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Enhanced biodegradation of sugarcane bagasse by *Clostridium thermocellum* with surfactant addition

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Clostridium thermocellum is a potential strain in consolidated bioprocessing (CBP) for lignocellulose biorefinery, while its large-scale utilization is not economically available yet due to a few hassles such as low hydrolysis efficiency. Biodegradation of sugarcane bagasse (SCB) accompanied with accumulation of reducing sugars was remarkably improved with the addition of non-ionic surfactant in CBP system using *Clostridium thermocellum*. The addition of 2.5 g L^{-1} Triton X-100 resulted in an accumulation of 3.65 g L^{-1} reducing sugars and a substrate degradation of 46.6%, which increased by approximately 36-fold and 1.2-fold, respectively, compared with control. Furthermore, the addition of Trition X-100 has little negative effect on hydrogen production in CBP using *C. thermocellum*. The result suggests that Triton X-100 is a most promising surfactant in improving the biodegradation of SCB, probably leading to a more efficient CBP in biorefinery.

Introduction

Biorefinery, analogous to today's petroleum refineries, provides us a new insight into the production of multiple fuels and products by biomass to help relieve the energy and environment crisis. Lignocellulosic material is considered to be a potential renewable resource for bioenergy production, meeting the needs of transportation fuel without threatening the food supply and biodiversity[1]. The first step in lignocellulose bioconversion is depolymerizing lignocellulosic biomass into monomeric constituents via enzymatic hydrolysis, providing a fermentable sugar stream for bioenergy production[2,3]. Despite extensive research efforts, several factors hindered the development of a coherent process of lignocellulosic bioethanol based on enzymatic hydrolysis. First, C-O-C bonds and other ester bonds presented between lignin and carbohydrate complex inhibit the rates of hydrolysis[4]. Besides, the crystalline parts in the lignocellulosic materials, consisting of parallel cellulose chains which are tightly held together by hydrogen bonds, make the hydrolysis more difficult[5]. To economically drive ethanol production from lignocellulose, finding a new approach to improving the catalytic efficiency is essential. Several methods can be used for an efficient enzymatic hydrolysis of lignocellulose, such as increasing the accessibility of substrate by modifying its structure and/or composition, decreasing the inhibitors of cellulases and improving the performance of the enzymes[2]. Finding an effective pretreatment method is a research hotspot in biorefinery[6]. NaOH pretreatment, a typical alkali pretreatment, can be carried out at a lower temperature and pressure under ambient conditions[7].Compared to acid or organosolv pretreatment, it can also dissolve silica, hydrolyse uronic and acetic esters, reduce crystallinity and significantly remove lignin [8,9]. Cao et al.[10] suggested that sodium hydroxide can disrupt the ester bonds between lignin and hemicellulose, leading to a higher lignin and hemicellulose removal. Our previous study confirmed that sugarcane bagasse (hereafter SCB) pretreated with sodium hydroxide showed significant advantages over raw SCB in substrate utilization and hydrogen production[11]. Besides pretreatment, high enzyme loading significantly increases the enzymatic hydrolysis of lignocellulose, but goes against the process economy[2,12]. Lignin present in the lignocellulose can lead to unproductive adsorption of cellulase[5] and an increase in cellulase loading[10,13].

To further lower biofuel production costs by improving catalytic efficiency, a variety of methods have been suggested for increasing hydrolysis yields, such as gradual substrate loading[14], advanced reactor configurations coupled with product removal to avoid inhibition[15], and surfactant addition[5]. Researches maintain that surfactants can change the substrate structure, contribute to the enzyme-substrate interaction and increase the stability of enzymes[2,16,17].

With their hydrophobic and hydrophilic properties, surfactants can decrease surface tension and help remove hydrophobic compounds[18] and thus have been widely investigated in the pretreatment and hydrolysis of lignocellulosic biomass[19,20]. Castanon et al.[19] found that after 48h hydrolysis with Tween 80, the conversion of newspapers increased by 14%. Börjesson et al.[21] reported that PEG 4000 had a stimulation effect on steam pretreated spruce hydrolysis

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at 50 °C. Positive effects of surfactants have also been reported in other lignocellulosic materials, such as pine wood chips[22], bagasse[17], and corn stover[16]. Several studies reported that surfactants could enhance the bioaccessibility of substrates and the stability of enzymes, resulting in more effective cellulose conversion[5,16].

Consolidated bioprocessing (CBP) shows great potential in biorefinery by combining all the biochemical steps involved in lignocellulosic bioenergy production, such as enzyme production, substrate hydrolysis, unbiased utilisation of the full-spectrum of the liberated sugars and bioenergy fermentation in one bioreactor[23]. *Clostridium thermocellum* is considered a potential anaerobic bacterial species for consolidated bioprocessing (CBP) of lignocellulose, but one of the problems for its economical availability of industrialization is low hydrolysis efficiency. Nevertheless, considerably more knowledge on the interactions among surfactants, enzymes and lignin is required for improving hydrolysis efficiency. The objectives of the present study were (i) to investigate the effects of several surfactants on SCB degradation using *C. thermocellum*; (ii) to reveal the promoting mechanism of surfactants in SCB hydrolysis by *C. thermocellum*; and (iii) to evaluate the effects of surfactants on xylanase from *C. thermocellum*.

Results and discussion

Effect of surfactant addition time on reducing sugar accumulation

Since surfactants had effects on bacterial growth and metabolism[24,25], the optimal time for surfactant addition was studied first. Triton X-100 could be adsorbed to the surface of SCB, leading to steric repulsion of enzyme from the lignin surface due to hydrophobic interaction between lignin and Triton X-100, further reducing the unspecific adsorption of cellulase from *C. thermocellum*[26]. Supplementation of Triton X-100 at a different time in fermentation significantly affected the accumulation of reducing sugars (Fig. 1a). Without adding Triton X-100, the glucose concentration was just 0.13 ± 0.01 g L^{-1} and nearly no xylose was detected after fermentation. Both the glucose and xylose accumulations were significantly enhanced with the addition of Triton X-100. Interestingly, adding Triton X-100 after inoculation seemed more effective. Zhang et al.[4] reported that the addition of PEG 4000 before cellulase can reduce wasteful cellulase adsorption on the lignin and resulted in higher saccharification efficiency. Since cellulosome and other hydrolytic enzymes were generated by the strain, we got different results. With the addition time of Triton X-100 delayed, the reducing sugar concentration increased while the pH value dropped. Adding Triton X-100 at the 96 h of fermentation (the pH value is 5.42 ± 0.08), the accumulation of reducing sugars (mainly glucose and xylose) reached the highest (1.93 ± 0.06 g L^{-1}), which was 15.32-fold that of the control. When Triton X-100 was added at the 120 h, the increasing trend was not maintained, probably due to the low pH (5.01 ± 0.03). Our previous study indicated that nearly no SCB degradation and hydrogen production were detected in the fermentation at a pH below 5.3[11].

Since hydrogen was another important product of this process, the effect of surfactants on hydrogen production was also studied (Fig. 1b). Results showed that the addition of Triton X-100 at 96 h gained 22.78 ± 0.34 mmol L^{-1} hydrogen, which was close to the result of the control (24.13 ± 0.78 mmol L^{-1}), indicating that surfactant addition had little negative effect on hydrogen production in this process.

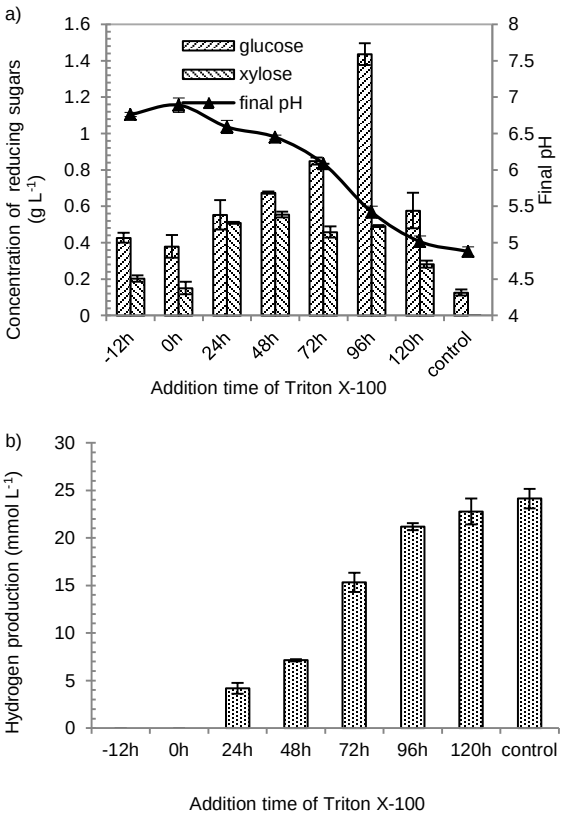


Fig.1. Effect of Triton X-100 addition time on pH, the accumulation of reducing sugars (a), and hydrogen production (b). Concentration of the added Triton X-100 was 1%. -12 h meant addition of Triton X-100 was at 12 h before inoculation; 0 h meant addition of Triton X-100 and inoculation of *C. thermocellum* were operated at the same time. 24, 48, 72, 96 and 120 h meant additions of Triton X-100 were at 24, 48, 72, 96 and 120 h after inoculation of *C. thermocellum*. Control meant the fermentation with inoculation of *C. thermocellum* at 0 h without addition of Triton X-100. All experiments were performed in triplicate and mean values and standard deviation are presented.

Non-ionic surfactant screening for enhanced residual sugar accumulation

Five different surfactants were compared with respect to their efficiency of enhancing the accumulation of reducing sugars during biodegradation of SCB by *C. thermocellum*. All the surfactants (10 g L^{-1}) were supplemented at the 96 h of the process (broth pH about 5.5) with a total fermentation time of 168 h.

Interestingly, all the surfactants tested showed a positive effect on the reducing sugar accumulation as compared with the control (Table 1). The positive effect of PEG on the accumulation of reducing sugars was not so significant as that of other surfactants. The recoveries of reducing sugars slightly increased from 2.06 ± 0.54% of the control to 3.82 ± 0.05% (PEG 4000) and 4.69 ± 1.69% (PEG 6000). Triton X-100 and Tween 20 showed more notable stimulating effects, with the reducing sugar recoveries reaching 42.78 ± 2.11% and 28.00 ± 0.94%, respectively. Hemicellulose hydrolysis was also found to be enhanced by surfactants, although not so pronounced as cellulose hydrolysis. However, only Triton X-100 and Tween 20 were found to improve the xylose accumulation in the broth (Table 1), which was consistent with the finding in the hydrolysis of wheat straw lignocellulose[26]. The xylose

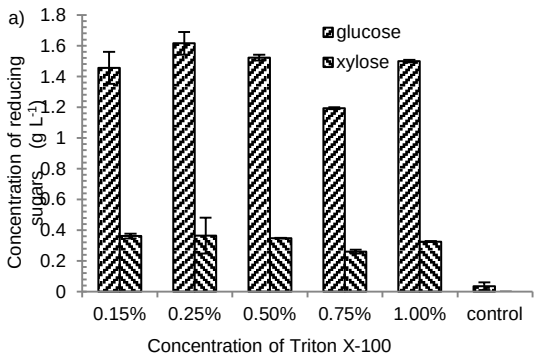
concentrations for Tween 20 and Triton X-100 reached 0.42 ± 0.03 g L^{-1} and 0.31 ± 0.05 g L^{-1}, respectively, indicating that Tween 20 was more conducive to xylose accumulation and had positive effects on xylanase. Triton X-100 was selected for further studies due to the high cellulose content of SCB.

Table 1 Effects of surfactants on SCB fermentation by *C. thermocellum*

Surfactant	Reducing sugars in the broth (g L^{-1})		Reducing sugar recovery (%)	Final pH
	Glucose	Xylose		
Control	0.08 ± 0.039	0.09 ± 0.011	2.06 ± 0.54	5.13 ± 0.04
Tween 20	0.57 ± 0.040	0.42 ± 0.033	28.00 ± 0.94	5.40 ± 0.16
Tween 80	0.14 ± 0.031	0.08 ± 0.012	6.60 ± 0.11	5.25 ± 0.04
PEG 4000	0.11 ± 0.077	0.10 ± 0.011	3.82 ± 0.05	5.22 ± 0.10
PEG 6000	0.10 ± 0.028	0.09 ± 0.013	4.69 ± 1.69	5.17 ± 0.03
Triton X-100	1.33 ± 0.066	0.31 ± 0.050	42.78 ± 2.11	5.37 ± 0.02

Effect of Triton X-100 concentration on reducing sugar accumulation

The effect of Triton X-100 concentration on reducing sugar accumulation was outlined in figure 2a. The cellulose conversion was reported to increase with the surfactant concentration[5,26], which was not consistent with the result of this study. The highest reducing sugar production (at a glucose concentration of 1.62 ± 0.04 g L^{-1} and a xylose concentration of 0.36 ± 0.02 g L^{-1}) was obtained at a Triton X-100 concentration of 2.5 g L^{-1}, which was similar to the finding by Kristensen[2624] that no significant effect of surfactant concentrations on xylan hydrolysis. The optimal surfactant concentration in the degradation process of SCB might mean that all the possible binding sites on the lignin of SCB were occupied by surfactant, irrespective of the ability of SCB to unspecifically bind enzymes[27]. A decrease in the concentration of reducing sugars occurred at a Triton X-100 concentration over 2.5g L^{-1}, which was consistent with the finding of the co-occurrence of hydrogen bonds and hydrophobic interactions between non-ionic surfactant and cellulase, leading to denaturation and inactivation of enzymes[3]. Besides, according to the figure 2b, 23.61±1.12 mol L^{-1} hydrogen was obtained which was 91.58% of the control (25.78±1.02 mmol L^{-1}). Therefore, 2.5 g L^{-1} was selected as the optimum Triton X-100 addition concentration.



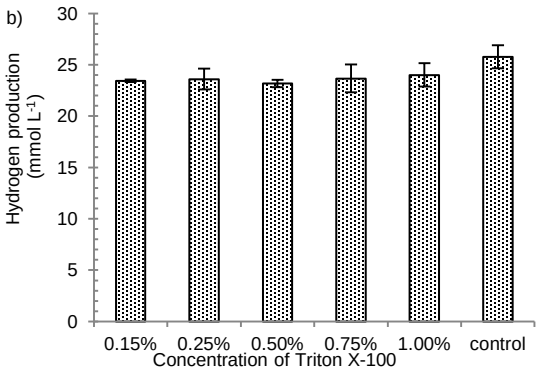


Fig.2. Influence of Triton X-100 concentration on reducing sugars accumulation (a) and hydrogen production (b). All experiments were performed in triplicate and mean values and standard deviation are presented.

Effect of Triton X-100 on the Zeta potential (ζ) of cell surface

Surfactants have been reported to have positive effects on enzyme–substrate interactions and lead to more effective conversion of cellulose[5]. The surface charge represented by zeta potential is an important parameter for a better understanding of the degradation of SCB by *C. thermocellum*, since hydrophobic aggregation decreases the bioaccessibility of fermenting bacteria to substrates[28]. Charge densities of *C. thermocellum* at different Triton X-100 concentrations were illustrated in Fig. 3. The zeta potentials of the cell surface were significantly affected by Triton X-100. Without Triton X-100, the charge density of the cell surface was -12.04 ± 0.27 mV, but with the addition of 0.5 g L^{-1} Triton X-100, the zeta potential increased to -7.23 ± 0.73 mV, which was similar to the finding by Hua[29]. The results indicated that surfactant supplementation increased the positive electric charge of the cell surface, leading to weakened mutual repulsion and a better adsorption between the cell and SCB[20,29].

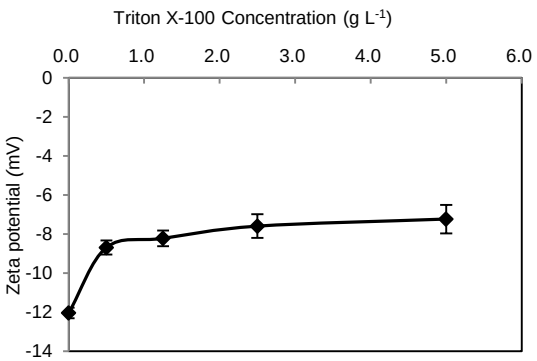


Fig.3. Influence of adding Triton X-100 on Zeta potential of *C. thermocellum* surface.
All experiments were performed in triplicate and mean values and standard deviation are presented.

Effect of Triton X-100 on the unproductive adsorption of cellulase

Prior studies found that surfactants could be adsorbed to lignin and prevent the unproductive adsorption of cellulase[5,16]. The addition of Triton X-100 significantly increased the production of reducing sugars both in Avicel and SCB biodegradation by *C.*

thermocellum (Fig. 4). The increment of glucose production in the SCB was significantly higher than that in Avicel. Approximately 1.62 g L^{-1} glucose was detected in the SCB run, but only 0.41 g L^{-1} in Avicel (Fig. 4a). Xylose accumulation was also detected in the SCB run and reached 0.50 g L^{-1} at the end of the fermentation (Fig. 4b).The results indicated that mutual interaction between Triton X-100 and lignin in SCB might play an important role in reducing sugar accumulation. The positive effects of surfactants on pure cellulose as well as lignocellulosic biomass were also reported by Li[30]. Researchers suggested that hydrophobic sites on lignin could be occupied by surfactants, and the hydrophilic portions of the surfactant would protrude into the aqueous solution, resulting in the steric repulsion of lignin surface from cellulase, thereby either releasing unspecifically bound cellulase or preventing the unproductive adsorption of enzymes[5,19].

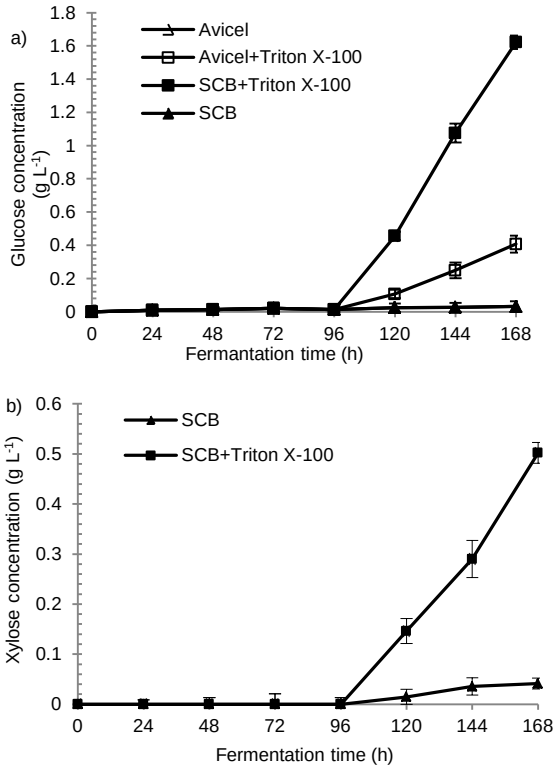


Fig.4. Time-dependent course of glucose (a) and xylose (b) accumulation during SCB and Avicel degradation using *C. thermocellum.*
The substrates were added at the same cellulose concentration (SCB 20 g L^{-1}, Avicel 10 g L^{-1}) and Triton X-100 addition time was 96 h of the fermentation without pH control. All experiments were performed in triplicate and mean values and standard deviation are presented.

Effect of Triton X-100 on cellulase and xylanase production

Figure 6 showed the changes of cellulase and xylanase produced by *C. thermocellum* activities during the process. The addition of Triton X-100 at the 60 h of the fermentation (the broth pH of 5.5) significantly increased both the activities of cellulase and xylanase and the accumulation of reducing sugars. The activities of cellulase and xylanase reached 1.35 U mL^{-1} and 0.49 U mL^{-1} at the 120 h and the 96 h (Fig. 5a), respectively, which were 3.75-fold and 1.71-fold of the control, indicating that Triton X-100 had a pronounced stimulatory effect on both cellulase and xylanase activities.

Furthermore, the cellulase and xylanase activities remained stable in the following process after reaching a peak. The final concentrations of glucose and xylose reached 2.99 g L^{-1} and 0.66 g L^{-1} (Fig. 5b), respectively. Surfactants have also been reported to have stabilizing effects on several enzymes[31]. Bálint et al.[32] reported that the addition of PEG resulted in an increase in the recovery of cellulase. In the previous study without added Triton X-100, the cellulase produced could not maintain the catalytic activity in the process[11].

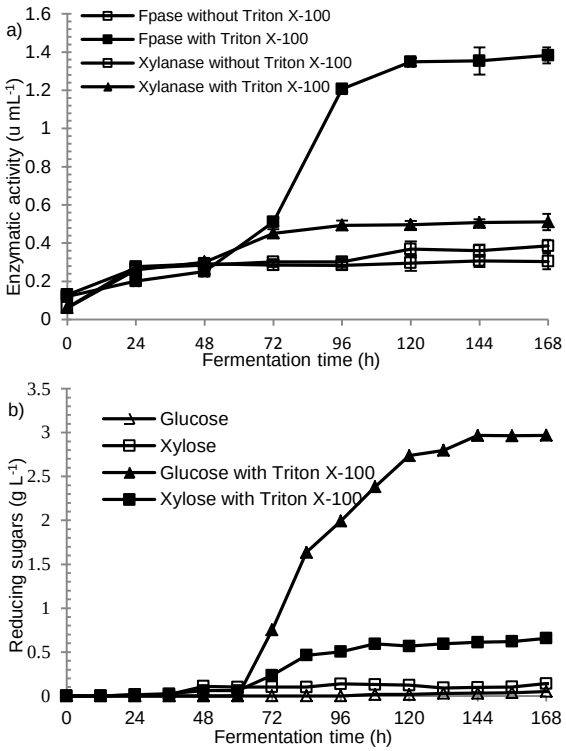


Fig.5. Influence of Triton X-100 on enzyme production (a) and reducing sugars (b) accumulation from SCB by *C. thermocellum.*
Triton X-100 was added at 60 h with the broth pH of 5.5 in 500 mL fermenters.

Changes in the content of cellulose and hemicellulose

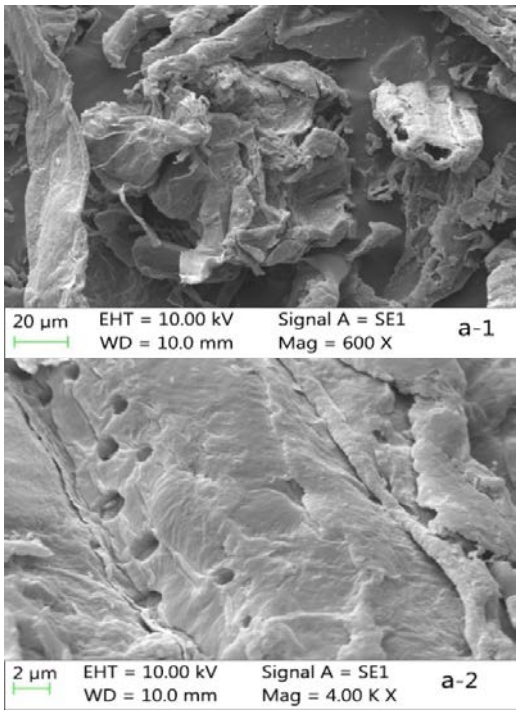
The biodegradation of cellulose and hemicellulose in SCB during the process was shown in Table 2. 66.51 ± 3.08% of cellulose and 85.10 ± 0.86% of hemicellulose were degraded by *C. thermocellum* in the system supplemented with Triton X-100, leading to a total degradation of 46.6% (Table 2). Although 39.2% SCB was degraded without added Triton X-100, few reducing sugars were accumulated (Fig. 5b). The addition of Triton X-100 enhanced the bioaccessibility and bioavailability of SCB degraded by *C. thermocellum*, leading to the increment of biomass hydrolysis. In short, the cellulose and hemicellulose degradation by *C. thermocellum* with added Triton X-100 had a pronounced advantage over that without Triton X-100. This could also be confirmed by the scanning electron microscopy (SEM) images (Fig. 6). Before fermentation, the structure of SCB was rather compact (Fig. 6a), while after fermentation, fibrous layers were exposed and large swelling and scaling appeared (Fig. 6b). This was even more notable in the SCB fermented with added Triton X-100, as more fibrous layers were degraded and smaller micropore structures appeared (Fig. 6c), indicating greater digestibility.

Table 2 Changes of cellulose and hemicellulose contents in SCB treated with and without added Triton X-100 in CBP.

Substrate	Glucan (%)	Xylan (%)	CD[a] ratio (%)	HD[b] ratio (%)	TD[c] ratio (%)
SCB before fermentation	50.61 ±0.48	15.76 ± 0.47			
SCB after fermentation without Triton X-100	36.47 ±0.80	5.20 ±0.12	56.18 ±1.21	79.95 ±0.52	39.20
SCB before fermentation withTriton X-100	31.75 ± 1.06	4.40 ± 0.16	66.51 ±3.08	85.10 ±0.86	46.60

a Cellulose degradation
b hemicellulose degradation
c Total degradation

Similarly, surfactants also showed significant improvement in rice straw hydrolysis with the most widely studied cellulase-producing strain, *Trichoderma reese i* [33]. 2.73 g L^{-1} reducing sugars were produced by *T. reesei* using 30 g L^{-1} rice straw as substrate[25]. In this study, higher reducing sugar concentration (3.65 g L^{-1}) was obtained by *C. thermocellum* at a SCB concentration of 20 g L^{-1}, indicating that *C. thermocellum* is a potential strain for the biodegradation of lignocelluloses.



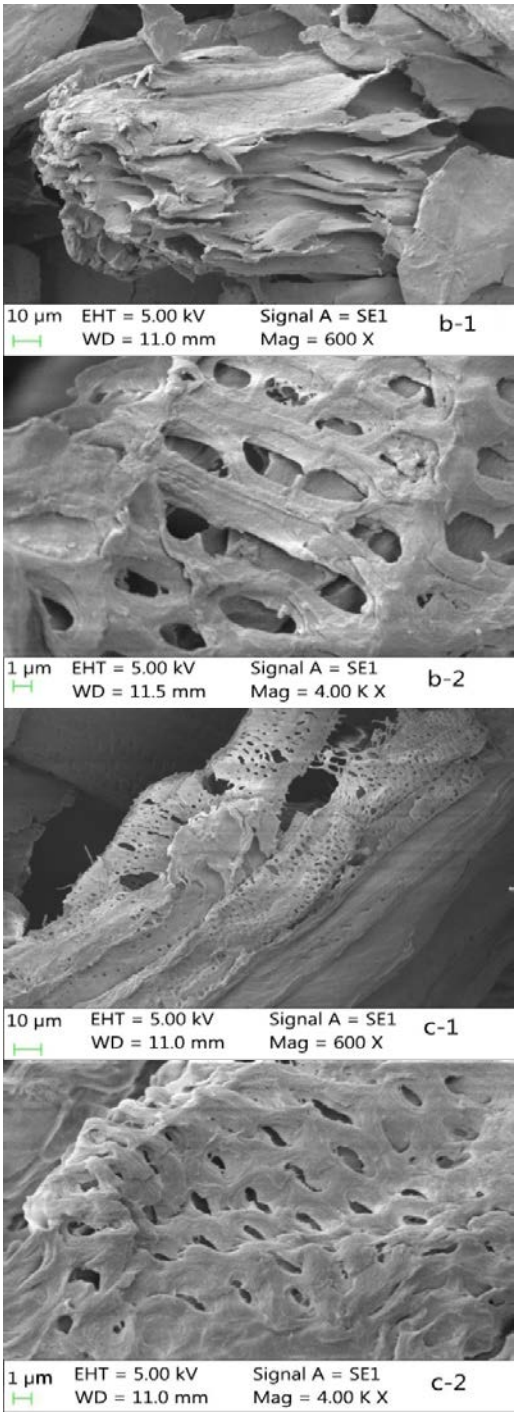


Fig.6. SEM images of the structural changes of SCB before and after degradation using *C. thermocellum* with and without Triton X-100.
SCB before fermentation, magnification: a-1: 600×, a-2: 4000×; b) SCB fermented without Triton X-100, magnification: b-1: 600×, b-2: 4000×; c) SCB fermented with Triton X-100, magnification: c-1: 600×, c-2: 4000×.

Conclusions

This is the first report concerning the surfactant supplement for enhancing lignocellulose degradation using *C. thermocellum.* Triton X-100 showed significant positive effects on SCB

degradation by *C. thermocellum* and reducing sugars accumulation, while little passive effect on hydrogen production. The optimal Triton X-100 adding time was 96 h of the fermentation for flask experiment and the optimal adding concentration was 2.5 g L^{-1}. Under these optimal conditions, the degradation of SCB could reach 46.6% and reducing sugars concentration was 3.65 g L^{-1} after 168h fermentation. Meanwhile, the promoting mechanism of Triton X-100 was explored, and can be interpreted as follows: Triton X-100 supplementation increased the positive electric charge of *C. thermocellum* surface, leading to weakened mutual repulsion and a better adsorption between the cell and SCB. Besides, it could occupy the hydrophobic sites on lignin and reduce unproductive cellulase adsorption, leading to a better cellulase and xylanase production and SCB degradation. The present research developed a novel method to improve the lignocellulose biodegradation and sugar accumulation by *C. thermocellum* with surfactant addition and certified the mechanism for the enhancement.

Experimental methods

Materials and methods

Cellulosic substrates

Microcrystalline cellulose, Avicel (9004-34-6) was purchased from Adamas Reagent Co., Ltd (Shanghai, China). The SCB was donated by the Guangzhou Sugarcane Industry Research Institute (Guangdong Province, China). SCB used in the study was soaked in sodium hydroxide solution (3%) for 3 h at 80 °C as described in the previous study[11]. The SCB composition was analysed using two-step acid hydrolysis according to the procedures published by the National Renewable Energy Laboratory[34]. The SCB consisted of 50.61 ± 0.48% glucan, 15.76 ± 0.47% xylan, 8.03 ± 0.49% Klason lignin, and 1.57 ± 0.14% acid soluble lignin, apart from some other components such as arabinose, moisture, ash, and benzene[35].

Surfactants

The tested additives were: Triton X-100 (BSA, Sigma–Aldrich, t. Louis, USA); poly(oxyethylene): Tween 20, Tween 80 (Merck); and poly(ethylene glycol): PEG 4000, PEG 6000 (Akzo Nobel, Stenungsund, Sweden). All surface active additives were referred to as surfactants for the sake of convenience. Other chemical reagents were all of analytical grade and used directly.

Microorganism strains, media and inoculum preparation

Clostridium thermocellum ATCC 27405 was donated by Professor Lynd (Dartmouth College). *C. thermocellum* ATCC 27405 was grown in the serum bottles with MTC Medium[36] (3 g L^{-1} cellulose as the carbon source) and was repeatedly transferred as the seed (over 10 generations continuously) for about 96 h at 55°C with rotary shaking at 150 rpm (C24KC refrigerated incubator shaker, Edison, New Jersey, United States) All the serum bottles were sealed with butyl rubber stopper and aluminum seals, and then each bottle was purged and degassed 3 times with 100% nitrogen.

Biodegradation of SCB

Surfactant screening and surfactant-addition method optimization were performed with 100 ml serum bottles in anaerobic conditions. The biodegradation of SCB were performed by microbial fermentation at 55 °C, 150 rpm, for 168 h. After fermentation, the broth was filtered through a 0.22-um syringe filter (Millipore, Bedford, MA). All experiments were performed in triplicate. Mean values and standard deviations were presented. The mechanism of surfactant effect on SCB degradation was investigated with a 500 ml fermenter (Applikon, Netherlands) under fermentation conditions similar to bottle experiments except at 300 rpm.

Analyses

Hydrogen production and reducing sugar accumulation were measured as described in the previous study[11].

For measurement of zeta potential, Triton X-100 were added into bacteria solutions at the logarithmic phase (5g L^{-1} cellobiose as carbon source) to reach various concentrations (0%~1%). After 4 hour-shaking (55°C, 150 rpm), zeta potentials were measured with purified suspensions of *C. thermocellum* at room temperature using a zeta meter as previously described in detail[37].

For the enzyme analysis, fermentation broth was collected and centrifuged to remove cells and residual substrate. The supernatant was used to measure the enzyme activity. The enzyme activity of the filter paper was determined according to International Union of Pure and Applied Chemistry[38]. Xylanase activity was determined as previously described[39].

One unit (IU) of filter paper enzyme activity was defined as the amount of enzyme required to produce 1 mg of glucose per hour and reported on basis of milliliter broth used in fermentation. One unit (IU) of xylanase was defined as the amount of enzyme required to release 1μmol of xylose per minute under the assay conditions.

The fermentation broth was centrifuged and washed repeatedly, and then the precipitates were dried at 50 °C for 72 h before gravimetrical determination. The SCB weight loss was calculated by subtracting the weight of the residual substrates from the total SCB weight before degradation. Reducing sugar recovery was calculated as follows：.

$$Reducing\ sugars\ recovery\ (\%) = \frac{C_R * V}{M_{SCB} * 1.1} \quad (1)$$

C_R: Concentration of reducing sugars, including glucose and xylose;
M_{SCB}: the SCB weight loss;
1.1: The coefficient of reducing sugars;

The morphology of the structural changes of SCB before and after fermentation using C. thermocellum with and without Triton X-100 was analyzed by SEM. All of the images were taken at a magnification of 600× and 4000× using a ZEISS EVO 18 electron microscope 51-XMX0003, Special Edition (Carl Zeiss Microscopy, Germany) equipped with an X-Max detector (Oxford Instruments, Oxford, UK) operating at 10 or 50 kV. The dried samples were coated with a 20-nm layer of gold by high vacuum metallisation using the SBC-12 Sputter Coater System (KYKY Technology Development Ltd., Beijing, China) before being stored in a desiccator until analysis.

Acknowledgments

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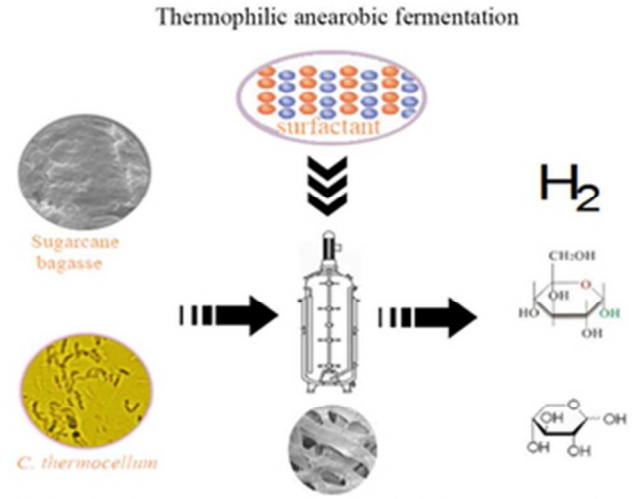

SCB biodegradation and reducing sugar accumulation were remarkably increased by adding surfactant in the CBP system using *C. thermocellum*.

36x26mm (300 x 300 DPI)