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Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed

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HIGHLIGHTS

- Steam explosion much reduces cellulose DP and largely extracts polymers in reed.
- Tween-80 is effective for high biomass saccharification in steam-exploded residues.
- Additional CaO pretreatment leads to the highest ethanol yield at 19% of dry matter.
- Tween specifically blocks lignin absorbing with cellulase for high biomass digestion.
- It provides an optimal biomass process approach for high ethanol yield in reed.

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ABSTRACT

In this study, eight physical and chemical pretreatments were compared in terms of their enhancements on biomass enzymatic saccharification in reed. Despite 8% NaOH pretreatment could result in 100% biomass enzymatic digestion while co-supplied with 1% Tween-80, it only produced bioethanol at 10% (% dry matter). By comparison, 10% CaO pretreatment with Tween-80 is a relatively low-cost biomass conversion with ethanol yield at 12%. Notably, the steam-explosion pretreatment with 1% Tween-80 could cause a complete biomass enzymatic hydrolysis with bioethanol yield at 17%. The sequential 5% CaO pretreatment with the steam-exploded residues could lead to the highest ethanol yield at 19% with an almost complete sugar–ethanol conversion rate. Due to much low-DP cellulose and less noncellulosic polymers (lignin, hemicelluloses) that increase biomass surfaces, the steam-exploded residues were specifically effective for Tween-80 either to block lignin absorbing with cellulases or to disassociate hemicelluloses, leading to an efficient lignocellulose enzymatic digestion. Compared with previously reported pretreatments in other C4-grasses (*Miscanthus*, corn, sweet sorghum, switchgrass), to our knowledge, this study has therefore provided three more applicable approaches for high ethanol production with relatively low cost, less contaminate release and efficient biomass conversion rates in reed.

1. Introduction

Lignocellulose has been increasingly considered for bioethanol production, due to large fossil energy consumption and environmental changes [1–3]. Common reed (*Phragmites australis*) is one of the most widespread wetland plants with high biomass yield for biofuel purpose. As a typical C4 grass, reed grows fast in water and lake margins, but also distributes in deserts and dry lands

Abbreviations: CrI, crystalline index; DP, degree of polymerization; Ara, arabinose; Xyl, xylose; H, *p*-coumaryl alcohol; G, coniferyl alcohol; S, sinapyl alcohol; GC–MS, gas chromatography–mass spectrometer.

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around the world. Over the past years, reed has been broadly used as a valuable raw fiber material in industry, agriculture and daily life. In particular, due to a high proportion of short fibers, reed is favor for paper production [4]. Despite physical and chemical pretreatments have been used in reed biomass process [5], it remains unknown about its optimal technology on biofuel production.

Principally, biomass process involves three major steps: physical and chemical pretreatments for wall polymer disassociation, enzymatic hydrolysis toward soluble sugar release, and yeast fermentation leading to ethanol production [6]. However, as lignocellulose recalcitrance could basically determine a costly biomass process, it becomes essential to find out an optimal pretreatment that not only enhances biomass enzymatic saccharification but also causes high ethanol production with less secondary pollution to the environment [7,8].

Acid and alkali such as H_2SO_4 and NaOH are the classical agents applied in chemical pretreatments, but CaO as a relatively low-cost chemical, can be re-used in industry, which has thus been considered as a relatively economical and environment-friendly chemical pretreatment [9–11]. In principle, alkali pretreatment can extract entire wall polymers by disassociation of hydrogen bonds among polymers, whereas acid pretreatment is able to release soluble sugars and lignin monomers [12,13]. By comparison, hot water and steam explosion are regarded as other relatively economical and environment-friendly physical pretreatments, due to less by-products release during biomass process [14,15]. Notably, the steam explosion pretreatments could largely reduce biomass particle size, extract wall polymers and alter lignocellulose features, leading to much enhanced biomass enzymatic digestibility distinctive in different biomass samples [16–18].

Furthermore, despite of a low cost, Tween has been found as a powerful surfactant for enhancing biomass saccharification by either distinctively disassociating wall polymers or largely increasing cellulases enzyme activity [19,20]. Despite Tween effects on biomass saccharification have been reported in different biomass samples [21,22], little is known about its specific roles in steam-exploded residues and other pretreated lignocelluloses, in particular on reed biomass. Hence, it remains to find out optimal technology of biomass pretreatment and sequential enzymatic hydrolysis for efficient biofuel production in reed.

Plant cell walls are composed mainly of cellulose, hemicelluloses and lignin. Cellulose crystallinity and degree of polymerization (DP) have been characterized as the negative factors on biomass digestibility, whereas hemicelluloses could reduce cellulose crystallinity for high biomass saccharification in many plant species examined [23,24]. By comparison, lignin may play dual roles in biomass enzymatic digestions, due to three monolignols proportions distinctive in different plant species [25].

In the present study, we performed various physical and chemical pretreatments with the mature stem materials of reed plants, and compared their distinct effects on biomass enzymatic saccharification and bioethanol production. Then, we found out optimal technology with relatively economical and environment-friendly biomass pretreatments that are capable for high bioethanol production by means of steam explosion or chemical (CaO) pretreatment followed by Tween-80 co-supply with cellulases into biomass enzymatic hydrolysis in reed.

2. Material and methods

2.1. Plant samples

Common reed (*P. australis*) was grown in lake margins of Tarim, Xinjiang, China. The mature stalks of 5–10 plants were harvested, dried at 50–55 °C and ground into powders through 40 mesh screen. The well-mixed powders were stored in a sealed dry container until in use.

2.2. Plant cell wall fractionation

The procedure of plant cell wall fractionation was used to extract wall polymers as described by Peng et al. [26] and Huang et al. [14]. After soluble sugars, lipids, starches and pectin of the biomass samples were successively removed, the remaining pellet was treated with 4 M KOH and 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and the combined supernatant was used as KOH-extractable hemicelluloses. The remaining one parallel non-KOH-extractable residue was sequentially extracted with TFA for monosaccharides. One parallel 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. One parallel was extracted with acetic–nitric acids–water (8:1:2; v/v/v) for 1 h at 100 °C and the remaining pellet was regarded as crystalline cellulose for DP detection. All experiments were carried out in biological triplicate.

2.3. Colorimetric assay of hexoses and pentoses

The anthrone/H_2SO_4 method [27] and orcinol/HCl method [28] were respectively used for hexoses and pentoses assay. D-glucose and D-xylose were used in drawing the standard curves, 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. All experiments were conducted in biological triplicate.

2.4. Total lignin and monolignol detection

Total lignin content was measured by the two-step acid hydrolysis method according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory as described by Wu et al. [6]. Monolignols were detected by HPLC according to the method described by Si et al. [29].

2.5. Hemicellulose monosaccharide determination

Total hemicelluloses were measured by accounting total hexoses and pentoses from KOH-extractable and non-KOH-extractable hemicelluloses. Monosaccharides of hemicelluloses were detected by GC–MS as described by Li et al. [30].

2.6. Cellulose CrI and DP measurement

The X-ray diffraction method was used for cellulose crystalline index (CrI) assay as described by Zhang et al. [24]. Standard error of the CrI method was detected at ±0.05 to approximately 0.15 using five representative samples in triplicate. The viscosity method was applied for cellulose DP detection using crystalline cellulose samples as described by Huang et al. [14].

2.7. Physical and chemical pretreatments

Steam explosion pretreatment: The dried reed stem materials were pretreated under steam explosion using Steam Explosion Reactor (QBS-200, Hebi Zhengdao Machine Factory, Hebi, China). All conditions were described by Huang et al. [14]. The steam-exploded reed residues were dried and ground into powders through 40 mesh screen, and used for further chemical pretreatments as described below.

Liquid hot water (LHW) pretreatment: The well-mixed raw materials or steam-exploded residues were added into well-

sealed stainless steel bombs, and heated at 200 °C under 15 rpm shaking for 2, 4, 8, 16 min, respectively. Then, the sealed bombs were cool down immediately.

H_2SO_4 pretreatment: The well-mixed biomass samples were treated with 6 mL H_2SO_4 at various concentrations (0.5%, 1%, 2%, v/v). The sample tubes were sealed and heated at 121 °C for 20 min in autoclave (15 psi), then at 50 °C for 2 h. 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 performed as control.

NaOH pretreatment: The well-mixed biomass samples were treated with 6 mL NaOH for 2 h at 50 °C at various concentrations (0.5%, 1%, 2%, 4%, 8%, 12%, 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 for 48 h at 50 °C at various concentrations (1%, 2.5%, 5%, 10%, 15%, w/w). 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.

The solid–liquid/water ratio for pretreatment is 1:20. The sealed samples were centrifuged at 3000*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 carried out in biological triplicate.

2.8. Detection of enzymatic hydrolysis

The remaining residues from steam explosion and chemical pretreatments were washed once with 10 mL of mixed-cellulases reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8), treated with 6 mL (2.0 g/L) of mixed-cellulases containing β-glucanase ($\geqslant 3.73 \times 10^4$ U), cellulase ($\geqslant 373$ U) and xylanase ($\geqslant 6 \times 10^4$ U) purchased from Imperial Jade Bio-technology Co., Ltd and shaken under 150 rpm for 48 h at 50 °C. The samples were centrifugation at 3000*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 regarded as the control. All experiments were carried out in biological triplicate.

2.9. Enzymatic hydrolysis with Tween-80

The biomass samples from steam explosion and chemical pretreatments were washed once with 10 mL of mixed-cellulases reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8), treated with 6 mL (2 g/L) of mixed-cellulases containing Tween-80 at various concentrations (0.5%, 1%, 2%, 4%, v/v). The sealed samples were shaken under 150 rpm for 48 h at 50 °C (solid–liquid ratio, 1:20). After centrifugation at 3000*g* for 5 min, the supernatants were collected for pentoses and hexoses assay.

2.10. Detection of cellulase adsorption

The soluble cellulase enzymes were obtained by collecting the supernatants from biomass enzymatic hydrolysis described above. Total cellulase proteins were determined by using Coomassie Brilliant Blue G250 assay [31]. The Coomassie Brilliant Blue G250 dye was prepared in solution containing ethanol (w/v; 2:1) and phosphoric acid (w/v; 1:1) and filtered through a 0.22 μm filter. The absorbance of the protein-dye complex was reading at 595 nm using UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China). Every 1 mL enzymatic hydrolysate was added into 10 mL ethanol for precipitating cellulase and reducing the Tween-80 interference. The precipitated protein was added into 1.0 mL distilled water and 3.0 mL Coomassie Brilliant Blue G-250, and the absorbance was measured 10 min later. All experiments were carried out by reading absorbance at time-course.

2.11. Yeast fermentation and ethanol measurement

Yeast fermentation was performed as described by Li et al. [13] using *Saccharomyces cerevisiae* powder (Angel yeast Co., Ltd., Yichang, 443000, China) and sugar-extracts obtained from various pretreatments and sequential enzymatic hydrolysis. 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 at 37 °C for 48 h in glass test tube sealed with rubber plugs to allow CO_2 liberation.

Ethanol was measured using $K_2Cr_2O_7$ method as described by Li et al. [13]. The fermentation liquid was distilled at 100 °C for 15 min, and appropriate amount of ethanol sample in 2 mL 5% $K_2Cr_2O_7$ was heated for 10 min in a boiling water bath. The samples were cooled down and added with distilled water to 10 mL. The absorbance was read at 600 nm, and absolute ethanol was used as the standard. All experiments were carried out in biological triplicate.

2.12. Listing of all methods performed in this study

Based on the methods described above, a list of all methods applied in this study was presented in Table S9.

2.13. Statistical calculation of correlation coefficients

Correlation coefficients were obtained 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. Large wall polymer extractions with steam explosion

In this study, three major wall polymers (cellulose, hemicelluloses and lignin) levels were initially determined at 35%, 26% and 21% in the mature stem tissues (raw materials) of reed (Table 1). Steam explosion was then performed with the raw materials, leading to hemicelluloses and lignin extractions by 76% and 41%, respectively. By contrast, cellulose level was significantly increased by 5% in the steam-exploded residue, compared with raw material. Hence, the data indicated that reed is the cellulose-rich biomass material, in particular on the steam-exploded residues, compared with other C4 grass plants examined, such as *Miscanthus* (ranged from 19.7% to 38.5%), switchgrass (28–37%), corn (37%) and sweet sorghum (15–27%) [24,32–34].

As a consequence, the steam explosion significantly altered wall polymers features (Table 2). In terms of cellulose features, cellulose CrI value was increased by 13%, whereas cellulose DP was reduced by 45% in the steam-exploded residues. Monosaccharide composition analysis indicated two major monosaccharides (xylose-xyl and arabinose-Ara) of hemicelluloses at high proportions (90%, 7.5%) in the raw material, similar to previous reports in *Miscanthus* and other grasses [16,18]. However, the steam explosion caused a high percentage of glucose (Glu) with relatively reduced Xyl and Ara proportions. As the hemicelluloses fraction was obtained from 4 M KOH extraction, the increased glucose should be derived from the low-DP cellulose co-extracted with hemicelluloses in the steam-exploded residues. In addition, despite total lignin was

Table 1
Cell wall composition (% dry matter) of the raw materials and steam-exploded residues in reed.

Samples	Cell wall composition (% dry matter)		
	Cellulose	Hemicelluloses	Lignin
Raw material	34.98 ± 0.35	26.47 ± 1.18	21.18 ± 1.88
Steam-exploded	36.65 ± 0.39 **	6.4 ± 0.13**	12.46 ± 0.11**
	(4.77% #)	(−75.8%)	(−41.2%)

** Indicated significant difference between the raw material and steam-exploded residue by *t*-test at $p < 0.01$ ($n = 3$).
# Percentage of increased or decreased level between the raw materials and steam-exploded residues by subtraction of two values divided by value of the raw materials. Data indicated mean as ± SD ($n = 3$).

reduced in the steam-exploded residues, three monomers proportions were not much altered, in particular on H-monolignol. Taken together, the steam explosion could not only extract large amounts of hemicelluloses and lignin, but also greatly reduce cellulose DP in reed.

3.2. Enhanced biomass saccharification under steam explosion and chemical pretreatments

Biomass saccharification (digestibility) has been measured by calculating the hexoses yield (% cellulose) released from cellulases hydrolysis of physical and chemical pretreated biomass [23]. In this work, we examined the hexoses yields released from enzymatic hydrolysis in raw materials and steam-exploded residues of reed samples (Fig. 1). As a result, the steam-exploded residue exhibited the hexoses yield at 52% (Table S1), which was more than 5-folds higher than that of the raw material (control, without steam explosion). Under the sequential pretreatment with 0.5% H_2SO_4, the steam-exploded residue remained an increased hexoses yield up to 83%, but it had a slightly reduced hexoses yield from high H_2SO_4 concentrations (1%, 2%). By comparison, the raw materials showed the hexoses yield up to 35% under H_2SO_4 pretreatment at various concentrations (Fig. 1A). Under NaOH pretreatments, the raw materials of reed could even have hexoses yields up to 84% at high concentration (8%), and the steam-exploded residue had the highest hexoses yield at 88% from 4% NaOH pretreatment (Fig. 1B). Thus, either the raw material or the steam-exploded residue of reed exhibited biomass enzymatic saccharification distinctive in H_2SO_4 and NaOH pretreatments, similar to the reports in *Miscanth*us, corn, sweet sorghum [12,25,35].

To find out relatively cost-effective pretreatments with less environmental pollution, CaO and liquid hot water (LHW) pretreatments were performed in raw material and steam-exploded residue of reed (Fig. 1C and D; Table S2). Pretreated with 10% CaO, the raw material of reed exhibited the highest hexoses yield at 43% from enzymatic hydrolysis, which was more than 4-fold higher than that of control (10%). By comparison, the steam-exploded residue had the highest yield at 70% upon 10% CaO pretreatment (Fig. 2C). Hence, the CaO pretreatment could also cause high biomass enzymatic saccharification in reed, in particular on the steam-exploded residue. Furthermore, despite that the raw material of reed only showed the hexoses yield at 37% under LHW pretreatment for 8 min, the steam-exploded residue could have the hexoses yield up to 71% under 2 min (Fig. 2B). However, LHW pretreatments at longer time caused a reduced hexoses yield, suggesting that such pretreatment may alter lignocellulose and soluble sugar structures and properties [36]. Thereby, the physical (steam-explosion) and chemical pretreatments performed in this study could exhibit a distinctive enhancement on biomass enzymatic saccharification in reed, but the steam explosion combined with other pretreatments (such as NaOH, H_2SO_4, CaO, LHW) could enhance hexoses yields from enzymatic hydrolysis in different degrees.

3.3. Tween-80 enhancement on biomass enzymatic hydrolysis

In this work, Tween-80 was directly applied into biomass enzymatic hydrolysis in raw materials and steam-exploded residues of reed by co-supplying with cellulase enzymes (Fig. 2A). Incubated with 0.5% Tween-80, the steam-exploded residue exhibited much higher hexoses yield (91%) by 1.7-fold from enzymatic hydrolysis, compared with control (0% Tween-80, Table S3). Notably, the steam-exploded residue could be almost hydrolyzed with hexoses yield at 98% from 1% Tween-80 treatment, indicating that the Tween-80 is extremely effective for steam-exploded biomass enzymatic hydrolysis in reed, compared with Tween applications in other biomass resources [21,37]. By comparison, the raw material of reed exhibited the highest hexoses yields at 19% from 1% Tween-80 supply. In addition, supplements with higher concentrations of Tween-80 (2%, 4%) could slightly reduce hexose yields in both raw material and steam-exploded samples. Similarly, the ethanol production is initially increasing and then becomes decreasing as Tween 40 concentrations remain raised up to the extremely high concentration, in which biochemical reaction condition may be altered, and/or more inhibition compounds may be released [21]. The results thereby suggest that 1% Tween-80 is the optimal concentration for enhancing biomass enzymatic digestibility in reed.

Based on those pretreatment enhancements on biomass saccharification as described above (Fig. 1), the raw materials and steam-exploded residues obtained from eight optimal pretreatments were respectively added with 1% Tween-80 during biomass enzymatic hydrolysis (Fig. 2B and C; Table S4). The raw materials with 1% Tween-80 exhibited much increased hexoses yields by 19–49% from four pretreatments, compared with the samples without Tween-80 (Fig. 2B). In particular, the raw material from 8% NaOH pretreatment showed a completely enzymatic hydrolysis. By comparison, the steam-exploded residues obtained from three pretreatments (0.5% H_2SO_4, 4% NaOH, 2 min LHW) also exhibited a completely enzymatic digestion while 1% Tween was added (Fig. 2C). Furthermore, the sequential 5% CaO pretreatment with the steam exploded residue supplied with 1% Tween-80, showed the hexose yield at 98%, similar to the control from 1% Tween-80

Table 2
Wall polymer features in the raw materials and steam-exploded residues of reed.

Samples	Cellulose features&		Monosaccharides of hemicelluloses (% total)							Three monolignols (% total)		
	CrI (%)	DP	Rha	Fuc	Ara	Xyl	Man	Glu	Gal	H	G	S
Raw material	53.27	254 ± 3	0.13	0	7.5	90.05	0.1	0.56	1.66	21.55	38.47	39.98
Steam-exploded	60.24	140 ± 3**	0.05	0	2.78	86.24	0.69	9.72	0.51	22.64	32.78	44.58
	(13%#)	(−45%)	(−62%)		(−63%)	(−4%)	(590%)	(1636%)	(−69%)	(5%)	(−15%)	(12%)

& Crystalline cellulose of raw materials and stem-exploded residues used for DP detection.
** Indicated significant difference between the raw material and steam-exploded residue by *t*-test at $p < 0.01$ ($n = 3$).
# Percentage of increased or decreased level between the raw materials and steam-exploded residues by subtraction of two values divided by value of the raw materials.

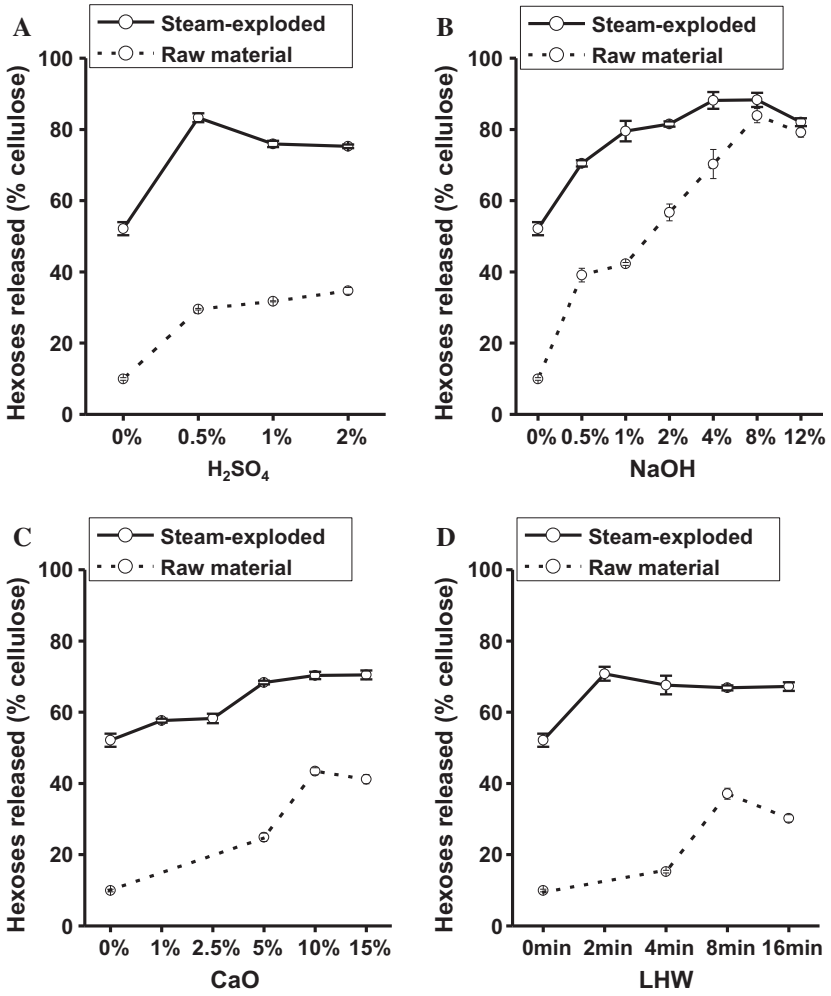

Fig. 1. Biomass saccharification under various pretreatments in raw materials and steam-exploded residues of reed. Hexoses yields (% cellulose) released from enzymatic hydrolysis after pretreatments with different concentrations of H_2SO_4 (A, Table S1), NaOH (B), CaO (C, Table S2) and with liquid hot water (LHW) at different times (D); The values indicated the means ± SD (n = 3).

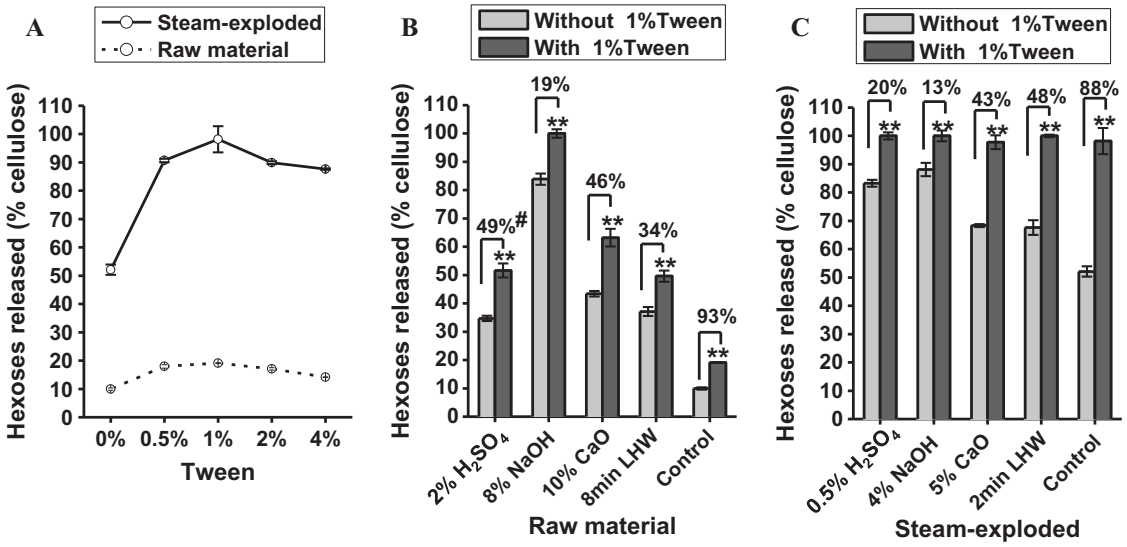

Fig. 2. Hexoses yields released from enzymatic hydrolysis supplied with Tween-80 after various pretreatments in raw materials and steam-exploded residues of reed. (A) Tween-80 effects on biomass saccharification at different concentrations (Table S3); (B and C) 1% Tween-80 effects on biomass saccharification under four pretreatments in the raw materials and steam-exploded residues, respectively (Table S4). The values indicated the means ± SD (n = 3); ** Indicated significant different hexoses yields between 1% Tween-80 supply and without Tween-80 at $p < 0.01$ level; # Indicated percentage of increased hexoses yield level between with 1% Tween-80 and without Tween-80 by subtraction of two values divided by value without Tween-80.

supplement. Thereby, the 1% Tween-80 supplement is extremely effective not only for enhancing biomass enzymatic saccharification of steam-exploded residues, but also for the raw materials from various pretreatments.

3.4. Tween-80 enhancement on bioethanol production

As physical and chemical pretreatments could produce the toxin compounds that inhibit yeast fermentation for ethanol production [13,35], we performed a classic yeast fermentation course to measure the ethanol production by using the sugars of supernatants obtained from pretreatment and sequential enzymatic hydrolysis (Fig. 3). Supplied with 1% Tween-80, the raw materials under four pretreatments exhibited much higher ethanol yields (Fig. 3A; Table S5). In particular, despite of 10% CaO pretreatment with 1% Tween-80 only resulting in 64% hexoses yield (Fig. 2B), it had the highest ethanol production at 12% among four pretreatments. By compared, the steam-exploded residues with 1% Tween-80 exhibited much higher ethanol yields ranged from 14% to 19% than those without Tween-80 with ethanol yields from 6% to 13% from other four pretreatments (Fig. 3B; Table S5). Notably, supplied with 1% Tween-80, the steam-exploded residues (control) could also have high ethanol yield at 17%, which is even higher than those from pretreatments with 8% NaOH and 2 min LHW. The results indicated that the steam-exploded residues with 1% Tween-80 supply are also effective for ethanol production.

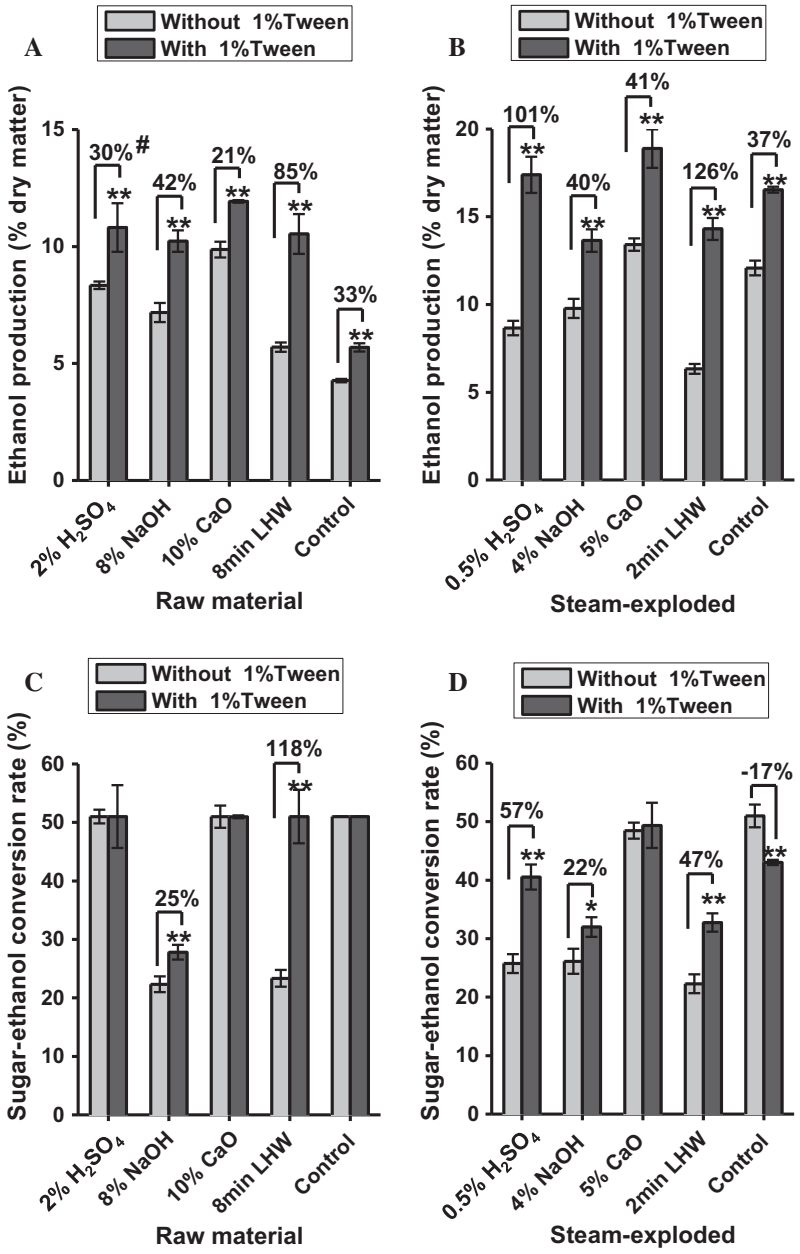

Fig. 3. Bioethanol production released from yeast fermentation using sugars obtained from pretreatments and enzymatic hydrolysis supplied with Tween-80 in raw materials and steam-exploded residues of reed. (A and B) 1% Tween-80 effects on ethanol productions under four pretreatments in raw materials and steam-exploded residues, respectively (Table S5); (C and D) 1% Tween-80 effects on sugar–ethanol conversion rates under four pretreatments in the raw materials and stem-exploded residues, respectively (Table S6). The values indicated the means ± SD ($n = 3$); * or ** indicated significant different ethanol yields between 1% Tween-80 supply and without Tween-80 at $p < 0.05$ or 0.01 level; # Indicated percentage of increased or decreased ethanol yields (A and B) or sugar–ethanol conversion rates (C and D) between with 1% Tween-80 and without Tween-80 by subtraction of two values divided by value without Tween-80.

Table 3
Bioethanol production in reed and other four C4 grasses.[a]

Plant species	Pretreatment	Ethanol production (% dry matter)	Reference
Common reed	10% CaO + 1% Tween-80	12%	This study
	Steam-explosion + 1% Tween-80	17%	
	Steam-explosion + 5% CaO + 1% Tween-80	19%	
Miscanthus	LHW	15%	[40]
Sweet sorghum	Mixing sorghum fibers, ammonia, and water at a ratio of 1:0.14:8 at 160 °C for 1 h under 140–160 psi pressure	21%	[41]
Cornb	Excess of calcium hydroxide (0.5 g $Ca(OH)_2$/g raw biomass) in oxidative conditions at 55 °C	15%	[42]
Switchgrass	AFEX	20%	[43]

[a] Ethanol production from yeast fermentation using sugars from various pretreatments and sequential enzymatic hydrolysis.

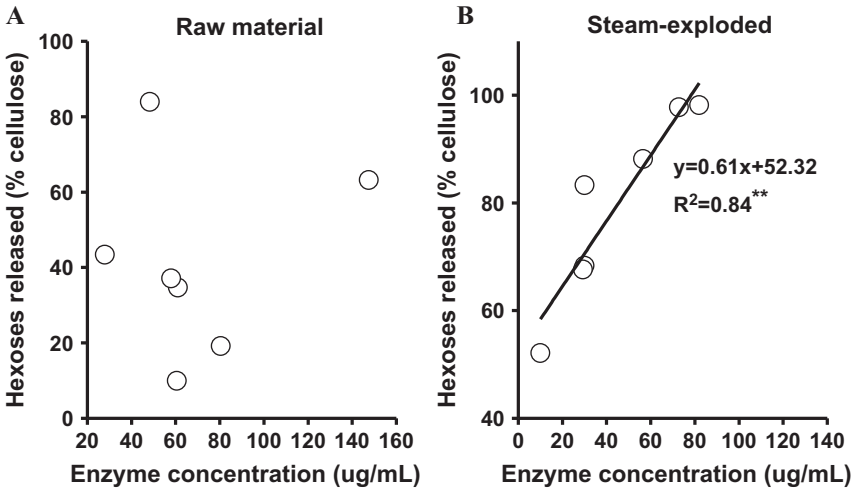

Fig. 4. Correlation analysis between the hexoses yields and cellulase enzyme concentrations in reed. The hexoses yields (Table S4) and cellulase enzyme concentrations (Table S7) were used for correlation analysis in raw materials (A) and steam-exploded residues (B); Data of 100% hexoses yields were not used for correlative coefficient calculations. ** Indicated the significant correlation at $p < 0.01$ ($n = 7$).

Furthermore, the sugar–ethanol conversion rates were calculated according to the hexoses yields (Table S4) and ethanol production (Table S5) in the raw materials and steam-exploded residues. The most pretreated raw materials, except the samples from pretreatments with 8% NaOH (with/without Tween-80) and 8 min LHW without Tween-80, showed the sugar–ethanol conversion rates at 51% (Fig. 3C; Table S6), indicating a almost complete sugar–ethanol conversion rate similar to the theory value. As contrast, only the steam-exploded residues from 5% CaO pretreatments and the control without Tween-80 had the sugar–ethanol conversion rates at 48%, 49%, 51% (Fig. 3D; Table S6), suggesting that the steam-explosion pretreatment should produce relatively more inhibitors on yeast fermentation than other pretreatments with raw materials in reed, which could be explained by the previous report that steam-explosion pretreatment largely degrades hemicelluloses-derived sugars and lignin-related compounds, thus producing furfural, aliphatic acids and phenolics as the toxic compounds inhibiting yeast fermentation [15]. The results also indicated that CaO pretreatment could reduce toxin inhibition to yeast fermentation in the steam-exploded residues, probably by absorbing and masking toxin compounds [38], or promoting precipitation of low molecular phenolics [39]. On the other hands, the other chemical (H_2SO_4, NaOH) and physical (LHW) pretreatments with steam-exploded residues performed in this work, should produce relatively more toxic compounds than those of CaO pretreatment, resulting in lower sugar–ethanol conversion rates and relatively less bioethanol production. In addition, the 1% Tween-80 supply could particularly increase sugar–ethanol conversion rates in the most pretreatments conducted in this study.

Taken all together, either the steam-explosion with 1% Tween-80 or the steam-explosion followed by 5% CaO with 1% Tween-80 is an optimal economical and environment-friendly technology for high ethanol production (17%, 19%) (Table 3), due to either relatively low-cost of both CaO and Tween-80 chemicals, or their directly re-use available in industry. In addition, the 10% CaO pretreatment with 1% Tween-80 in the raw materials of reed, other than the steam-exploded residues, should be an additional choice for the relatively low-cost biomass process into bioethanol yield (12%), because it does not use the initial steam-explosion. Furthermore, compared with other C4 grasses examined, the reed applied with steam-explosion and 1% Tween-80 supply, exhibited much higher bioethanol production than that of *Miscanthus* and corn (Table 3). Despite of a similar ethanol production to sweet sorghum and switchgrass, the steam explosion used in reed should be relatively less cost, compared with the ammonia fiber explosion (AFEX) and longer-time high pressure and temperature applied in those grasses [40–43].

3.5. Mechanism of optimal technology on biomass saccharification enhancement

To understand the steam explosion and Tween-80 that are optimal for enhancing biomass enzymatic hydrolysis in reed, we further measured the soluble cellulase enzymes that were applied in the biomass enzymatic hydrolysis. The steam-exploded residues under four pretreatments exhibited a significantly positive correlation between the soluble cellulase enzymes concentrations and hexoses yields at $p < 0.01$ level with high R^2 value at 0.84, whereas

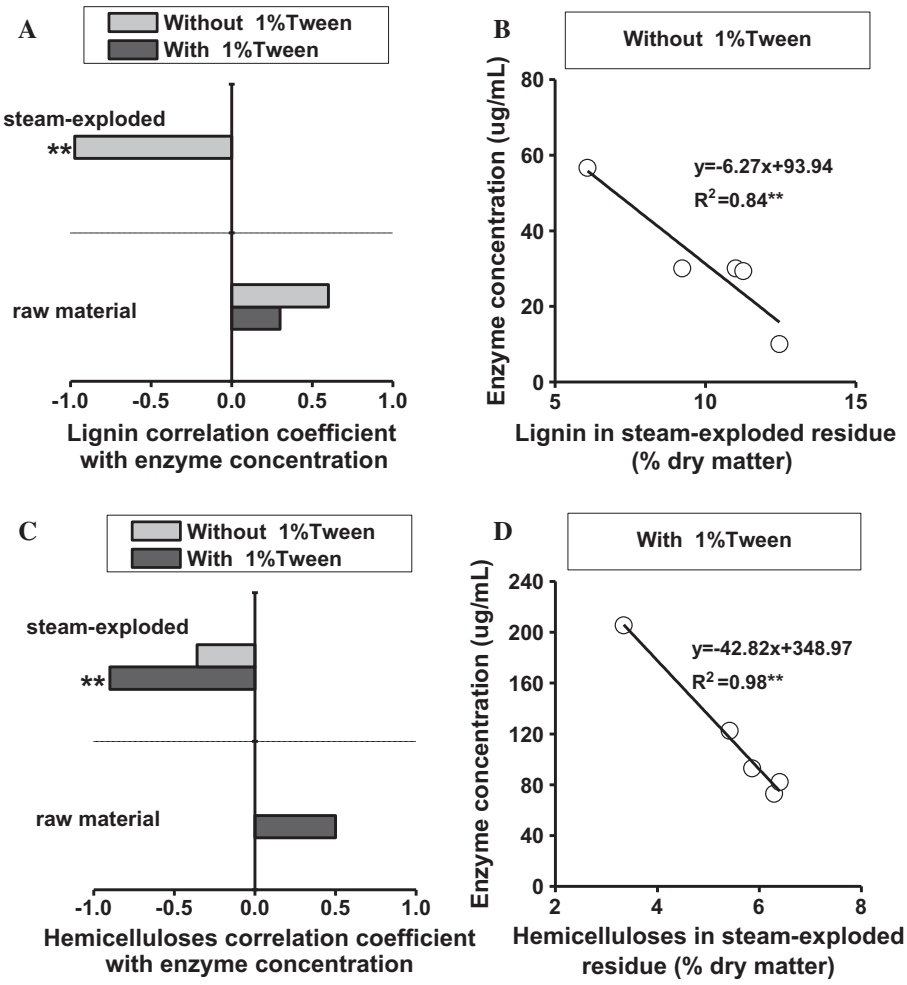

Fig. 5. Correlation analysis between lignin and hemicelluloses levels and cellulase enzyme concentrations in reed. Lignin (A/B; Table S8) and hemicelluloses levels (C/D) under four pretreatments were used for correlative coefficient calculations with cellulase enzyme concentrations (Table S7) in raw materials and steam-exploded residues, respectively. ** Indicated the significant correlation at $p < 0.01$ ($n = 5$).

the raw materials had a non-correlation (Fig. 4; Table S7). It suggested that the soluble cellulase enzymes should be not much absorbed with noncellulosic polymers (lignin, hemicellulose) in the steam-exploded residues, leading to an effective enzymatic hydrolysis of lignocelluloses [19]. On the other hands, due to its binding with much noncellulosic polymers, the cellulase enzymes may not much interact with cellulose residues in the pretreated raw materials, leading to relatively low hexoses yields as reported above (Fig. 2).

Furthermore, lignin levels of the steam-exploded residues after various pretreatments, showed a negative correlation with the soluble cellulase enzyme concentrations at $p < 0.01$ level with high R^2 value at 0.84, but a non-correlation was found in the residues with Tween-80 supply (Fig. 5A and B, Table S8), indicating that the Tween-80 should play a role in blocking lignin absorbing with cellulase enzymes in the steam-exploded residues, other than the pretreated raw materials. Hence, although it has been reported about Tween roles in disassociation between lignin and cellulases in different plant species [22], this study has found that the Tween-80 is specifically effective for the steam-exploded residues, other than the raw materials, probably due to its much low-DP cellulose and less lignin that lead to more surface space with Tween-80. As contrast, hemicelluloses of the steam-exploded residues with Tween-80, were negatively correlated with the cellulase enzymes at $p < 0.01$ level with extremely high R^2 value at 0.98, whereas correlation was not found in the steam-exploded residues without Tween-80 supply (Fig. 5C and D). It indicates that the Tween-80 could disassociate hemicelluloses with lignin in the steam-exploded residues, leading to relatively more cellulase enzymes interaction with hemicelluloses, in particular on xylanase. However, due to much low hemicelluloses level in the steam-exploded residues (Table 1), the hemicelluloses association with cellulases should not much affect biomass enzymatic hydrolysis. By comparison, lignin and hemicelluloses of the pretreated raw materials did no exhibit any correlation with cellulase enzymes no matter Tween-80 was supplied or not. It suggests that the lignocellulose residues could not well expose to the cellulase enzymes and Tween-80 in the pretreated raw materials.

Therefore, due to much lower-DP cellulose and less noncellulosic polymers, the steam-exploded residues should expose more surface spaces for Tween-80 either to block cellulase enzymes absorbing with lignin or to disassociate hemicelluloses from lignin, compared with the pretreated raw materials. It has also interpreted why the steam explosion with Tween-80 supply is optimal for high biomass enzymatic saccharification and ethanol production in reed.

4. Conclusions

Using various physical and chemical pretreatments in reed, the steam explosion pretreatment with 1% Tween-80 co-supply with cellulases is optimal for enhancing both hexoses yield from

enzymatic hydrolysis and bioethanol production from yeast fermentation. Additional 5% CaO pretreatment with the steam-exploded residues could have the highest ethanol production at 19% (% dry matter), while 1% Tween-80 is co-supplied. Due to much low-DP cellulose and less noncellulosic polymers in the steam-exploded residues, it has also demonstrated that Tween-80 is effective for blocking lignin interaction with cellulase enzymes, leading to a high biomass enzymatic digestion.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2016.04.104.

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