Distinct mechanisms of enzymatic saccharification and bioethanol conversion enhancement by three surfactants under steam explosion and mild chemical pretreatments in bioenergy Miscanthus

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\begin{abstract}
Miscanthus is a leading bioenergy crop that represents an enormous lignocellulose resource for biofuels and bioproducts. However, as lignocellulose recalcitrance leads to financially inviable bioethanol production and the potential of secondary wastes into the environment, it becomes crucial to explore green-like and cost-effective biomass processing technologies. To address these issues, low doses of chemical surfactants have been added to enhance biomass enzymatic hydrolysis and bioethanol conversion, but much remains unknown about the mechanism of enhancement. For first time, in this study, a novel chemical surfactant (1\% Silwet L-77) was applied to enhance the enzymatic hydrolysis of raw Miscanthus straw, and 40\% cellulose digestion was achieved, which is 1.2- and 4.5-fold higher than that of two well-known surfactants (PEG-4000 and Tween-80), respectively. Using lignocellulose substrates obtained from Miscanthus biomass samples that were pretreated by green-like steam explosion followed by mild chemical (NaOH or H\textsubscript{2}SO\textsubscript{4}) pretreatments, supplementation with the three surfactants led to significantly enhanced enzymatic saccharification. The 2\% Tween-80 supply resulted in a hexose yield of 99\% (% cellulose) from enzymatic hydrolysis, followed by 95\% with 0.5\% PEG-4000 and 71\% with 1\% Silwet L-77. Despite the slightly lower hexose yield, Silwet L-77 resulted in consistently higher sugar-ethanol conversion rates in all lignocellulose substrates examined. Furthermore, based on the enzyme profiling of mixed cellulase adsorption on lignocellulose and the chemical analysis of wall polymer features and lignocellulose accessibility, this study proposed multiple hypothetical models to interpret the distinct enhancement roles of three surfactants in the enzymatic hydrolyses of diverse lignocellulose substrates. These models also provide a powerful strategy for low-cost bioethanol production with the potential for high-value bioproducts by using desirable surfactants in Miscanthus and other bioenergy crops.
\end{abstract}

1. Introduction

Lignocellulose represents the most abundant renewable resource that is convertible for biofuel and chemical production (Pauly and Keegstra, 2010; Ragauskas et al., 2006). In particular, cellulosic ethanol has been evaluated as an excellent additive for petrol fuel to reduce net carbon release (Xie and Peng, 2011; Huang et al., 2011). In principle, the biochemical conversion of lignocellulose involves three major steps:
initial physical and chemical pretreatments for wall polymer destruction, sequential enzymatic hydrolysis for soluble sugar release and yeast fermentation for ethanol production (Wang et al., 2016; Nakashima et al., 2011; de Souza et al., 2014). However, lignocellulose recalcitrance requires a high-cost pretreatment and results in low-efficiency enzymatic hydrolysis along with the formation of various toxic compounds that inhibit yeast fermentation, leading to bioethanol production that is financially inviable for commercial marketing (Palmqvist and Hahn-Hägerdal, 2000). Hence, it becomes crucial to discover the optimal technology for biomass hydrolysis and bioethanol conversion.

As the initial step in biomass processing, cost-effective and environmentally-friendly pretreatments have been explored to enhance lignocellulose hydrolysis (Yang et al., 2019; Gu et al., 2012). Acids and alkalis such as H₂SO₄ and NaOH are the classic agents used in chemical pretreatment by respectively digesting hemicellulose and partially extracting lignin and non-cellulosic polysaccharides, but it is difficult to avoid the release of secondary waste when high concentrations of acid or碱 are required to overcome lignocellulose recalcitrance under intense conditions (Si et al., 2015; Xu et al., 2012; Zhang et al., 2013; Samuel et al., 2011). By comparison, steam explosion has been regarded as relatively economical and environment-friendly pretreatment that reduces lignocellulose particle size and partially extracts wall polymers, leading to remarkably enhanced enzymatic saccharification in different biomass residues (Sun et al., 2017; Huang et al., 2015; Zahoor et al., 2017).

In recent years, chemical surfactants, particularly nonionic chemicals such as polysorbate (Tween) and polyethylene glycol (PEG), have been applied to enhance enzymatic lignocellulose saccharification (Tu and Saddler, 2010; Vaidya et al., 2014). For example, Tween-80 can specifically block cellulase adsorption to lignin in steam-exploited lignocellulose, leading to enhanced enzymatic saccharification and bioethanol conversion in the common reed (Jin et al., 2016). PEG is another synthetic polymer with low toxicity and high solubility in aqueous solutions that can effectively bind to the lignin surface, resulting in enhanced enzymatic digestion of cellulose (Vaidya et al., 2014; Nasipour and Mousavi, 2018). More recently, it has been reported that nonionic surfactants enhance biomass digestibility via several different mechanisms: (i) the surfactant changes the structure of the lignocellulose substrate, making the cellulose more available to cellulase attack (Kaar and Holtzapple, 1998); (ii) surfactants increase cellulose stability (Qi et al., 2010); (iii) surfactants decrease the nonproductive adsorption of cellulase to lignin (Eriksson et al., 2002); and (iv) surfactants enhance the cellulase-substrate interaction (Brosse et al., 2012). However, the distinct mechanisms by which different surfactants enhance enzymatic lignocellulose hydrolysis and bioethanol conversion remain to be discovered.

Silwet L-77 is a semi-synthetic mixture of polyalkyleneoxide composed of 84% heptamethyldithioloxane and 16% allyloxypolyethylene glycol methyl ether. Due to its effective penetration and low damage to plant cells, Silwet L-77 has been used as an ingredient in infiltration media for agrobacterium-based gene transformation in Arabidopsis and other plants (Clough and Bent, 1998; Zhang et al., 2006). However, little has been reported regarding whether Silwet L-77 could act as an active surfactant in biomass enzymatic hydrolysis and bioethanol conversion under various physical and chemical pretreatments.

Miscanthus is a leading bioenergy crop due to its rapid growth, high lignocellulose yield and adaptability to various environments (Xie and Peng, 2011). Based on previously performed analyses of large populations of Miscanthus accessions (Li et al., 2014a; Wang et al., 2015; Li et al., 2013), this study collected Miscanthus straw samples that showed variation in biomass enzymatic digestibility. We then performed steam explosion followed by a mild chemical (acid or alkali) pretreatment and evaluated three surfactants (TWEEN-80, PEG-4000, and Silwet L-77) with distinct enhancement roles in biomass enzymatic saccharification and bioethanol conversion in both raw materials and various pretreated lignocellulose substrates. This study represents the first application of Silwet L-77 as a unique surfactant specific for enhancing either direct enzymatic hydrolysis of raw biomass materials or the sugar-ethanol conversion efficiency of all pretreated lignocellulose substrates examined. In addition, this study presents an enzyme profile of the mixed cellulases applied to enzymatic hydrolysis and determines the major wall polymer features and interlinkings, leading to the proposal of multiple hypothetical models for understanding how the three surfactants could play distinct enhancement roles in biomass enzymatic hydrolyses under various physical and chemical pretreatments. This study provides a green-like and cost-effective strategy for bioethanol production and other potential value-added bioproducts from bioenergy Miscanthus.

2. Material and methods

2.1. Biomass sample collection

The five/six-years-old Miscanthus accessions were respectively grown in Hanchuan and Wuhan experimental fields, and the mature stem were harvested, dried at 50 °C, ground into powder through 40 mesh screen and stored in sealed dry container until in use.

2.2. Wall polymer extraction

Plant cell wall fractionation procedure was used to extract hemicelluloses and cellulose as previously described by Peng et al. (2000). Hemicelluloses was calculated based on total hexoses and pentoses determined in the hemicellulose fraction, and hexoses were measured as cellulose in the cellulose fraction. All experiments were carried out in independent triplicate.

2.3. Colorimetric assay of hexoses and pentoses

UV-vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co.) was used for hexoses and pentoses assay as previously described by Huang et al. (2012). For cellulose assay, the biomass sample was dissolved in 67% H₂SO₄ and hexoses were calculated by the anthrone/H₂SO₄ method. The pentoses were measured by the orcinol/HCl method. The standard curves for hexoses and pentoses assay were drawn by using α-glucose and β-xylene as standards (purchased from Sinopharm Chemical Reagent Co., Ltd.). Regard less, the high pentose levels that affect the absorbance reading at 620 nm for hexoses assay, the deduction from pentoses readings at 660 nm was carried out for final hexoses calculation. All experiments were conducted in independent triplicate.

2.4. Cellulose features and accessibility detection

The degree of polymerization (DP) of crude cellulose sample was measured as recently described by Sun et al. (2017). All experiments were conducted in biological triplicate. Cellulose crystallinity index (CrI) was determined using X-ray diffraction (XRD) method (Rigaku/D-MAX, Ultima III; Japan) as previously described by Xu et al. (2012). Congo Red (CR) stain was applied to estimate cellulose surface area as described by Cheng et al. (2019). All experimental analyses were performed in independent triplicates.

2.5. Scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy observation

The biomass morphology was observed using SEM (SEM JSM-IT300, Akishima, Tokyo, Japan). Well-mixed sample residues collected after pretreatments and enzymatic hydrolysis were sputter-coated with gold in a JFC-1600 ion sputter (Mito City, Japan) and visualized for 5–8 times to acquire representative images. FTIR spectroscopy was performed to observe the chemical linkages in the biomass residues using
2.6. Biomass pretreatment and enzymatic hydrolysis

The dried Miscanthus stem samples were pretreated under steam explosion as previously described by Huang et al. (2015). The Miscanthus stem tissues were cut into the 5–6 cm size and sprayed with deionized water to remain its moisture at 50%. The cut stem samples (200 g, dry matter) were loaded into 5-L steam explosion reactor (QBS-200, Hebi Zhengdao Machine Factory, Hebi, China), and treated at 225 °C (2.5 MPa) for 3 min (severity factor: R<sub>0</sub> = 4.22). The steam-exploded (SE) Miscanthus residues were ground into powders through 40 mesh screen and used for enzymatic hydrolysis experiments as described below.

H<sub>2</sub>SO<sub>4</sub> and NaOH pretreatments were respectively performed as described by Li et al. (2018). For acid pretreatments, the well-mixed biomass powders (0.3 g) were added with 6 mL H<sub>2</sub>SO<sub>4</sub> at various concentrations, heated at 121 °C for 20 min in an autoclave (15 psi), and shaken under 150 rpm for 2 h at 50 °C. The samples were then centrifuged at 3000g for 5 min, and the pellets were washed with 10 mL distilled water for 4–5 times until no sugar release. For alkali pretreatments, the well-mixed biomass powders (0.3 g) were incubated with 6 mL NaOH at various concentrations, shaken under 150 rpm for 2 h at 50 °C, and centrifuged at 3000g for 5 min. The pellets were washed with 10 mL distilled water for 4–5 times. All supernatants were combined for pentoses and hexoses assay and the remaining pellets were used for enzymatic hydrolysis as described below. Meanwhile, the well-mixed biomass powders were added only with 6 mL distilled water and shaken under 150 rpm for 2 h at 50 °C as a control.

For enzymatic hydrolysis, the pretreated biomass samples were washed once more with 10 mL of mixed-cellulase reaction buffer (0.2 mol L<sup>−1</sup> acetic acid-sodium acetate, pH 4.8). The washed samples were added with mixed-cellulases (containing β-glucanase ≥ 5.96 × 10<sup>4</sup> U and cellulase ≥ 596 U and xylanase ≥ 9.6 × 10<sup>4</sup> U, purchased from Imperial Jade Bio-technology Co., Ltd., China) and surfactants, with the final enzyme concentration at 1.6 g/L and Tween-80 concentration at 2% (v/v), PEG-4000 (0.5%) and Silwet L-77 (1%), respectively. The samples were then shaken under 150 rpm at 50 °C for 48 h, and the sample was only added with 6 mL of reaction buffer and shaken under 150 rpm at 50 °C for 48 h as a control. After reaction, all samples were centrifuged at 3000 g for 5 min. The supernatants were collected for determining total pentose and hexose yields released from enzymatic hydrolysis, and the pentoses and hexoses yields were expressed based on the same amounts of dry biomass used for pretreatments. All samples were carried out in independent triplicate.

2.7. Yeast fermentation and ethanol measurement

Yeast fermentation was performed as described by Zahoor et al. (2017). Using Saccharomyces cerevisiae strain (Angel yeast Co., Ltd., Yichang, China) and ethanol was measured using K₂Cr₂O₇ method as described by Wu et al. (2019) and Zahoor et al. (2017). The yeast fermentation used total hexoses released by enzymatic hydrolysis of pretreated biomass residues or total soluble sugars obtained from plant cell wall fractionation procedure. The fermentation liquid was distilled at 100 °C for 15 min, and appropriate amount of ethanol sample in 2 mL 5% K₂Cr₂O₇ 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. The bioethanol yields were expressed based on the same amounts of dry biomass used for pretreatments and sequential enzymatic hydrolyses. All experiments were carried out in independent triplicate.

The sugar-ethanol conversion rate was calculated by the following equation as described by Huang et al. (2015), 

\[ S - E = E/A/H \times 100\% \]

where S is the sugar-ethanol conversion rate; E is the total ethanol weight (g) at the end of fermentation; A is the conversion rate at 51.11% (92/180) in the case that glucose is completely converted to ethanol according to the Embden-Meyerhof-Parnas pathway in S. cerevisiae; and H is the total hexoses weight (g) at the beginning of fermentation. All experiments were performed in technological triplicate.

2.8. Enzyme adsorption detection

The soluble mixed-cellulases enzymes were obtained by collecting the supernatants from biomass enzymatic hydrolysis described above. Total cellulase proteins were measured using Coomassie Brilliant Blue G250 assays described by Jin et al. (2016). Total proteins were then loaded into 12% SDS-PAGE gel and separated as previously described by Li et al. (2017).

2.9. Statistical analysis

Correlation coefficients were generated by performing spearman rank correlation analysis for all the measured traits across biomass samples from different treatments. The analysis used average values calculated from all original determination values. Superior Performance Software Systems (SPSS version 16.0, Inc., Chicago, IL) were applied for any types of calculations. Means were separated by least significant difference (LSD) test at p = 0.05. Pair-wise comparisons were conducted between two measurements by Student’s t-test. The line graphs were generated using Origin 8.5 software (Microcal Software, Northampton, MA). The average values were calculated from the original triplicate measurements for these analyses.

3. Results and discussion

3.1. Specific enhancement of direct enzymatic hydrolysis of Miscanthus straw using Silwet L-77

In this study, we initially performed direct enzymatic hydrolysis of raw straw from a Miscanthus accession (Mfl12) to measure the hexoses yields against cellulose (% cellulose) released during incubation with three surfactants (Tween-80, PEG-4000, and Silwet L-77) under a series of concentrations (Fig. 1A–C). As a control without any surfactant supplementation, the raw Mfl12 straw sample showed a hexose yield of 7% (% cellulose) from direct enzymatic hydrolysis. Incubation with 1% Tween-80 produced a 9% hexose yield, which was slightly higher than that of the control (p < 0.05, Fig. 1A). Supplementation with PEG-4000 and Silwet L-77 at optimal concentrations produced hexose yields of 33% and 40% (% cellulose), respectively. To confirm this finding, we examined another two Miscanthus accessions (Mfl40 and Mfl02) that were previously reported to show distinct enzymatic hydrolyses. The addition of Silwet L-77 produced significantly higher hexose yields than that of PEG-4000 (p < 0.05) from direct enzymatic hydrolysis of raw straw from both Miscanthus accessions, whereas the Tween-80 continued to exhibit the lowest hexose yields (Fig. 1D). Thus, despite the large variation in hexose yields among the three different Miscanthus accessions, the addition of the novel surfactant (Silwet L-77) consistently produced remarkably higher biomass saccharification from direct enzymatic hydrolysis of raw straw compared with two well-known surfactants (Tween-80 and PEG-4000), probably due to the well penetration of Silwet L-77 into the cell walls of intact biomass tissues, allowing enzyme access and loading on the cellulose surface (Clough and Bent, 1998; Zhang et al., 2006). In addition, the significantly higher enhancement shown by PEG-4000 relative to that of Tween-80 was assumed to be due to PEG-4000 having relatively better penetration into the plant cell walls.

Because supplementation with 1% Silwet L-77 produced 40% cellulose digestion in the desirable Miscanthus accession (Mfl12), this
study also examined that the most hemicelluloses were digestible by direct enzymatic hydrolysis of raw straw using mixed-cellulase enzymes (data not shown). Hence, the pentoses released from hemicellulose hydrolysis could be directly collected for use as a diet additive and other valuable products without any additional chemical application (Nabarlatz et al., 2004; Sørensen et al., 2007). Furthermore, the remaining crystalline cellulose should be completely separable from the lignin, which could be used for high-value cellulose nanofibers and lignin-derived chemicals in a low-cost and green-like process (Liu et al., 2014; Eichhorn, 2011).

3.2. Enhanced enzymatic saccharification of diverse lignocellulose substrates with the application of three surfactants

Biomass enzymatic saccharification (digestibility) has been defined by calculating hexoses yields against cellulose (% cellulose) released from the enzymatic hydrolyses of pretreated biomass residues (Alam et al., 2019). Using our previously established steam explosion approach (Zahoor et al., 2017; Jin et al., 2016), this study examined enzymatic saccharification of the steam-exploded (SE) biomass residues of three Miscanthus accessions that were supplemented with each of the three surfactants (Fig. 2). In the absence of any surfactant supplementation, all SE residues of the three Miscanthus accessions showed increased hexose yields compared with those of their raw materials, which is consistent with previous reports in other plant species (Huang et al., 2015; Zahoor et al., 2017; Jin et al., 2016). However, optimal concentrations of the Tween-80 and PEG-4000 supplements respectively led to hexose yields of 77% and 71% (% cellulose) from enzymatic hydrolysis of the SE residues of the Mfl12 sample, which were 1.8- and 1.9-fold higher than the SE control with a hexose yield of 40% (without surfactant; Fig. 2A and B). By comparison, supplementation with SilwetL-77 caused relatively less enzymatic saccharification with a hexose yield of 56% (Fig. 2C). Furthermore, the Tween-80 supplementation showed a significantly higher hexose yield than those of PEG-4000 and Silwet L-77 in the Mfl40-SE sample, but the three surfactants had similar hexose yields in the Msa02-SE sample ($p > 0.05$, Fig. 2D). Therefore, supplementation with the three surfactants remarkably enhanced enzymatic saccharification of the SE residues of Miscanthus straw. However, the degree of enhancement varied among the three surfactants, probably because the SE residues had distinct cell wall compositions and polymer features as discussed below.

3.3. Complete enzymatic saccharification under combined steam explosion and chemical pretreatments

To achieve complete biomass enzymatic saccharification, this study

Fig. 1. Distinct enhancements of three surfactants (Tween-80, PEG-4000, Silwet L-77) for direct enzymatic saccharification of raw straws in three Miscanthus accessions (Mfl12, Mfl40, Msa02) by measuring hexose yield (% cellulose) released from direct enzymatic hydrolysis. The data indicated the mean ± SD (n = 3), Small letters (a–d) represent multiple significant difference by LSD-test at $p < 0.05$ by t-test.

Fig. 2. Large enhancements of three surfactants (Tween-80, PEG-4000, Silwet L-77) for enzymatic saccharification of steam exploded (SE) residues in three Miscanthus accessions (Mfl12, Mfl40, Msa02) by measuring hexose yield (% cellulose) released from enzymatic hydrolysis. The data indicated the mean ± SD (n = 3), Small letters (a–c) represent multiple significant difference by LSD-test at $p < 0.05$ by t-test.
performed chemical pretreatments with the SE residues of Miscanthus accessions using acid (H₂SO₄) and alkali (NaOH) chemicals at a series of concentrations (Fig. S1†). When pretreated with 4% H₂SO₄ (the optimal concentration), the raw material and SE residues of Mf40 showed hexose yields of 26% and 51% (% cellulose), respectively, but under 8% NaOH pretreatment, there was almost complete biomass saccharification with hexose yields of close to 100%. Furthermore, when incubated with Tween-80 or PEG-4000, two Miscanthus accessions (Mf40 and Msa02) showed substantially enhanced enzymatic saccharification with hexose yields of 85% and 72%, respectively, in the SE residues after pretreatment with 4% H₂SO₄ (Fig. 3A), which is almost 2-fold higher than those of their controls (34%). By comparison, the Silwet L-77 supplementation only produced hexose yields of 72% and 42% in the SE residues of Mf40 and Msa02, respectively. However, the Tween-80 supplementation produced almost complete biomass enzymatic saccharification with a hexose yield of 99% (% cellulose) in the SE residue of Mf40 after mild alkali pretreatment (1% NaOH at 50 °C), and the PEG-4000 achieved a hexose yield of 95% (Fig. 3B). The Silwet L-77 supplementation continued to produce a relatively low hexose yield from the 1% NaOH pretreatment. In addition, the three surfactants showed variation in the enhancement of enzymatic saccharification of SE residues between the two Miscanthus accessions after pretreatment with 1% NaOH, which may also be due to their distinct lignocellulose features.

3.4. Consistently increased sugar-ethanol conversion of diverse lignocellulose substrates with Silwet L-77 supplementation

Using the total hexose released from the enzymatic hydrolysis of the SE residues, this study performed a classic yeast fermentation for bioethanol production (Fig. 4 and Table S1†). The SE residues of two Miscanthus accessions (Mf40 and Msa02) supplied with the three surfactants under 1% NaOH pretreatment showed significantly higher bioethanol yields from yeast fermentation of the total hexose released from enzymatic hydrolysis, ranging from 12% to 16% (% biomass), respectively (Table S1†), consistent with their relatively higher hexoses yields reported above. In addition, this study calculated the sugar-ethanol conversion rates based on hexose and bioethanol yields obtained from all SE residues examined (Fig. 4). In general, supplementation with the three surfactants led to considerably enhanced sugar-ethanol conversion rates in all SE residue samples of the two Miscanthus accessions compared with those of the controls with acid or alkali pretreatments (without surfactants). In particular, Silwet L-77 supplementation produced higher sugar-ethanol conversion rates for almost all SE residue samples, ranging from 72% to 88%; in comparison, the Tween-80 and PEG-4000 supplementations exhibited conversion rates of 44–72% (Fig. 4A–F). The Tween-80 supplementation produced the lowest sugar-ethanol conversion rates in most of the SE residues examined, but the Tween-80 and PEG-4000 supplementations exhibited variable conversion rates in the SE residues of two Miscanthus accessions pretreated with NaOH (Fig. 4E and F). Because it has been reported that numerous toxic compounds released from both pretreatment and sequential enzymatic hydrolysis can inhibit yeast fermentation (Li et al., 2014b; Bellido et al., 2011; Luo et al., 2002), the results suggest that Silwet L-77 supplementation could either reduce the formation of toxic compounds from the enzymatic hydrolysis or enhance the blockage of the interaction between the toxic compounds and yeast cells during ethanol fermentation compared with Tween-80 and PEG-4000 supplementation. Therefore, it would be interesting to further explore the role of Silwet L-77 in the bioethanol conversion of various lignocellulose residues in the future. It also remains to evaluate the economic benefit of bioethanol production using Silwet L-77 in a large-scale biomass process.

3.5. Blockage of cellulase enzyme adsorption by surfactant supplementation

To examine the roles of the three surfactants in the enzymatic hydrolysis of diverse SE residues from the two Miscanthus accessions, this study measured the total soluble protein/enzymes collected in the supernatant after enzymatic hydrolysis at 50 °C for 48 h (Table S2†). In general, the three surfactants led to considerably higher levels of collectable soluble protein in the supernatant than in those of the controls (without surfactant/SE only) for all the SE residues examined, with increases in soluble protein of up to 11-fold (Table S2†). However, approximately 2- to 3-fold more soluble protein was observed in the Tween-80 supplementation than in the PEG-4000 and Silwet L-77 treatments, consistent with the relatively higher biomass enzymatic saccharification from the Tween-80 supplementation described above. Similarly, the three surfactants produced much more soluble protein in the SE residues pretreated with 1% NaOH, with increases of 1- to 2-fold, compared with samples of SE only or SE residue pretreated with 4% H₂SO₄ (SE + 4% H₂SO₄), which is consistent with the significantly higher biomass enzymatic saccharification in the SE residue pretreated with 1% NaOH (SE + 1% NaOH). In confirmation of this finding, we found a significant positive correlation between the soluble protein level and hexose yield (p < 0.01, R² = 0.90; Fig. 5A), indicating that the soluble protein/enzymes should be available for lignocellulose enzymatic hydrolysis.
Furthermore, this study created separation profiles of total soluble proteins using SDS-PAGE (Fig. 5B). With regard to the commercial mixed-cellulases applied to the enzymatic hydrolysis performed in this study, we observed three strong bands and three minor bands, which should correspond to all major types of enzymes such as β-glucanases, cellulases, xylanases and others (Zahoor et al., 2017; Wu et al., 2019). All six protein bands were observed in all nine samples supplemented with the three surfactants, but they were faint or almost absent in the three control samples (without surfactants), providing direct evidence that the three surfactants could block mixed-cellulase enzyme adsorption to lignin and other wall polymers (Jin et al., 2016). In addition, the Tween-80 supplementation led to relatively stronger bands than those of Silwet L-77, which is consistent with more soluble enzymes in the supernatants of the nine SE residues with Tween-80. Taken together, these experiments not only confirmed the role of the three surfactants in enhancing enzymatic saccharification of diverse lignocellulose substrates by blocking adsorption but also identified Silwet L-77 as a novel surfactant for improving the enzymatic hydrolysis of both raw materials and diverse pretreated biomass substrates in Miscanthus.

3.6. Distinct wall polymer extractions from diverse lignocellulose substrates

To understand how the three surfactants enhance enzymatic saccharification in diverse lignocellulose substrates of the two Miscanthus accessions, this study examined the extraction and destruction of major wall polymers during steam explosion and subsequent chemical (acid or alkali) pretreatment (Fig. 6). Following the first step of steam explosion, the SE residues of the two Miscanthus accessions showed increases in hemicellulose extraction of 2- to 3-fold with small amounts of lignin removal, particularly in the Mfs accession (Fig. 6A–C). The second step of acid (+ 4% H$_2$SO$_4$) pretreatment furthered hemicellulose extraction, leading to relatively higher cellulose and lignin levels in the SE + 4% H$_2$SO$_4$ residues. By comparison, the 1% NaOH pretreatment removed almost 2–3 times the amount of lignin, resulting in a 1- to 2-
fold increase in cellulose levels in the SE + 1% NaOH residues. Because it was assumed that the surfactants may block cellulase enzyme adsorption to lignin (Jin et al., 2016; Börjesson et al., 2007), the high level of lignin extraction is likely the primary reason for the high levels of soluble enzymes detected in the supernatant following enzymatic hydrolysis in the SE + 1% NaOH residues supplemented with the three surfactants (Table S2†).

In regards to the wall polymer extraction, this study used fourier transform infrared (FTIR) spectroscopy to detect alterations in polymer interlinkages in the diverse SE residues of the two Miscanthus accessions (Fig. 6D and E and Table S3†). The SE and SE + 4% H2SO4 samples exhibited similarities in most of the peaks observed within the region of 1728–829 cm⁻¹; however, the peak at 1247 cm⁻¹ for the C–C linkage was absent in the SE + 4% H2SO4 samples. Notably, the four peaks (829, 1460, 1515, 1603 and 1735 cm⁻¹) corresponding to the C–C, C−CH₃, CC and CC—C groups, which are associated with the...
lignin interlinkages with hemicelluloses (Alam et al., 2019), were almost absent in the SE + 1% NaOH samples, suggesting an effective extraction of hemicellulose-lignin complexes following the combined steam explosion and alkali pretreatments performed in this study.

3.7. Significantly increased cellulose accessibility in diverse lignocellulose substrates

To further understand the enhanced enzymatic saccharification and bioethanol conversion of the diverse SE residues, this study examined cellulose features (crystalline index/CrI, degree of polymerization/DP, and surface area) in two Miscanthus accessions (Fig. 7). The SE + 1% NaOH samples showed a much reduced CrI value compared with those of the other three samples (Fig. 7A), whereas the SE + 4% H2SO4 samples had the lowest cellulose DP values among the four samples (Fig. 7B). The two Miscanthus accessions showed variation in the CrI and DP values among the four samples, confirming the diverse lignocellulose substrates described above.

Although cellulose CrI and DP are the major factors negatively affecting enzymatic hydrolysis (Wang et al., 2016), cellulose accessibility has more recently been identified as the final positive determinant for biomass enzymatic saccharification supplemented with Tween-80 (Alam et al., 2019). Hence, this study examined the cellulose surface area, which is a major parameter affecting cellulose accessibility (Fig. 7C). The SE + 1% NaOH samples showed a much greater increase in cellulose surface area than those of the other three samples, and the raw material samples had the lowest surface area in the two Miscanthus accessions, which is consistent with the finding of effective extraction of hemicellulose-lignin complexes from the combined steam explosion and alkali pretreatments described above. This finding also accounts for the enhanced biomass enzymatic saccharification achieved in the SE + 1% NaOH samples. In particular, the SE + 1% NaOH sample of Mfl40 had the highest surface area, which accounts for its complete enzymatic saccharification as described above (Fig. 3). In addition, we observed the morphology of the pretreated biomass residues using scanning electron microscopy (Fig. 7D). The three types of substrates (SE, SE + 4% H2SO4, and SE + 1% NaOH) exhibited different morphologies; in particular, the SE + 1% NaOH samples showed few small lignin-like particles on the surface of the cellulose microfibrils compared with the other two samples, which supports the findings of greater lignin extraction in the SE + 1% NaOH samples.

Taken together, the results of this study have demonstrated that combined steam explosion and mild alkali pretreatments can extract most of the lignin for substantially increased cellulose accessibility and effectively enhanced surfactant blocking of cellulase enzyme adsorption, which may lead to integrated enhancement of biomass enzymatic saccharification in Miscanthus.

3.8. Distinct mechanisms of the three surfactant enhancements of biomass enzymatic saccharification

To determine how each of the three surfactants enhances direct enzymatic saccharification, this study examined six other Miscanthus samples showing diverse cell wall compositions and varied hexose
Fig. 8. (A–C) Correlation analysis between wall polymer contents and hexoses yields (% cellulose) released from direct enzymatic hydrolysis of raw straws of Miscanthus accessions supplied with three surfactants; *indicated significant correlation as \( p < 0.05 \) (\( n = 9 \)). (D) A hypothetic model highlighting that Silwet L-77 was of more effective penetration into plant cell walls of intact stem tissues than the PEG-4000 did, and then both surfactants could block mixed-cellulases adsorption with lignin for enhanced enzymatic hydrolysis of intact cell walls.

Fig. 9. (A–C) Correlation analysis between wall polymer levels and soluble protein contents (Table S2) collected from the supernatants of enzymatic hydrolysis of diverse lignocellulose substrates in two Miscanthus accessions supplied with three surfactants; * and ** indicated significant correlation as \( p < 0.05 \) and \( p < 0.01 \) level (\( n = 6 \)). (D) A hypothetic model interpreting that three surfactants distinctively enhanced biomass enzymatic saccharification of diverse lignocellulose substrates by blocking mixed-cellulases adsorption with lignin.
yields from direct enzymatic hydrolysis of raw straw supplemented with the three surfactants. Using the data obtained from all nine Miscanthus samples, this study performed a correlation analysis between the levels of the three major wall polymers and hemicellulose yield from the direct enzymatic hydrolysis of raw straw (Fig. 8A–C). The Silwet L-77 and PEG-4000 supplements showed a significant correlation between cellulose level and hemicellulose yield ($p < 0.05$, Fig. 8A), which is consistent with the findings that approximately 20–40% of cellulose could be digested from use of these two surfactant supplements, in particular from the Silwet L-77 supplement (Fig. 2). In comparison, there was no correlation between cellulose and hemicellulose for Tween-80 or the control (without surfactant), which could account for the much lower hemicellulose yields released from direct enzymatic hydrolysis supplemented with Tween-80. Similarly, the Silwet L-77 and PEG-4000 supplementation led to a significant negative correction between lignin level and hemicellulose yield (Fig. 8C), which would suggest that the lignin negatively affected the direct enzymatic hydrolysis of raw straw. This provides indirect evidence that Silwet L-77 and PEG-4000 can block lignin from adsorbing cellulases and thereby enhance enzymatic hydrolysis. Surprisingly, the hemicellulose levels were not correlated with supplementation of the three surfactants or the control (Fig. 8B), suggesting that hemicellulose may not be the primary factor affecting direct enzymatic hydrolysis. This finding may also explain why almost all hemicellulose could be digested by direct enzymatic hydrolysis of the raw straw in the desirable Miscanthus accession when supplemented with Silwet L-77. Therefore, this study proposed a hypothetical model regarding Silwet L-77 and PEG-4000 enhancement of the direct enzymatic hydrolysis of raw straw (Fig. 8D). Nevertheless, compared with PEG-4000, Silwet L-77 should more effectively penetrate the plant cell walls of intact stem tissues to block lignin adsorption of cellulase enzymes (Clough and Bent, 1998; Zhang et al., 2006). In addition, we assumed that the penetration of Silwet L-77 may open a path to allow cellulase enzymes into the cell walls for effective enzymatic hydrolysis, whereas Tween-80 may have little effect on enzyme access and loading in the raw straw.

This study also performed a correlation analysis between the three major wall polymers and the level of soluble protein collected from the supernatants of the enzymatic hydrolyses of diverse lignocellulose substrates supplied with the three surfactants (Fig. 9). The lignin levels of diverse lignocellulose substrates were significantly negatively correlated with the soluble protein contents ($p < 0.05$ or 0.01), particularly for the Tween-80 supplementation, which showed an extremely high $R^2$ value of 0.96 (Fig. 9C); neither cellulose nor hemicellulose showed any significant correlations with soluble protein levels (Fig. 9A and B), indicating that the increased soluble protein contents are primarily due to the blocking of lignin adsorption of enzymes by surfactants in the lignocellulose substrates examined in this study (Table S2?). This finding could also explain why the highest soluble protein content was detected in the enzymatic hydrolyses of the SE + 1% NaOH substrate compared with other substrates, leading to complete enzymatic saccharification (Fig. 3B). Therefore, we proposed another hypothetical model regarding surfactant enhancements of the enzymatic hydrolysis of the diverse lignocellulose substrates obtained in this study (Fig. 9D). Due to high levels of hemicellulose and lignin extractions from SE and following chemical pretreatments, Tween-80 was more effective at blocking cellulase enzyme adsorption to lignin compared with PEG-4000 and Silwet L-77, which allowed more cellulase loading for biomass enzymatic saccharification (Jin et al., 2016). In addition, Silwet L-77 supplementation showed lower biomass saccharification than those of the other two surfactants, probably due to relatively less adsorption with lignin. However, it may explain why Silwet L-77 supplementation could consistently maintain much higher sugar-ethanol conversion rates in all the pretreated lignocellulose substrates: more Silwet L-77 remained in the supernatants of enzymatic hydrolyses to block the interaction between toxin compounds and yeast cells during ethanol fermentation (Si et al., 2015; Sun et al., 2017); this should be clarified in future studies.

4. Conclusion

In this study, a novel surfactant (1% Silwet L-77) was evaluated its ability to enhance the biomass enzymatic saccharification of raw straw in Miscanthus accessions compared with two well-known surfactants (TWEEN-80 and PEG-4000). Using diverse lignocellulose substrates generated from combined steam explosion and mild chemical (H$_2$SO$_4$ or NaOH) pretreatments, the three surfactant supplements led to distinct patterns of enhanced biomass enzymatic saccharification, and complete enzymatic hydrolysis was achieved with the 2% Tween-80 supplement. However, the 1% Silwet L-77 consistently produced higher sugar-ethanol conversion rates compared with the other two surfactants in all lignocellulose substrates examined, probably due to the release of fewer toxic compounds from enzymatic hydrolysis or the effective blocking of the interaction between toxic compounds and yeast cells. This study proposes multiple hypothetical models to interpret how these three surfactants could play distinct enhancement roles in the enzymatic saccharification of diverse lignocellulose substrates, providing a green-like and cost-effective biomass processing technology for bioethanol production and other potential valuable bioproducts from bioenergy Miscanthus and other substrates.

Acknowledgements

This work was in part supported by the guidance project of Science and Technology Department of Hubei Province [2019CFCB568], open fund and doctoral research foundation project of Hubei University of Technology [BSQD2017020; 2018BEB07], scientific research program guidance project of Hubei Provincial Department of Education [B2019044], the Earmarked fund for China agriculture research system and the key laboratory of development and application of rural renewable energy [CARS-31-02] and the project of Ministry of Agriculture and Rural Affairs of China [2019004].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112559.

References


