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Enhanced enzymatic xylose/cellulose fractionation from alkaline liquor-pretreated corn cob by surfactant addition and separate fermentation to bioethanol

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Abstract: Xylanase xylose/cellulose fractionation can efficiently improve the utilization of xylose and cellulose. Improvement of xylanase performance during hemicellulose hydrolysis can increase xylose/cellulose fractionation efficiency. To utilize xylose and cellulose efficiently, an enzymatic xylose/cellulose fractionation and a separate fermentation bioethanol process were performed. Alkaline liquor-pretreated corn cob was subjected to xylose/cellulose fractionation with xylanase in the presence or absence of the following mixed surfactants: polyoxyethylene(20)-sorbitan-monooleate (0.15% v/v), polyethylene glycol 6000 (0.15% w/v), bovine serum albumin (0.15% w/v), and rhamnolipid (0.005% v/v). The hemicellulose hydrolysis yield (HHY) in the presence of mixed surfactants was 70.5%, which was 66% higher than that of the control (no surfactants added). Optimization of other hydrolysis conditions, such as pH, temperature, liquid-to-solid ratio, xylanase loading, and incubation time, increased the HHY to 86.62%. The xylose in the enzymatic hydrolysate was fermented to ethanol by *Candida shehatae*, and the cellulose remaining in the solid residues was converted to ethanol by simultaneous saccharification and fermentation with *Saccharomyces cerevisiae*. In total, 40.67% of the hemicellulose and 76.14% of the cellulose in the raw corn cob was converted to ethanol.

Key words: Xylose, hemicellulose, cellulose, bioethanol, surfactants, corn cob

1. Introduction

With population growth and industrial prosperity, exploring alternative energy sources and materials is urgent because of the shortage of fossil resources (Sun and Cheng, 2002; Farrell et al., 2006). Lignocellulosic biomass can become a major source of fermentable sugars for the production of bioethanol and other biochemicals (Duff and Murray, 1996; Sarkar et al., 2012). However, lignocellulose has a highly complex structure with features that limit the hydrolysis of carbohydrate polymers into fermentable sugars. Consequently, lignocellulose bioconversion can still be difficult. For example, the high cost of the enzymatic hydrolysis and low efficiency of xylose utilization are limiting factors for lignocellulosic bioethanol industrialization (Zhao et al., 2008).

To efficiently utilize xylose and cellulose, we developed a xylose/cellulose fractionation and separate fermentation (XCFSF) process (Chen et al., 2010). Xylose and cellulose can be fractionated by xylanase or other chemicals, such as diluted sulfuric acid. Compared with chemical fractionation, the lack of inhibitors produced during enzymatic hydrolysis under the XCFSF method benefits

yeast fermentation. For efficient enzymatic xylose/cellulose fractionation, new methods that can increase xylanase efficiency are required.

The addition of surfactants after pretreatment can enhance enzymatic hydrolysis and reduce the amount of enzyme required (Helle et al., 1993; Kristensen et al., 2007). Surfactants, such as nonionic surfactants and biosurfactants, can improve the enzymatic conversion of cellulose into soluble sugars (Fendler et al., 1975; Alkasrawi et al., 2003). However, little is known about the effect of surfactants on the enzymatic conversion of hemicellulose to xylose. Several surfactants, including nonionic polyoxyethylene(20)-sorbitan-monooleate (Tween-80) (Ballesteros et al., 1998), polyethylene glycol (PEG) (Eriksson et al., 2002), biosurfactant bovine serum albumin (BSA) (Park et al., 1992), and rhamnolipid (RH) (Zhang et al., 2009), can improve cellulose activity.

Several mechanisms underlying the positive effect of surfactant addition on the enzymatic hydrolysis of lignocellulose have been presented (Olsen et al., 2011; Wang et al., 2011). These mechanisms include changing the nature of cellulose and improving the accessibility of

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enzymes (Helle et al., 1993; Kaar and Holtzapple, 1998), preventing unproductive binding of enzymes to lignin and reducing unproductive enzyme adsorption to the lignin part of cellulose (Castanon and Wilke, 1981; Helle et al., 1993; Eriksson et al., 2002; Zhang et al., 2011), improving cellulase stability and preventing enzyme denaturation during hydrolysis (Kim et al., 1982; Kaar and Holtzapple, 1998), and increasing contact between cellulose and enzymes, as well as allowing the enzyme to reach previously inaccessible areas (Malmsten and Van Alstine, 1996; Kaar and Holtzapple, 1998; Eriksson et al., 2002). In addition, Ooshima et al. (1986) demonstrated that surfactants prevent the adsorption of endoglucanase from cellulose and change the adsorption balance of endoglucanase and exoglucanase, thereby increasing the concentration of endoglucanase in the liquid phase. Surfactants effectively improve enzyme activity during hydrolysis by binding to the tertiary structure of the enzyme protein (Yoon and Robyt, 2005). Okino et al. (2013) showed that Tween-80 can improve and stabilize not only cellulase enzyme production but also cellulose hydrolysis. However, an explanation that can consistently illustrate how surfactants increase enzymatic hydrolysis of lignocelluloses has not yet been developed.

In this study, alkaline liquor-pretreated corn cob (Qin et al., 2009), from which 88.17% of lignin had been removed to enhance the enzymatic digestibility of hemicelluloses, was efficiently fractionated into xylose hydrolysate and cellulosic residues by commercial xylanase in the presence or absence of surfactants. Xylose-containing hydrolysate was fermented to ethanol by *Candida shehatae*. The cellulose remaining in the solid residues was converted to ethanol using a simultaneous saccharification and fermentation (SSF) method with *Saccharomyces cerevisiae*. The effects of surfactant additives on the enzymatic fractionation of xylose and cellulose and, ultimately, on the fermentation of xylose and cellulose to ethanol were investigated.

2. Materials and methods

2.1. Materials

For alkaline liquor-pretreated corn cob residue, corn cobs were purchased from Pingyao (a city in Shanxi, China) and were ground to a particle size of 1–2 mm with a straw stalk knife mill (Shandong, China). The ground biomass was dried overnight at 60 °C, and the dried corn cob (10 g) was mixed with a solution (230 mL) comprising 2.5% (v/v) NH_4OH , 0.6% (v/v) H_2O_2 , 5% (w/v) Na_2SiO_3 , and 0.05% (w/v) MgSO_4 . The corn cob solution was incubated in a shaker at 70 °C for 16 h. After pretreatment, the solid residue was collected by filtration, washed with deionized water until a neutral pH was reached, and then dried. The compositions of the initial and pretreated corn cob were measured.

The surfactants used were BSA (Sigma, USA), PEG 6000 (Merck, USA), Tween-80 (Merck, USA), and RH (Engster Biotech, China). The rest of the chemicals and medium components were purchased locally.

The enzymes used were xylanase (840 IU/g) from *Aspergillus niger* and cellulase (320 FPU/g) from *Trichoderma reesei*, and both were purchased from Shenzhen Leveking Biology Engineering Co., Ltd., China.

The microorganisms used were *Candida shehatae* CICC1766 (China Center of Industrial Culture Collection) and *Saccharomyces cerevisiae* TCCC34074 (Tianjin University of Science and Technology's Center of Culture Collection).

2.2. Methods

2.2.1. Enzymatic xylose/cellulose fractionation

The enzymatic saccharification of hemicellulose via xylanase in the alkaline liquor-pretreated corn cob residue (3 g dry weight) was performed with gentle shaking (150 rpm) at 50 °C in a 100-mL Erlenmeyer flask after adjusting the pH to 5.0 using a citric acid- NaH_2PO_4 buffer solution (0.1 mol/L). After centrifugation, most of the xylose was in the liquid, and most of the cellulose was in the solid. The liquid and the solid portions were fermented separately. The concentrations of glucose and xylose after 45 h of hydrolysis were determined by high-performance liquid chromatography (HPLC). In the experiments reported here, optimization of different factors was performed. The factors and their levels were as follows: time (36, 48, and 72 h), xylanase loading (90, 120, and 150 IU/g), and liquid-to-solid ratio (10:1, 15:1, and 20:1). All experiments were performed in triplicate and the average values were recorded.

2.2.2. Addition of surfactants

Surfactants enhance the enzymatic conversion of cellulose (Kumar et al., 2009). The surfactants Tween-80, PEG 6000, BSA, and RH were tested for their effect on hemicellulose hydrolysis in alkaline liquor-pretreated corn cob with xylanase. Each surfactant was simultaneously added to the alkaline liquor-pretreated corn cob residue with xylanase. The loading amounts for Tween-80 (v/v), PEG 6000 (w/v), and BSA (w/v) were 0.25%, 0.5%, 1%, 2.5%, and 4%, whereas those for RH (v/v) were 0.02%, 0.25%, 0.5%, 1%, 2.5%, and 4%. An orthogonal test was performed to investigate the effect of mixed surfactants on the enzymatic hydrolysis of hemicelluloses. To study the effects of time of the surfactants' addition to the compound on the enzymatic hydrolysis of hemicelluloses, the mixed surfactants were added at 8, 5, and 2 h before or 0, 2, 5, and 8 h after the beginning of enzymatic hydrolysis of the pretreated corn cob.

2.2.3. Seed culture of microorganisms

Liquid inoculum of *C. shehatae* was prepared by inoculation in culture medium containing 20 g/L xylose,

10 g/L yeast extract, 3 g/L malt extract, and 20 g/L peptone. The culture was maintained at 28 °C for 24 h on an orbital shaker agitated at 120 rpm. The liquid seed of *C. shehatae* was subcultured in fresh culture medium of the same composition and cultivated for an additional day under the same conditions. *S. cerevisiae* liquid inoculum was grown in a medium containing 40 g/L glucose, 3 g/L yeast extract, 5 g/L peptone, and 2 g/L $(\text{NH}_4)_2\text{SO}_4$ at 30 °C (Chen et al., 2010). The other cultivation details were the same as those used for *C. shehatae*.

2.2.4. Ethanol fermentation of hemicellulose enzymatic hydrolysate by *C. shehatae*

The enzymatic hydrolysate of hemicelluloses was further supplemented with the following additional nutrients: 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 2 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g/L yeast extract, and 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The pH of the solution was adjusted to 4.5. The corn cob hydrolysate and the nutrients were autoclaved separately and were combined after sterilization. Fermentation medium was inoculated with 10% (v/v) cultures of *C. shehatae*. The enzymatic hydrolysate was fermented at 28 °C in a 250-mL Erlenmeyer flask containing 100 mL of the culture medium with shaking at 160 rpm for 72 h (Chen et al., 2010). Liquid samples were obtained for ethanol determination. All experiments were performed in triplicate.

2.2.5. SSF by *S. cerevisiae*

SSF of cellulosic residues was performed as previously described (Krishna et al., 2001). The inoculum medium was composed of 2 g/L $(\text{NH}_4)_2\text{SO}_4$, 5 g/L KH_2PO_4 , 0.4 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g/L yeast extract. The pH of the substrate was adjusted to 5.0, and the substrate was autoclaved for 15 min at 121 °C before the addition of enzymes and inocula. The inoculum amount was 10% (v/v) of the SSF medium. SSF processes were performed statically with a substrate concentration of 10% (w/v) at 35 °C in an incubator for 72 h, and the cellulase loading was 20 FPU/g substrate. Samples were obtained every 12 h at regular intervals, and the solid substrate was removed by centrifugation. The supernatant was used for ethanol determination, and the cellulose content of the remaining solid substrate was measured (Chen et al., 2010). All experiments were performed in triplicate.

2.2.6. Analytical methods and calculations

The cellulose, hemicellulose, and lignin contents of the dry corn cob were determined as previously described (Wang and Qi, 1987). Sugars (D-glucose and D-xylose) were quantitated on an Agilent HPLC system (Agilent Corporation, USA) equipped with an SCL-10A system controller, a CTO-AS column oven, an RID-10A refractive index detector, an Aminex HPX-87H column, and a Shimadzu refractive index detector. Before injection, samples were passed through a 0.22- μm filter and diluted

appropriately by an eluent (i.e. 5 mmol/L H_2SO_4). The column temperature was fixed at 65 °C, and the mobile phase had a flow rate of 0.6 mL/min. The amount of ethanol was quantified as previously reported (Chen et al., 2010).

$$\text{HHY} = \frac{V \times C \times 0.9}{M \times W \times 1000} \times 100\%$$

HHY, Hemicellulose hydrolysis yield; V, hemicellulose hydrolysate volume (mL); C, concentration of xylose in the hydrolysates (g/L); M, weight of alkaline liquor-pretreated corn cob used in the experiment (g); W, percentage of hemicellulose in alkaline liquor-pretreated corn cob (%).

$$\text{CHY} = \frac{V \times C \times 0.9}{M \times W \times 1000} \times 100\%$$

CHY, Cellulose hydrolysis yield; V, cellulose hydrolysate volume (mL); C, concentration of glucose in the hydrolysates (g/L); M, weight of solid residue after enzymatic hydrolysis of hemicelluloses (g); W, percentage of cellulose in solid residue after enzymatic hydrolysis of hemicelluloses (%).

Hemicellulose conversion rate (HCR) = Ethanol output (g) / [raw material (10 g) \times hemicellulose content \times theoretical yield of ethanol to hemicellulose (0.523 g/g)] \times 100%.

Cellulose conversion rate (CCR) = Ethanol produced from cellulose (g) / [raw material (10 g) \times cellulose content \times theoretical yield of ethanol to cellulose (0.567 g/g)] \times 100%.

3. Results

3.1. Enzymatic fractionation of alkaline liquor-pretreated corn cob

3.1.1. Effect of single surfactant on enzymatic fractionation

The initial composition of the corn cob was as follows: 33.56% (w/w) cellulose, 34.65% (w/w) hemicellulose, 13.96% (w/w) lignin, and 1.6% (w/w) ash. The pretreated corn cob contained 48.43% (w/w) cellulose, 32.83% (w/w) hemicellulose, 2.76% (w/w) lignin, and 11.9% (w/w) other materials. The pretreated corn cob was subsequently used for enzymatic xylose/cellulose fractionation.

As shown in Figure 1, the conversion of hemicellulose was higher in the presence of, rather than in the absence of, surfactants. The highest HHYs of enzymatic hydrolysis were as follows: 57.32% in the presence of PEG 6000, 55.31% in the presence of BSA, 54.77% in the presence of Tween-80, and 49.94% in the presence of RH. The highest HHY of the control was 42.45%. However, the increase in the rate of enzymatic hydrolysis of hemicellulose gradually decreased with increasing surfactant concentration.

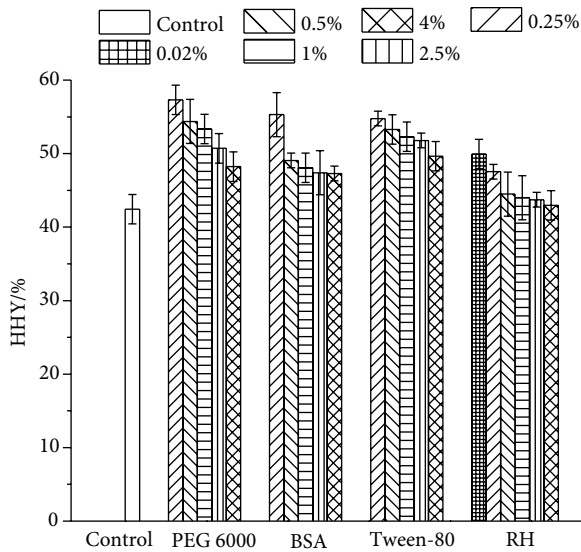


Figure 1. Effect of surfactants on enzymatic hydrolysis of hemicelluloses.

3.1.2. Combined effect of mixed surfactants on enzymatic fractionation

An orthogonal test was performed to investigate the effect of mixed surfactants on the enzymatic hydrolysis of hemicellulose (Table 1).

Based on the results of the orthogonal tests (Table 2), we found that the effect of the different surfactants on HHY was in the following order: Tween-80 > BSA > RH > PEG 6000. Considering the 4 surfactants comprehensively, we concluded that the optimal dosages were 0.15% (v/v) for Tween-80, 0.15% (w/v) for PEG 6000, 0.15% (w/v) for BSA, and 0.005% (v/v) for RH. The HHY was 70.50% under the optimal condition in the presence of mixed surfactants. This value was 66% higher than that under the original condition.

3.1.3. Determination of the time of addition of mixed surfactants and optimization of enzymatic fractionation condition

As shown in Figure 2, the HHY from hemicellulose gradually decreased when the addition of the mixed surfactants was delayed. When the surfactants were added 8 h before the beginning of the enzymatic hydrolysis, the HHY was 72.02%, which was slightly higher than that obtained in the treatment where the xylanase and surfactants were added simultaneously (70.50%). When addition was delayed until 8 h after the start of the xylanase treatment, the obtained HHY was 55.22%, which was still 12.77% higher than that of the control.

Considering the significant delay and the small gain in HHY when the surfactants were added 8 h before xylanase, we employed the strategy of simultaneously adding these compounds in subsequent experiments. We optimized

Table 1. Experimental factors and their levels.

Factor	Level		
	1	2	3
Tween-80 (%)	0.25	0.15	0.05
PEG 6000 (%)	0.05	0.25	0.15
BSA (%)	0.05	0.15	0.25
RH (%)	0.005	0.01	0.02

the conditions for hemicellulose hydrolysis, starting from the abovementioned mixed surfactant strategy. Aside from the simultaneous addition of xylanase (150 IU/g) with Tween-80 (0.15% v/v), PEG 6000 (0.15% w/v), BSA (0.15% w/v), and RH (0.005% v/v), the other factors were optimized as follows: pH 5.0, temperature of 50 °C, liquid/solid ratio of 15:1, and incubation time of 48 h. Under these conditions, the HHY reached 86.62%.

3.2. Effect of hemicellulose content on the enzymatic hydrolysis of cellulose

High HHY results in low hemicellulose content in the cellulosic residues. The cellulosic residues with different HHYs were selected for the enzymatic cellulose hydrolysis experiment to investigate the effect of hemicellulose content on cellulose enzymatic hydrolysis. The HHYs of the selected cellulosic residues were 0%, 35.5%, 42.54%, 51.54%, 57.34%, 68.04%, and 74.83%. As shown in Figure 3, the CHY increased with increasing HHY. When the HHY of cellulosic residues reached approximately 51.54%, the obtained CHY reached 90.93%. However, a substantial increase in HHY beyond 51.54% had no further positive effect on the enzymatic hydrolysis of cellulose. In conclusion, lignin and hemicellulose contents can affect the enzymatic hydrolysis of cellulose. However, enzymatic hydrolysis of cellulose was improved only marginally after a threshold amount of hemicelluloses (51.54%) was removed.

3.3. Xylose fermentation

The conversion of the xylose from hemicellulose in the hydrolysate to ethanol can greatly enhance the overall yield of cellulosic ethanol production and reduce the cost of ethanol per unit amount (Chen et al., 2010). Before fermentation, the hemicellulosic hydrolysate was at concentrations of 30 and 50 g xylose/L. Fermentation with *C. shehatae* was performed as previously described, and pure xylose was used as a control carbon source. The results are shown in Table 3.

For the xylose concentration of 30 g/L, a higher amount of ethanol (11.29 g/L) was obtained from the hydrolysate than from pure xylose fermentation (10.85 g/L) after 60 h. The small amount of glucose in the hydrolysate can probably promote yeast growth and ethanol fermentation.

Table 2. Results of the orthogonal tests for surfactants.

No.	Tween-80 (%)	PEG 6000 (%)	BSA (%)	RH (%)	HHY (%)
1	0.25	0.05	0.05	0.005	61.92
2	0.25	0.25	0.15	0.01	68.20
3	0.25	0.15	0.25	0.02	58.47
4	0.15	0.05	0.15	0.02	66.80
5	0.15	0.25	0.25	0.005	67.59
6	0.15	0.15	0.05	0.01	68.65
7	0.05	0.05	0.25	0.01	59.99
8	0.05	0.25	0.05	0.02	56.67
9	0.05	0.15	0.15	0.005	68.68
â1	62.863	62.903	62.413	66.063	
â2	67.680	64.153	67.893	65.613	
â3	61.780	65.267	62.017	60.647	
R	5.900	2.364	5.876	5.416	

For a xylose concentration of 50 g/L, the amount of ethanol obtained from the hydrolysate (11.29 g/L) was similar to that from pure xylose fermentation (11.04 g/L) after 60 h. Ethanol production from the hydrolysate was slightly decreased when fermentation time extended until 72 h, whereas that from pure xylose significantly increased with time extension. The component(s) in the hydrolysate with a high concentration (50 g xylose/L) probably inhibited xylose fermentation. When other conditions remained constant, the optimal hydrolysate fermentation condition included a xylose concentration of 30 g/L and a fermentation time of 60 h.

Under the abovementioned optimal conditions, 11.29 g/L ethanol was produced from 10 g of corn cob

fermentation with a yield of 0.4094 g/g xylose, which was 89% of the theoretical yield (0.46 g ethanol/g xylose). This finding indicated that 40.67% of hemicelluloses in the raw material was converted to ethanol (Table 4).

3.4. SSF of cellulosic residues into ethanol

The cellulose in lignocellulosic material is the greatest contributor to ethanol production. To avoid end-product inhibition caused by the accumulation of glucose during cellulase hydrolysis and to enhance ethanol yield, SSF was performed for ethanol fermentation of cellulosic residue (Chen et al., 2010).

We examined the ethanol yields through SSF as a function of time. The ethanol yield significantly increased

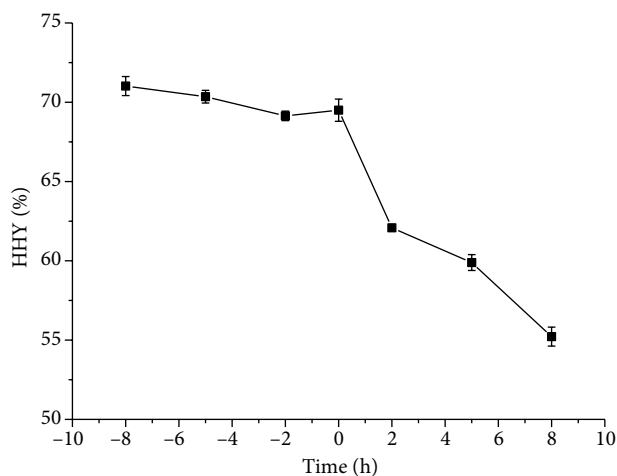


Figure 2. Effect of timing of the surfactant supplementation on hemicellulose hydrolysis.

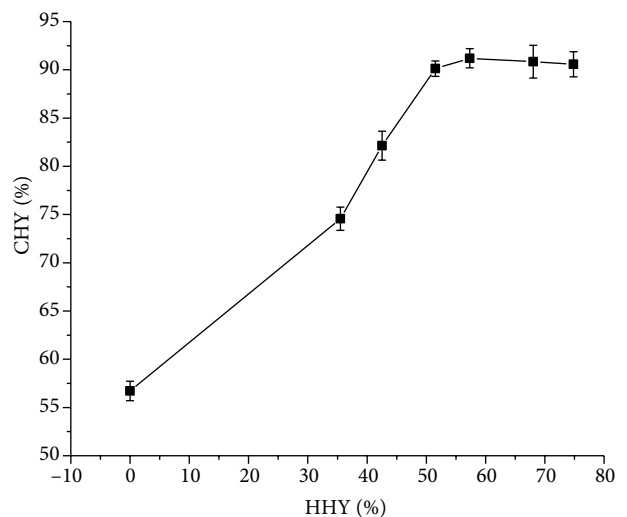


Figure 3. Effect of the hemicellulose content on cellulose enzymatic hydrolysis.

Table 3. Ethanol fermentation of hemicellulose hydrolysate.

	Initial xylose concentration (g/L)	Time (h)	Ethanol (g/L)	Residual sugar (g/L)
Pure xylose	30	60	10.85	0.1462
Hydrolysate	30	60	11.29	0.4585
Pure xylose	50	60	11.04	19.02
Hydrolysate	50	60	11.29	19.86
Pure xylose	50	72	17.71	0.1200
Hydrolysate	50	72	10.72	15.66

from 0 to 36 h and slightly increased from 36 to 60 h. Nevertheless, ethanol yields were not substantially changed from 60 to 72 h. Therefore, the optimal fermentation time for SSF was 60 h. Under the abovementioned optimal conditions, 28.8 g/L ethanol was produced by fermenting 10 g of corn cob, and the yield was 0.5005 g/g cellulose. According to the theoretical yield (0.5667 g ethanol/g cellulose), 88.33% of cellulose in the cellulosic residue and 76.14% of cellulose in the raw material was converted to ethanol (Table 4).

4. Discussion

Lignin forms a shield around cellulose and hemicelluloses and protects the polysaccharides from enzymatic degradation. Several studies (Sutcliffe and Saddler, 1986; Yang et al., 2006) have showed that lignin has negative effects on cellulase activity. Such effects originate from both physical blockage of enzymatic access to the substrate and adsorption caused by favorable enzyme–lignin interaction. These 2 mechanisms reduce the population of active enzymes and slow down saccharification. The enzymatic mechanism and molecular structure of xylanase and cellulase are similar. Thus, we hypothesize that the effects of surfactants on both enzymes are also similar. Surfactants with an affinity to lignin can be added prior to enzymatic hydrolysis to improve the efficiency of adsorption of xylanase and thereby improve the effectiveness of xylanase.

The reversible adsorption between hemicellulose and xylanase is probably the key to the enzymatic hydrolysis of hemicellulose. Hemicellulose and xylanase form a complex during enzymatic hydrolysis in order to complete the reaction, after which xylanase desorbs from this hemicellulose functional group to adsorb another hemicellulose functional group. Therefore, the rates of adsorption and desorption of xylanase to hemicellulose are crucial to the efficiency of xylanase. Xylanase, which may have been adsorbed first by the hemicellulose

Table 4. Ethanol yield from 10 g of corn cob after fermentation.

Ethanol from xylose (g)	HCR (%)	Ethanol from cellulose (g)	CCR (%)	Total ethanol output (g)
0.7203	40.67	1.448	76.14	2.168

functional group, did not desorb. Surfactants of low concentrations can create a hydrophilic environment and affect the desorption of xylanase from hemicellulose functional groups, thereby enhancing the hydrolysis of hemicellulose. However, surfactants at high concentrations may weaken the adsorption of xylanase to hemicellulose, thereby inhibiting the hydrolysis of hemicellulose. Enzyme accessibility to cellulose may have been impeded by the lignin and hemicellulose coating of cellulose.

Compared with the effects of a single surfactant, a mixture of surfactants can better promote the enzymatic reaction. The action mechanisms of the surfactants are different, and mixtures of different surfactants may elicit a synergistic effect on the enzymatic hydrolysis of hemicellulose.

In this study, alkaline liquor-pretreated corn cob was used for ethanol production. Surfactants can enhance the enzymatic hydrolysis of hemicellulose and ultimately improve the utilization of corn cob. Mixed surfactants caused higher hemicellulose hydrolysis than any single surfactant. The HHY (86.62%) and xylose fermentation yield (89% of theoretical yield) were high, but the notable loss of hemicelluloses during the alkaline liquor pretreatment resulted in a low overall conversion rate (40.67%) of the hemicellulose in the raw material. Although the contribution of hemicellulose to ethanol production is not as significant as that of cellulose, effectively promoting the development of lignocellulosic ethanol is still important. For efficient enzymatic xylose/cellulose fractionation and ethanol bioconversion, the development of pretreatment methods must be prioritized.

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