

# Enhanced Sugars Production with High Conversion Efficiency from Alkali-pretreated Sugarcane Bagasse by Enzymatic Mixtures

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Complementary enzymes can considerably enhance the hydrolysis effectiveness of cellulase. The influence of hemicellulase supplementation on high solids saccharification of alkali-pretreated sugarcane bagasse was assessed. Hemicellulase addition of 1200 IU/g substrate with cellulase loading of 10 FPU/g substrate achieved high sugars yield with glucose and xylose conversion efficiency of 95.4% and 87.4%, respectively. To further improve the substrate conversion efficiency based on high sugars production, fed-batch hydrolysis was employed with high solids loading of 20% (w/v) to 25% (w/v). After 96 h hydrolysis with 25% solids loading at cellulase and hemicellulase loading of 20 FPU/g and 1200 IU/g substrate, respectively, the obtained highest total sugars was 242 g/L, with glucose and xylose conversion efficiencies of 98.6% and 94.9%, respectively. An increase in substrate digestibility upon supplementation of mixture enzymes with high sugars production can be realized in high solids fed-batch system with proper cellulase and hemicellulase synergism.

*Keywords:* Complex enzymes; High sugars; Substrate digestibility; Fed-batch hydrolysis; High solids loading

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## INTRODUCTION

The depletion of fossil fuel and increasing concerns of environmental issues have brought increased attention to the development of renewable energy. Lignocellulosic biomass, which is rich in carbohydrates, is considered as an ideal raw material for bioethanol production (Himmel *et al.* 2007; Gao *et al.* 2011). Deconstruction of biomass needs to overcome the lignocellulosic recalcitrance to break the carbohydrate-lignin intricate links and disrupt the crystalline structure of cellulose, and hence, improve the subsequent enzymatic hydrolyzability (Van and Pletschke 2012; Liu *et al.* 2016). Bioconversion of lignocellulose residues to fermentable sugars requires delignification pretreatment followed by enzymatic hydrolysis of complex carbohydrates (Hendriks and Zeeman 2009).

For cost-effective production of bioethanol, one of the main challenges is the saccharification of biomass to fermentable sugars (Sharma and Horn 2016). It is important to use high concentration sugars as the yeast carbon source to increase the final product concentration and thus reduce the ethanol purification costs (Wang *et al.* 2012; Luis *et al.* 2014). Previous studies reported that high yields of sugars from both cellulose and hemicellulose components could be produced from the proper combination of chemical and enzymes. Physical and chemical pretreatments followed by enzymatic hydrolysis with

enzymes to produce sugars are common methods used in the saccharification process (Wyman *et al.* 2005; Lau and Dale 2009). In chemical pretreatment, various methods have been explored to enhance the accessibility of substrates. Among them, alkali-pretreatment has been widely studied for its effective solubilization of lignin. Meanwhile, it causes swelling, increasing the internal surface of cellulose, and provokes lignin structure disruption (Liu *et al.* 2015a). These structural changes are conducive for the subsequent enzymatic hydrolysis.

Enzymatic hydrolysis, especially operated at high-solids loading, is a convenient and direct technology to produce high sugars. Under high solids conditions, a significant portion of sugars produced is in oligosaccharide form, which cannot be directly used by yeast. Enzyme supplementation and alternative organism sources for cellulase to promote the degradation of monosaccharides have been investigated (Lau and Dale 2009; Zambare *et al.* 2011). Supplementing cellulase with  $\beta$ -glucosidase or xylanase has long been used to minimize end-product inhibition of cellulase and achieved higher substrate conversions (Yang and Wyman 2008). Typically, cellulase, xylanase,  $\beta$ -glucosidase,  $\beta$ -xylosidase, and other additional enzymes are required to hydrolyze polysaccharides effectively, with their optimum proportions (Mosier *et al.* 2005). It is recognized that the hydrolytic efficiency determined using a model cellulosic substrate does not present a reliable indication on pretreated biomass (Berlin *et al.* 2007). Other components, especially hemicellulose and lignin, exert restraints on cellulose hydrolysis. After alkali-delignification, the majority of xylan is left in the solids; it is possible to compensate cellulase by supplementing with xylanase to realize high sugars yield and meanwhile improve the solids conversion efficiency (Kumar and Wyman 2009).

It has been reported that enzymatic removal of hemicellulose (xylan) enhances cellulose degradation by disrupting the coating linkages of xylan to cellulose (Ishizawa *et al.* 2007). With the aim to obtain a high substrates conversion efficiency and meanwhile achieve higher glucose and xylose yields, this paper investigates the complex enzymes addition modes under high solids loading conditions with alkali-pretreated sugarcane bagasse.

## EXPERIMENTAL

### Materials

Sugarcane bagasse (SCB) was kindly provided by Guangxi Fenghao Sugar Co., Ltd., Nanning, China. It was pre-milled and screened, and the fraction between 40- and 60-meshes was collected for alkali-pretreatment. Cellulase CTec2 and hemicellulase HTec2 with the activity of approximately 200 FPU/mL and 8000 IU/mL, respectively, were generously provided by Novozymes A/S (Bagsaevrd, Denmark). The cellulase activity was determined according to the description by International Union of Pure and Applied Chemistry (IUPAC) (Liu *et al.* 2015a), and hemicellulase activity was measured as described elsewhere (Lin and Thomson 1991).

### Alkali Pretreatment

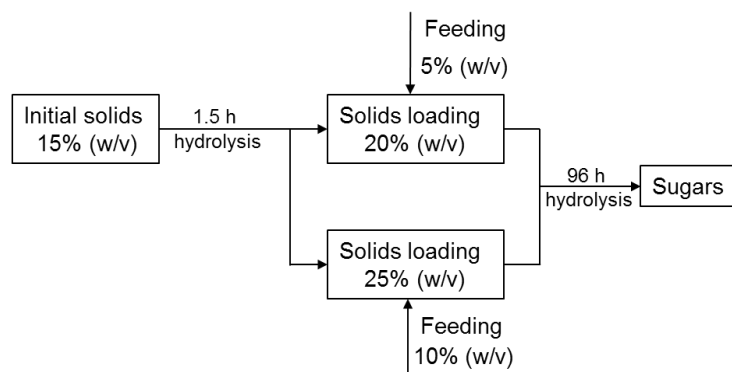
Alkali pretreatment of SCB was conducted as described in the authors' previous work (Liu *et al.* 2015b). The SCB was impregnated with 0.5 M NaOH solution at a solid to liquid ratio of 1:20 in a round-bottom flask at 80 °C for 2 h with stirring. The reaction was immediately terminated when the designed time was reached. The solid fraction was

separated by filtering and then washed with water to remove residual alkali until a neutral pH value was reached, then dried at  $50 \pm 5$  °C for 24 h. After delignification, the substrate became fractured and showed a rough structure with obvious cracks, ravines, and holes. These changes could facilitate the subsequent enzymatic hydrolysis. All experiments were repeated two times, and the given numbers are the mean values.

### Enzymatic Hydrolysis

The hydrolysis experiments were initiated with 15% (w/v) solids loading in 100 mL sodium acetate buffer (50 mM, pH 5.0). The reaction mixtures were incubated at 50 °C, 150 rpm in 150-mL Erlenmeyer flasks, and sealed with rubber stoppers under orbital agitation for 96 h. Five sets of hydrolysis were conducted. The required cellulase loading was 10 FPU/g substrate, and the effect of hemicellulase HTec2 was assessed by increasing its supplemented loadings with 0, 500, 800, 1200, and 1500 IU/g substrate. After sampling, the sugars concentration was analyzed by High Performance Liquid Chromatography (HPLC).

High solids enzymatic hydrolysis with improved substrate conversion efficiency was started with 15% (w/v) solids loading. After the slurries were totally liquefied, new 5% or 10% (w/v) substrate was added to reach the final solids loading of 20% and 25% (w/v) (Fig. 1). The hydrolysis was performed in 150-mL Erlenmeyer flasks sealed with rubber stoppers, at 50 °C, 150 rpm, and pH 5.0 (0.2 M acetate buffer). For the reactions, hemicellulase HTec2 supplementation was 1200 IU/g substrate based on the above optimized experiments. Cellulase loading was varied from 20 to 30 FPU/g substrate, and the dosing was based on the final total amount of DM loaded into the reaction. After sampling, the enzymes were inactivated (boiled at 100 °C for 5 min) and the released sugars were analyzed by HPLC.



**Fig. 1.** Fed-batch high solids hydrolysis process

The hydrolysis yields were calculated in relation to the amount of glucan (cellulose) and xylan present in the substrate. Glucan and xylan conversion efficiency were estimated by the following equations:

$$\text{Glucan conversion efficiency} = \frac{\text{Glucose produced (g)} \times 0.9}{\text{Glucan amount in enzymatic substrate}} \quad (1)$$

$$\text{Xylan conversion efficiency} = \frac{\text{Xylose produced (g)} \times 0.88}{\text{Xylan amount in enzymatic substrate}} \quad (2)$$

## Analytical Methods

### Composition assay

The chemical compositions of SCB before and after pretreatment were determined in duplicate according to the standardized methods of the National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2008). The values determined are described in Table 1.

**Table 1.** Chemical Composition of SCB Before and After Alkali-pretreatment

Sample	Xylan (%)	Glucan (%)	Klason Lignin (%)	Other Components (%)
Raw SCB	23.63	38.61	21.21	5.11
Alkali-treated	25.52	60.11	7.97	5.63

### Sugar analysis

Monomeric sugars in enzymatic hydrolysate were analyzed by HPLC (Waters model 2695, Waters, Wilford, Massachusetts, USA) using a Shodex sugar SH-1011 column coupled with a refractive index detector at 50 °C. The column temperature was 50 °C. The mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub> in demineralized water was used at a flow rate of 0.5 mL/min. Standard and hydrolyzed samples were filtrated by a 0.22- $\mu$ m filter before analysis. Duplicate runs of enzymatic hydrolysis were performed and the average values were reported.

## RESULTS AND DISCUSSIONS

### Effect of Hemicellulase Supplementation on Enzymatic Hydrolysis of SCB

To assess the influence of hemicellulase addition on enzymatic hydrolysis of alkali-pretreated SCB, hydrolysis reactions were performed with hemicellulase supplementation to various degrees at cellulase loading of 10 FPU/g substrate, and the reaction was started with 15% (w/v) solids loading for 96 h.

Because alkali pretreatment retains almost the entire carbohydrate intact in biomass, higher sugar yields are able to be obtained with low protein demands by reconstructing cellulase to include other enzymes than just cellulase.

As shown in Fig. 2, 500 IU/g substrate hemicellulase supplementation increased glucose and xylose concentrations to 74.3 g/L and 34.3 g/L, respectively after 96 h hydrolysis. With the pure cellulase loading, *i.e.*, in the absence of hemicellulase, the obtained glucose and xylose concentration were 69.0 g/L and 32.0 g/L, respectively. The released sugars after 96 h of hydrolysis continued to increase with increased hemicellulase loading.

At hemicellulase addition of 1200 IU/g substrate, the produced xylose concentration reached its highest value of 36.4 g/L. Meanwhile, the glucose concentration reached 82.1 g/L, with sugars conversion efficiencies of 95.4% (xylose) and 87.4% (glucose) (Fig. 3).

A further increase in hemicellulase loading beyond 1200 IU/g substrate did not obviously improve either cellulose or hemicellulose hydrolysis. To facilitate the subsequent high solids hydrolysis, 1200 IU/g substrate of hemicellulase supplementation was selected as the complementary enzymes to improve the high solids digestibility.

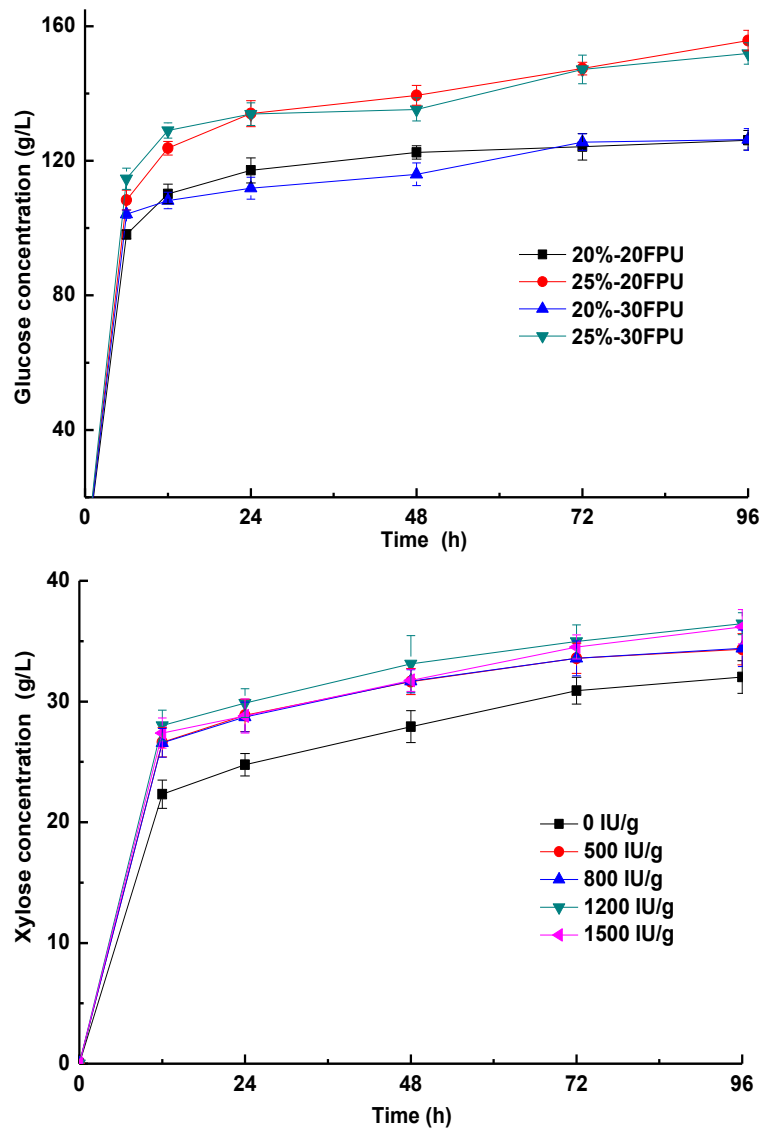
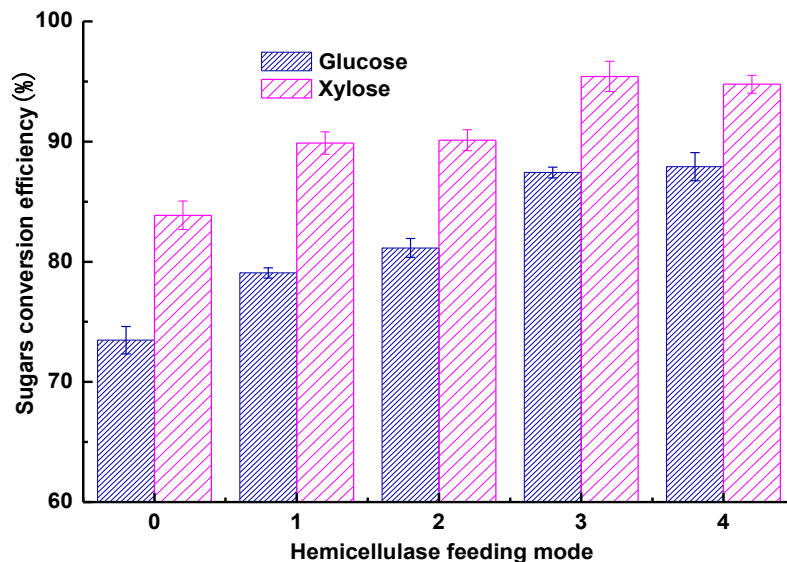


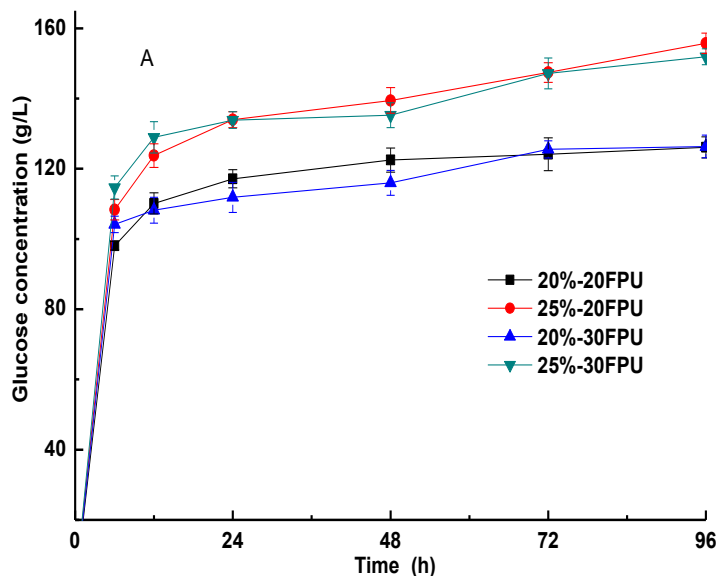
Fig. 2. Effect of different hemicellulase loadings on glucose and xylose concentration

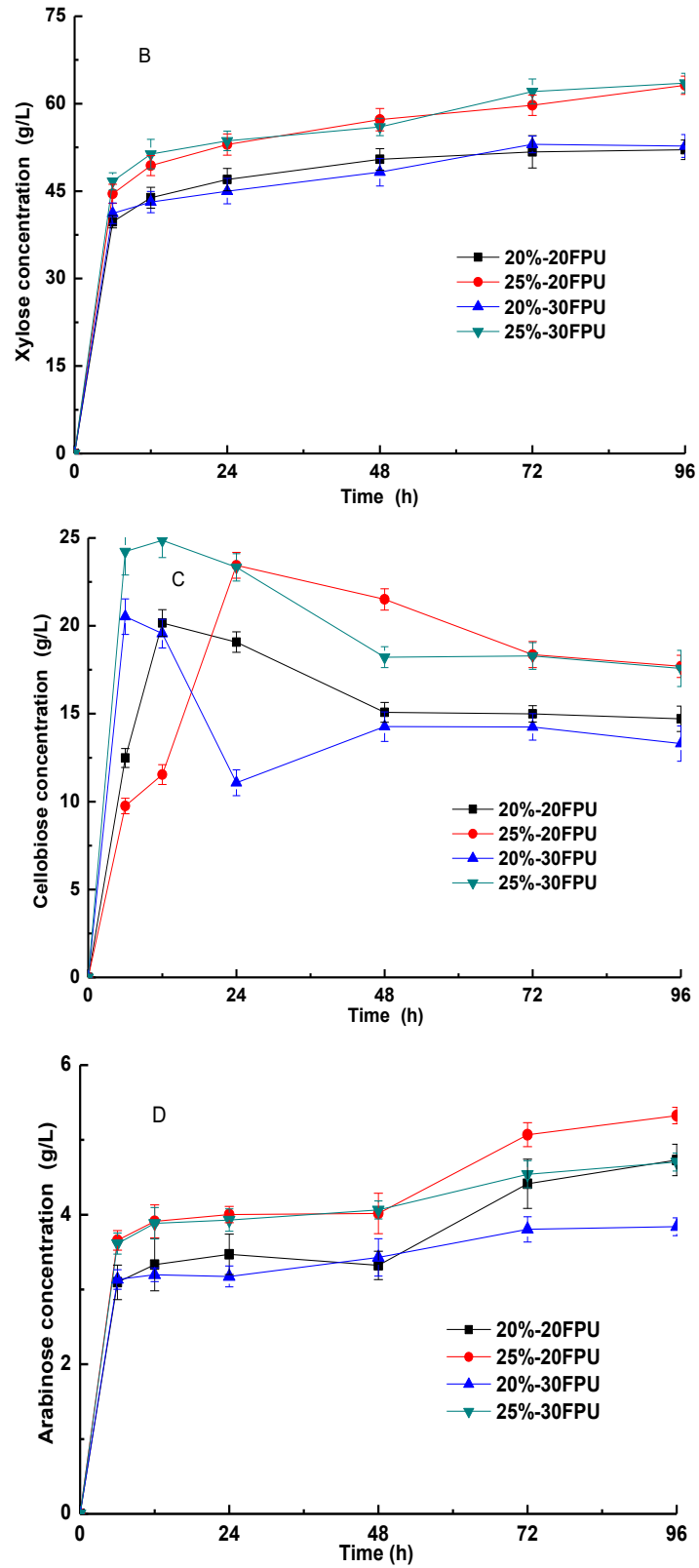


**Fig. 3.** Sugars conversion rate after 96 h enzymatic hydrolysis of sugarcane bagasse: 0, 1, 2, 3, 4 represent hemicellulase loading of 0, 500, 800, 1200, 1500 IU/g substrate, respectively

### High Sugars Production with High Solids Conversion Efficiencies

It is indisputable that the proper supplementation of cellulase with hemicellulase could not only substantially increase the sugars production, but also promote the extent of substrate hydrolysis. To further enhance the substrate digestibility based on the produced high sugars, four sets of hydrolysis experiments were conducted, as shown in Table 2. Fed-batch hydrolysis was performed with increased solids loading of 20% to 25% (w/v), where, the cellulase dosage was increased to 20 to 30 FPU/g substrate with the previously determined optimum loading of hemicellulase supplementation.





**Fig. 4.** Effect of different cellulase and substrates loadings on glucose (A), xylose (B), cellobiose (C), and arabinose (D) concentration

It was apparent that as the cellulase loading increased in the enzymes mixture, sugars concentration increased in both the 20% and 25% (w/v) hydrolysis systems. Under the tested conditions, the highest synergistic effect was observed under the cellulase and hemicellulase loadings of 20 FPU/g substrate and 1200 IU/g substrate, respectively, after 96 h hydrolysis with 25% (w/v) solids loading. The produced total sugars were as high as 242 g/L (Fig. 4, Table 2), in which the glucose and xylose concentrations were 156 g/L and 63.2 g/L, respectively.

**Table 2.** Sugars Concentration and Conversion Efficiency in Different Hydrolysis Systems

No.	Solid concentration (%)	Cellulase (FPU)	Hemicellulase (IU)	CGI/96(g/L)	CXyl/96(g/L)	CTs/96(g/L)	Y <sub>GI/120</sub> (%)	Y <sub>Xy120</sub> (%)
1	20	20	1200	126.10	52.09	197.63	99.78	97.80
2	25	20	1200	155.73	63.16	241.91	98.58	94.87
1o	20	30	1200	126.34	52.75	196.22	99.96	99.04
2o	25	30	1200	151.89	63.54	237.71	96.15	95.44

CGI (g/L): Glucose concentration; C<sub>Xy</sub> (g/L): Xylose concentration; 1, 2, 1o, 2o: Cellulase loading of 20, 20, 30, and 30 FPU/g substrate, hemicellulase loading of 1200 IU/g substrate; solids concentration of 20%, 25%, 20%, and 25%

The obtained total sugars content so far as we know is higher than 195 g/L, which was reported previously (Gao *et al.* 2014). With the above high sugars yield, the substrate conversion efficiency reached 98.6% and 94.9% for glucose and xylose, respectively (Fig. 4, Table 2), which suggested that almost all the substrates participating in hydrolysis were effectively degraded, and the observed improvement in substrate hydrolyzability probably benefited more from the increased enzyme loading. With these enzyme mixtures, cellulase and hemicellulase worked synergistically to hydrolyze SCB, and the experiments performed in this paper achieved solids conversion efficiencies more than 90%, which was consistent with the results reported by the previous work in this area (Van and Pletschke 2012). The promotion of substrate digestibility upon supplementation of the mixture enzymes with high sugars production can be realized in the high solids fed-batch system with proper cellulase and hemicellulase synergism.

The major advantage of hemicellulase supplement to cellulase might be the formed enzymatic mixtures that could promote the removal of the coating xylan on surface of the substrate fibers and increase the accessibility of enzyme to cellulose fibers. Therefore, the degradation process of substrate could be accelerated under the help of the hemicellulase. Despite the potential to enhance the digestibility of the substrate by hemicellulase supplementation, the major reservation in using enzyme mixture was that it would substantially increase the required protein loading, which will undoubtedly increase the enzyme costs. To improve the substrate conversion efficiency based on the high sugars production, the cellulase loading in this paper was not as low as possible to reduce the sugar production costs. Production of high efficient saccharification enzymes remains as the big challenge that has inhibited the industrialization process of bioethanol. In this study, the authors' initial approach was to find the optimum additive proportion of cellulase and hemicellulase to effectively improve hydrolysis efficiency of alkali-pretreated SCB. More work need to focus on how to enhance the conversion efficiency using low enzyme loading.



## CONCLUSIONS

1. The influence of hemicellulase loading on enzymatic hydrolysis of alkali-pretreated SCB was investigated. Hemicellulase supplementation of 1200 IU/g substrate combined with cellulase loading of 10 FPU/g substrate achieved high sugars yield with approximately 95.4% and 87.4% xylose and glucose conversion efficiency, respectively.
2. To further enhance the substrate digestibility, fed-batch hydrolysis was conducted with increased solids loading of 20% and 25% (w/v). After 96 h of hydrolysis with 20% (w/v) solids loading at cellulase and hemicellulase loadings of 20 FPU/g and 1200 IU/g substrate, the achieved final highest total sugars was 242 g/L, with glucose and xylose conversion efficiencies of 98.6% and 94.9%, respectively.
3. Hemicellulase supplementation substantially increased sugars production and enhanced enzymatic hydrolysis at a given cellulase loading. A promotion of substrate digestibility upon supplementation of enzyme mixtures with high sugars production can be realized in high solids fed-batch system with proper cellulase and hemicellulase synergism.

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