 10% CaO and 4 min LHW pretreatments for final yeast fermentation to produce bioethanol in three pairs of *Saccharum* species. To further increase bioethanol yields, this study attempted to co-supply different concentrations of Tween-80
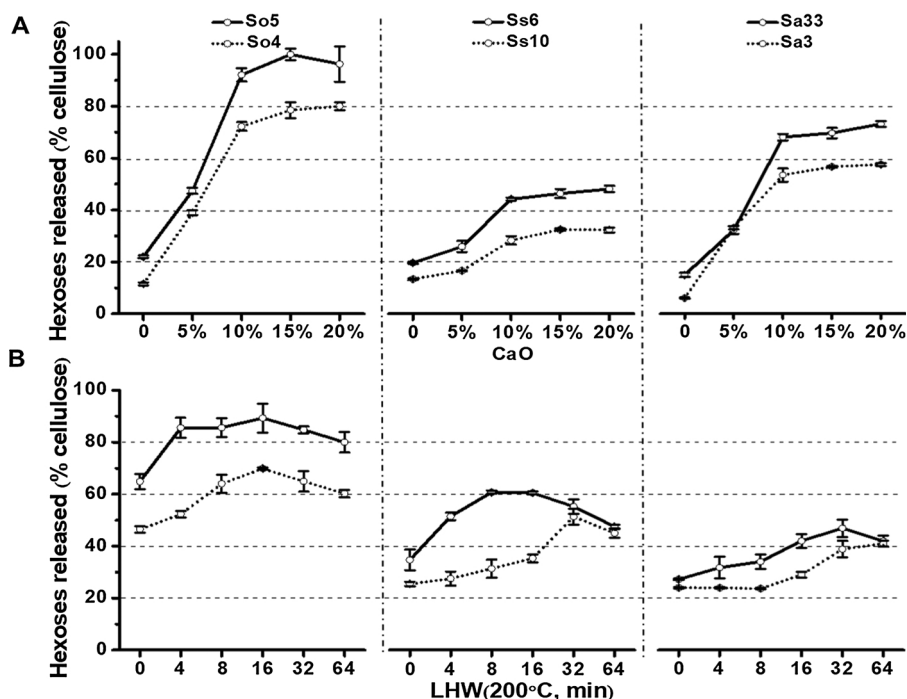
Fig. 3. Hexoses yields (% cellulose) released from enzymatic hydrolysis after CaO and liquid hot water (LHW) pretreatments: (A) CaO pretreatment with a series of concentrations; (B) A time course of LHW pretreatment; Bar as mean ± SD (n = 3).

Table 1
Bioethanol yields from yeast fermentation under CaO and LHW pretreatments in five sugarcane bagasse and other *Saccharum* species

Samples		Bioethanol yield (% dry matter)				Sugar-ethanol conversion rate	
		10% CaO		4 min LHW		10% CaO	4 min LHW
So5	Without Tween-80	14.06 ± 0.31		13.28 ± 0.34		83.59%	80.43%
(H)	1.6% Tween-80	17.88 ± 0.08 **	27%	16.86 ± 0.25**	27%	99.59%	90.70%
So4		12.01 ± 0.23		9.66 ± 0.34		79.39%	83.47%
(L)		15.87 ± 0.37**	32%	12.8 ± 0.14**	33%	93.88%	88.47%
Ss6		11.08 ± 0.15		13.09 ± 0.43		83.10%	93.43%
(H)		14.82 ± 0.13**	34%	13.74 ± 0.08	5%	98.65%	94.47%
Ss10		8.78 ± 0.22		9.18 ± 0.30		84.78%	98.31%
(L)		11.62 ± 0.08**	32%	9.79 ± 0.25	7%	97.90%	100.00%
Sa33		12.44 ± 0.23		10.69 ± 0.34		81.87%	97.01%
(H)		16.94 ± 0.53**	36%	12.64 ± 0.18*	18%	99.67%	100.00%
Sa3		9.47 ± 0.15		6.64 ± 0.31		85.53%	98.00%
(L)		11.96 ± 0.15**	26%	7.79 ± 0.34*	17%	95.74%	100.00%
So1		12.45 ± 0.17		9.93 ± 0.2		85.21%	93.05%
		16.85 ± 0.17**	35%	12.32 ± 0.15**	24%	100.00%	100.00%
So2		13.71 ± 0.25		10.05 ± 0.39		87.21%	92.50%
		17.33 ± 0.45**	26%	14.11 ± 0.57**	40%	100.00%	97.29%
So6		14.54 ± 0.23		10.18 ± 0.26		86.09%	97.26%
		18.45 ± 0.52**	27%	10.96 ± 0.13*	8%	99.28%	100.00%

The data as means ± SD (n = 3).
* and ** indicated significant difference by t-test between control and 1.6% Tween-80 at p < 0.05 and p < 0.01 levels (n = 3), respectively, the percentage calculated by subtracting the Tween-80 sample and the control (without Tween-80) divided by the control value.
(H) and (L) Indicated relatively high and low biomass saccharification in the pair.

into biomass enzymatic hydrolysis and yeast fermentation under the 4 min LHW pretreatment in the three pairs of *Saccharum* species and other three sugarcanes bagasse (So1, So2, So6) samples (Fig. S1). As a result, the most biomass samples showed the highest bioethanol yields from 1.6% Tween-80 supply. Notably, co-supplement with 1.6% Tween-80 could largely enhance bioethanol yields and sugar-ethanol conversion rates from both 10% CaO and 4 min LHW pretreatments in all biomass samples, compared with the control without Tween-80 (Table 1). Furthermore, the desirable sugarcane bagasse (So5) had the highest bioethanol yields at 17.88 and 16.86% (% dry matter), consistent with its highest hexoses yield released from enzymatic

hydrolysis (Fig. 3). In addition, the Tween-80 supplement also caused much enhanced bioethanol yields in other three sugarcane bagasse samples (So1, So2, So3), in particular from CaO pretreatment (Table 1).

Over the past years, many physical and chemical pretreatments have been performed in biomass process of sugarcane bagasse (Table 2) (Amores et al., 2013; Cao & Aita, 2013; Carvalho et al., 2016; Chandel et al., 2007, 2014; Hernandez-Salas et al., 2009; Martins et al., 2015; Mesquita et al., 2016; ; You et al., 2016; Yu et al., 2013; Zhang et al., 2015), but this study used an inexpensive chemical (10% CaO) under mild incubation condition (50 °C) for alkali pretreatment, and the 4 min LHW pretreatment was also a relatively cost-effective and

Table 2

Bioethanol production from sugarcane bagasse fermentation in this study and a comparison with previous reports

Pretreatment	Ethanol yield (% dry matter)	Sugar-ethanol conversion rate (%)	Reference
200 ℃, 4 min LHW & 1.6%Tween-80	12%-17%	88%-90.7%	This study
50 ℃, 48 h 10%CaO & 1.6%Tween-80	16%-19%	94%-99.6%	
Steam explosion 215 ℃, 5 min	ND[a]	65%	Amores et al. (2013)
Steam explosion 210 ℃, 5 min	ND	93%	Zhang et al. (2015)
8%(w/w)Na₂SO₃ & 4%(w/w)NaOH, 120 ℃, 2h	ND	56%	Mesquita et al. (2016)
1%H₂SO₄, 121 ℃, 20 min & 1%NaOH, 121 ℃, 1h	ND	74%	Chandel et al. (2014)
2.5% (v/v) HCl & Ion exchange	ND	94%	Chandel et al. (2007)
1.2% (v/v)HCl & 2%(w/v)NaOH, 121 ℃, 4h	ND	64%	Hernandez-Salas et al. (2009)
15%(w/w) NaOH, 175 ℃, 15 min	10%	ND	Carvalho et al. (2016)
0.4%H₂SO₄, 5%acidified glycerol solution, 150 ℃, 15min	14%	84%	Zhang et al. (2015))
7% (v/v) AHP(NaOH adjusted, pH11.5), 90 ℃, 1h	14%	85%	Martins et al. (2015)
Sono-assisted 2%H₂SO₄, 50 ℃, 45min	17%	92%	Velmurugan and Muthukumar (2011)
3%(w/w)Tween-80 & 0.65%(w/w) ammonia, 160 ℃, 1h	18%	69%	Cao & Aita (2013)
3%(w/w)PEG-4000 & 0.65%(w/w) ammonia, 160 ℃, 1h	20%	73%	

[a] ND: non-data available.

environmental-friendly method (Table 2). More importantly, although the 10% CaO and 4 min LHW pretreatments only led to the second high bioethanol yield at 18%-19% in the two desirable sugarcane bagasse, both pretreatments could cause the highest sugar-ethanol conversion rate in the most samples, which reached to the theoretical value (100%), compared with the previous reports that show the highest bioethanol yield at 20% (% dry matter) and the low conversion rates from 56% to 94% (35-45; Table 2). Hence, the experimental data suggested that the CaO and LHW pretreatments may release relatively less inhibition compounds to yeast fermentation in this study.

3.5. Distinct wall polymer extraction from CaO and LHW pretreatments

To understand why the desirable sugarcane bagasse had much enhanced biomass saccharification and bioethanol production from the 10% CaO and 4 min LHW pretreatments, we determined cell wall compositions between the raw materials and pretreated biomass residues (Table 3). Under both CaO and LHW pretreatments, all *Saccharum* samples showed significantly increased cellulose levels at $p < 0.05$ and $p < 0.01$ levels (n = 3), compared to the raw materials. In particular, the desirable sugarcane bagasse (So5) had the highest cellulose level (51% dry matter) with the increased rate at 57% from the 4 min LHW pretreatment. Accordingly, the So5 sample had the lowest

hemicellulose level (16% dry matter) with the reduced rate at 48% and all other samples also exhibited significantly reduced hemicellulose contents at $p < 0.01$ levels from the 4 min LHW pretreatments. By comparison, the CaO pretreatment did not cause much altered hemicellulose levels in all *Saccharum* samples, but it significantly reduced lignin contents by 14%-28% at $p < 0.01$ levels. In addition, the LHW pretreatment only relatively increased lignin levels in the Ss6 and Ss3 samples at $p < 0.01$. Hence, in terms of relatively increased cellulose levels in all biomass samples, the 4 min LHW pretreatment predominately extracted hemicellulose, whereas the 10% CaO mainly removed lignin, consistent with the previous reports (Li, Ren et al., 2013; Yu et al., 2013).

3.6. Largely altered cellulose features from CaO and LHW pretreatments

Despite the CaO and LHW pretreatments could distinctively extract lignin and hemicelluloses in biomass samples, much remains unknown about both pretreatments impacts on two major cellulose features (DP, CrI), which are the major factors on biomass enzymatic digestibility (Li, Feng et al., 2014; Zhang et al., 2013). In this study, we determined significantly reduced cellulose DP values from both 10% CaO and 4 min LHW pretreatments in all *Saccharum* samples at $p < 0.05$ and $p < 0.01$ levels (Table 4). Notably, the desirable sugarcane bagasse (So5) had the

Table 3

Cell wall components (% dry matter) of the raw material and pretreated residues in three pairs of Saccharum species[a]

Samples	Pretreatment	Cellulose		Hemicelluloses		Lignin	
So5	Raw material	32.49 ± 0.12		30.86 ± 0.33		19.22 ± 0.33	
(H)	10% CaO	38.45 ± 2.56*	18%	31.87 ± 0.29	3%	15.89 ± 0.72**	-17%
	4 min LHW	50.89 ± 2.61**	57%	16.03 ± 0.22**	-48%	19.37 ± 0.35	1%
So4		37.63 ± 0.44		29.96 ± 0.39		20.23 ± 0.74	
(L)		43.10 ± 2.18*	15%	32.01 ± 0.79	7%	17.37 ± 0.50**	-14%
		42.84 ± 1.44*	14%	20.43 ± 0.42**	-32%	21.38 ± 0.37	6%
Ss6		30.13 ± 0.49		27.09 ± 0.58		17.78 ± 0.59	
(H)		3 6.81 ± 0.42**	22%	30.42 ± 0.3	12%	15.11 ± 0.73**	-15%
		44.75 ± 1.35**	49%	19.91 ± 0.07**	-27%	21.75 ± 0.19**	22%
Ss10		36.69 ± 0.24		26.84 ± 0.29		21.51 ± 0.78	
(L)		40.10 ± 1.45*	9%	28.71 ± 1.31	7%	16.79 ± 0.24**	-22%
		38.48 ± 0.71*	5%	20.69 ± 0.26**	-23%	22.86 ± 0.51	6%
Sa33		34.55 ± 0.45		27.96 ± 0.61		17.99 ± 0.34	
(H)		40.86 ± 0.25**	18%	28.34 ± 0.91	1%	15.35 ± 0.33**	-15%
		49.47 ± 2.00**	43%	21.86 ± 0.30**	-22%	21.89 ± 0.78**	22%
Sa3		35.11 ± 0.49		28.51 ± 1.00		23.06 ± 0.44	
(L)		39.29 ± 0.04*	12%	29.86 ± 0.56	5%	16.49 ± 0.42**	-28%
		43.26 ± 2.57**	23%	23.75 ± 0.23**	-17%	21.91 ± 0.30	-5%

[a] The data as mean ± SD (n = 3).

* and ** indicated significant difference by t-test between raw material and pretreated residue at p < 0.05 and p < 0.01 levels (n = 3), respectively, the percentage calculated by subtracting the raw material and the pretreated residue values divided by the raw material value.

(H) and (L) Indicated relatively high and low biomass saccharification in the pair.

Table 4

Cellulose features (DP, CrI) of raw material and pretreated residues in three pairs of *Saccharum* species

Samples	So5 (H)		So4 (L)		Ss6 (H)		Ss10 (L)		Sa33 (H)		Sa3 (L)	
	DP	CrI	DP	CrI	DP	CrI	DP	CrI	DP	CrI	DP	CrI
Raw material	634	36.61	809	38.82	913	45.81	998	49.07	698	44.44	818	52.19
10 % CaO	435	46.36	592	52.13	803	57.19	815	60.66	662	52.83	718	58.80
	-31%	+ 27%	-27%	+ 34%	-12%	+ 25%	-18%	+ 24%	-5%	+ 19%	-12%	+ 13%
4 min LHW	340	39.30	542	44.85	660	49.28	829	52.15	501	50.03	662	57.85
	-46%	+ 7%	-33%	+ 16%	-28%	+ 8%	-17%	+ 6%	-28%	+ 13%	-19%	+ 11%

(H) and (L) Indicated relatively high and low biomass saccharification in the pair.

The percentage calculated by subtracting the raw material and the pretreated residue values divided by the raw material value.
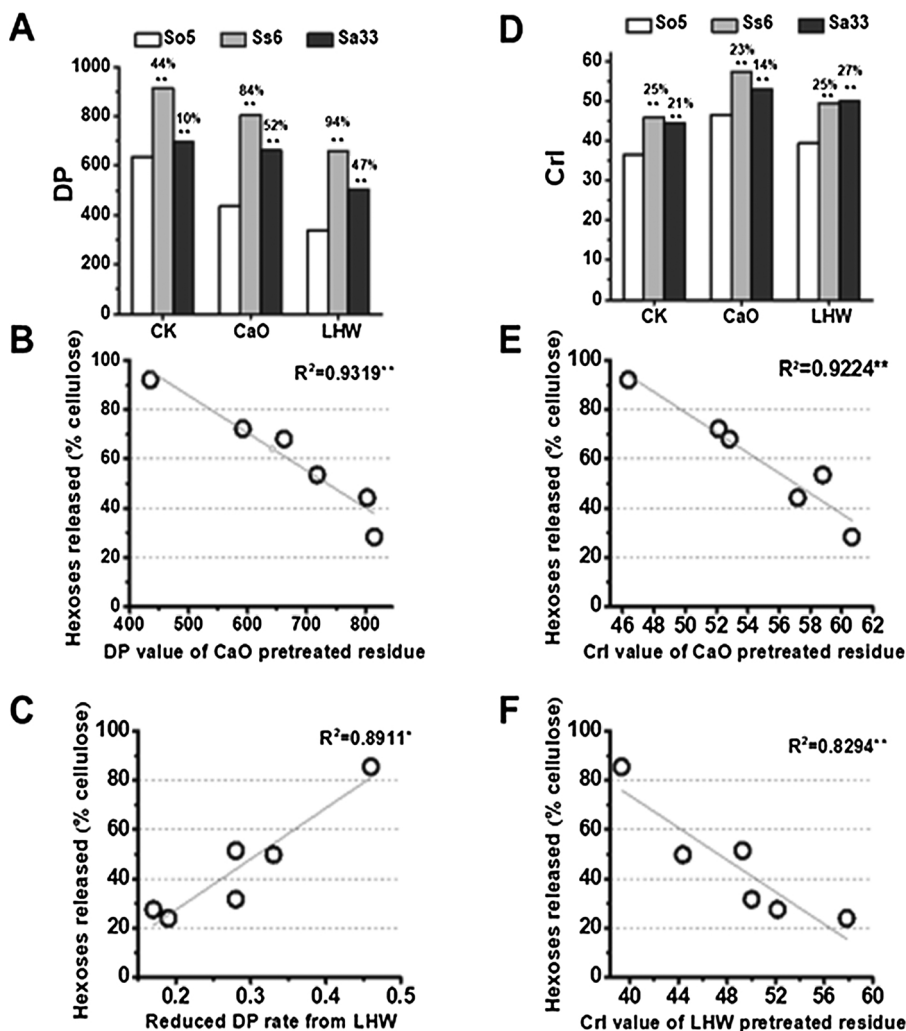
Fig. 4. Cellulose features (DP, CrI) impacts on biomass enzymatic digestibility in *Saccharum* species: (A) Cellulose DP values in the raw material (CK) and the pretreated residues of *Saccharum* species; (B) Correlation between the cellulose DP values of the pretreated residues and the hexoses yields released from enzymatic hydrolysis after the 10% CaO pretreatment; (C) Correlation between the reduced cellulose DP rates and the hexoses yields released from enzymatic hydrolyses after the 4 min LHW pretreatment, the reduced rate calculated by subtracting DP values of the CK and the LHW pretreated residues divided by the CK values; (D) Cellulose CrI values in the CK and the pretreated biomass residues; (E) Correlation between the cellulose CrI values of the pretreated residues and the hexoses yields released from enzymatic hydrolysis after the 10% CaO pretreatment; (F) Correlation between the cellulose CrI values and the hexoses yields released from enzymatic hydrolysis after the 4 min LHW pretreatment. ** (A, D) Indicated significant difference between the So5 sample and the other sample by *t*-test at $p < 0.01$ level (n = 3); * and ** (B, C, E, F) Indicated significant correlations at $p < 0.05$ and $p < 0.01$ levels (n = 6), respectively.

lowest cellulose DP values (435, 340) with the reduced rates at 31% and 46% from both pretreatments, compared to all other samples (Fig. 4A; Fig S2). Because cellulose DP is a negative factor on biomass saccharification (Huang et al., 2015; Jiang, Wang, Zhang, & Wolcott, 2017) a correlation analysis was performed between cellulose DP values and hexoses yields released from enzymatic hydrolyses of three *Saccharum* species samples examined in this study. Significantly, the hexoses yields were negatively correlated with the cellulose DP values of the CaO pretreated biomass samples at $p < 0.01$ level (Fig. 4B). Despite of non-correlation with the DP values of the LHW pretreated biomass residues, the hexoses yields were positively correlated by the reduced DP rate at $p < 0.05$ levels (Fig. 4C). Notably, two correlation analyses respectively showed much high R^2 values at 0.93 and 0.89,

indicating that the reduced DP value or rate should significantly enhance biomass enzymatic saccharification under the CaO and LHW pretreatments.

By contrast, this study determined significantly increased cellulose CrI values in all samples from the 10% CaO and 4 min LHW pretreatments (Table 4, Fig. 4D, Fig S2B). Because cellulose CrI is negatively affected by wall polymer interaction with cellulose microfibrils via hydrogen bonds (Das & Sarmah, 2015; Li, Feng et al., 2014; Wang et al., 2016), the increased cellulose CrI values should be mainly due to hemicellulose and lignin extraction from the CaO and LHW pretreatments. Despite that the cellulose CrI of raw material has been well characterized to negatively affect biomass enzymatic saccharification in all biomass samples examined, much remains unknown about cellulose

CrI impacts of the pretreated biomass residues. Based on the correlation analyses, this study examined that the hexoses yields were negatively correlated with the CrI values of both CaO and LHW pretreated biomass residues at $p < 0.01$ levels with much high R^2 values at 0.92 and 0.83, respectively (Fig. 4E and F), indicating that the pretreated biomass CrI should also be an important factor accounting for biomass enzymatic hydrolysis. Notably, because the desirable sugarcane sample (So5) had much lower CrI values than other *Saccharum* species in both raw material and pretreated biomass residues (Fig. 4D; Fig S2B), it should well explain why the So5 sample had the highest hexoses yields from enzymatic hydrolysis under the CaO and LHW pretreatments. On the other hands, as cellulose CrI is fundamentally determined by cellulose DP, the relatively lower cellulose CrI value of the sugarcane So5 sample should be mainly due to its much reduced cellulose DP from the CaO and LHW pretreatments, compared to other *Saccharum* species samples. Hence, this study has also indicated that both cellulose CrI and DP values of the pretreated biomass residues were accountable for biomass enzymatic saccharification.

3.7. Distinct altered hemicellulose and lignin features from CaO and LHW pretreatments

Because the 10% CaO and 4 min LHW could distinctively extract wall polymers, this study further examined hemicellulose monosaccharide composition in both raw materials and pretreated biomass residues in all *Saccharum* species samples (Table S4). Under the 10% CaO pretreatment, all biomass samples did not show much altered monosaccharide compositions of hemicelluloses relative to their raw materials (Table S3), consistent with only small amounts of hemicelluloses extracted with the CaO pretreatment (Table 3). By contrast, the 4 min LHW pretreatment led to much altered monosaccharide compositions in all samples, probably due to much hemicellulose extraction from the LHW pretreatment. In particular, all samples showed much higher Xyl/Ara ratios by more than 2-fold in the LHW pretreated residues, compared to the raw materials. In addition, the desirable sugarcane sample (So5) showed relatively lower Xyl/Ara value than those of other *Saccharum* species samples in the LHW pretreated residues (Fig. 5A and B; Table S3). As the Xyl/Ara ratio has been reported to negatively affect biomass enzymatic hydrolysis, the relatively lower Xyl/Ara should be a cause for higher biomass saccharification in the desirable sugarcane sample (So5) (Li et al., 2017; Wang et al., 2016).

Furthermore, this study determined much altered monomer compositions of lignin in all samples from the 10% CaO pretreatments (Table S4), probably due to much lignin extraction from this pretreatment (Table 3). Compared to the raw materials, the pretreated biomass residues showed relatively higher S-monomer and lower H-monomer proportions with small alteration of G-monomer, leading to much increased S/H ratios. Meanwhile, the 4 min LHW pretreatments did not cause much altered monomer composition in all samples, consistent with small amounts of lignin extracted from this pretreatment (Table 3). As it has been reported that the S-monomer negatively affects biomass enzymatic hydrolysis but H-monomer is a positive factor in some biomass samples examined (Li, Si et al., 2014; Wu et al., 2013), this study assumed that the S/H ratio should be the negative factor on the biomass enzymatic digestibility in the pretreated biomass residues. However, the desirable sugarcane sample (So5) showed relatively lower S/H ratio than those of other *Saccharum* species in the CaO pretreated biomass residues (Fig. 5C, D; Table S4), suggesting that the relatively lower S/H ration should be another cause for its higher biomass saccharification in the desirable sugarcane sample (So5).

3.8. Mechanism of enhanced biomass enzymatic hydrolysis under CaO and LHW pretreatments

To sort out the mechanism of how the CaO and LHW pretreatments largely enhance biomass saccharification and bioethanol production in
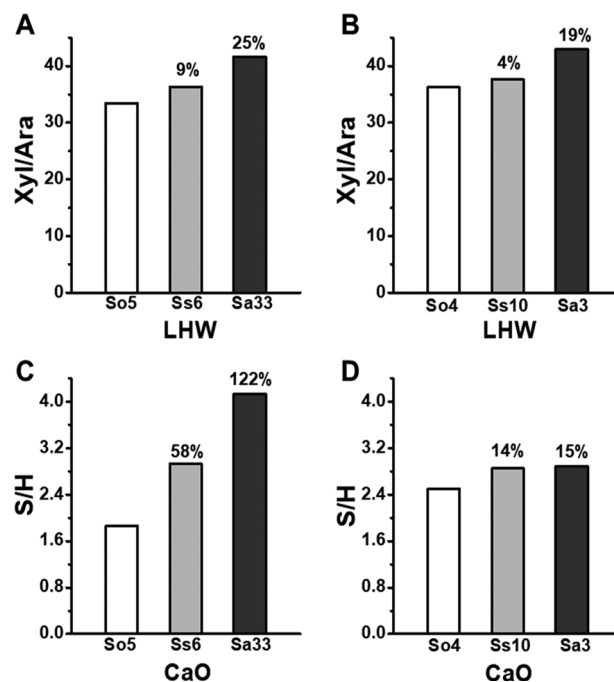

Fig. 5. Wall polymer features in the pretreated biomass residues in three typical pair of *Saccharum* species: (A, B) Hemicellulosic Xyl/Ara ratios of the 4 min LHW pretreated biomass residues in the three samples with relatively high (A) and low (B) biomass saccharification; (C, D) Lignin S/H ratios of the 10% CaO pretreated biomass residues in the three samples with relatively high (C) and low (D) biomass saccharification. Percentage value was calculated by subtracting sugarcane bagasse samples (So5, So4) and other *Saccharum* species samples divided by sugarcane bagasse samples.
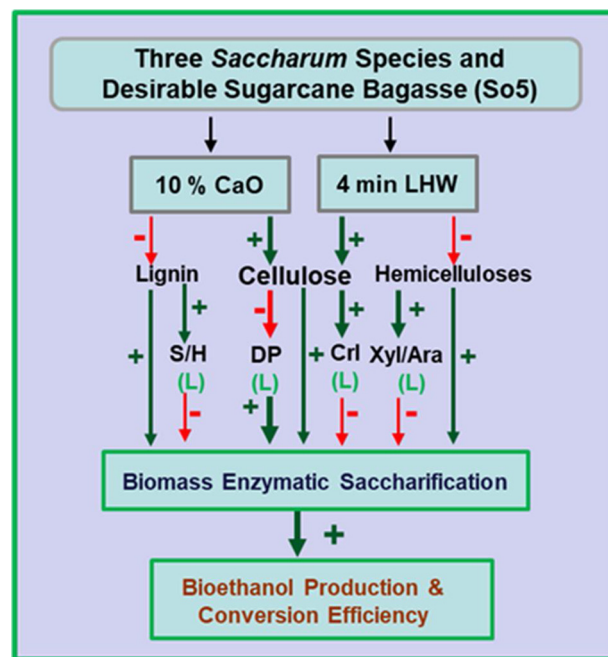

Fig. 6. A hypothesis model outlining how 10% CaO and 4 min LHW pretreatments largely enhance biomass saccharification and bioethanol production in three *Saccharum* species and the desirable sugarcane bagasse (So5): The red arrow and "-" mark indicate a reduction of wall polymer level or polymer feature or biomass saccharification, and the green arrow and "+" mark highlight an increase of those factors. The light green "(L)" indicates a relatively low value of wall polymer feature in the desirable sugarcane bagasse, compared to other *Saccharum* species samples.

the desirable sugarcane bagasse and other *Saccharum* species samples, we proposed a mechanism model that highlights the major factors on biomass enzymatic hydrolysis, based on the novel findings of this study and the previous reports (Fig. 6). (1) The 10% CaO and 4 min LHW pretreatments could respectively extract lignin and hemicelluloses, which should allow much more cellulases enzymes accession and loading onto cellulose microfibrils for enhancing lignocellulose enzymatic hydrolysis. (2) Both hemicellulose and lignin extractions relatively increased cellulose levels for higher ethanol production (% dry matter), and they also largely reduced cellulose DP for significantly enhanced biomass enzymatic digestibility probably by providing more reducing sites of cellulose microfibrils for cellulase enzyme initial loading and digestion. (3) Both pretreatments could lead to distinct increase of other three major wall polymer features (cellulose CrI, hemicellulose Xyl/Ara, lignin S/H), which were the negative factors on the biomass enzymatic saccharification. (4) The desirable sugarcane bagasse sample (So5) showed relatively lower values of those three negative wall polymer features, compared to other *Saccharum* species samples.

Therefore, this hypothetic model has pointed out four positive and three negative factors on biomass enzymatic hydrolysis from the 10% CaO and 4 min LHW pretreatments in all biomass samples. It has also highlighted that the desirable sugarcane bagasse has relatively lower values of three negative factors, which should be additional causes for its highest hexoses yield, second high bioethanol production and the highest sugar-ethanol conversion rate, compared to other *Saccharum* samples and the previously reported bioethanol yields in sugarcane bagasse.

4. Conclusion

Chemical (NaOH, CaO, H$_2$SO$_4$) and physical (LHW) pretreatments were performed in three *Saccharum* species. The 10% CaO and 4 min LHW pretreatments, while co-supplied with 1.6% Tween-80, led to much enhanced biomass enzymatic saccharification and bioethanol production, mainly due to distinct hemicellulose and lignin extractions and much reduced cellulose DP. Notably, the desirable sugarcane bagasse (So5) showed much high bioethanol yield (19% dry matter), partially due to relatively low values of three negative factors (CrI, Xyl/Ara, S/H) on biomass hydrolysis. Hence, this study has provided a cost-effective biomass process technology for high bioethanol production.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.08.039.

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