Monosaccharides and Ethanol Production from Superfine Ground Sugarcane Bagasse Using Enzyme Cocktail

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In this work, the effect of particle size on the enzymatic hydrolysis of milled and sieved sugarcane bagasse (SCB) was studied. The enzymatic hydrolysis and fermentability of superfine ground SCB (SGP400) using an enzyme cocktail strategy were also explored. Particle size reduction improved the enzymatic hydrolysis. The highest glucose yield was 44.75%, which was obtained from SGP400. The enzyme cocktail strategy greatly enhanced the glucose and xylose yield. The maximum glucose and xylose yield was from the enzyme cocktail of cellulase, xylanase, and pectinase. Synergistic action between xylanase and pectinase as well as cellulase and pectinase was quite noticeable. Hydrolysis times affected the degree of synergism. Ethanol production was carried out by employing simultaneous saccharification and fermentation (SSF) and semi-SSF using enzymes and their cocktails. Semi-SSF was found to be the better one compared with SSF. Xylanase and pectinase aided the ethanol production in both fermentation modes. Ethanol yield was 7.81 and 7.30 g/L for semi-SSF and SSF, respectively by using an enzyme cocktail of cellulase, β-glucosidase, pectinase, and xylanase.

Keywords: Enzymatic hydrolysis; Ethanol production; Superfine grinding; Enzyme cocktail; Synergistic effect

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INTRODUCTION

Considering the food crisis, bioethanol from lignocellulosic biomass is considered a promising solution (Fujii *et al.* 2013). Pretreatments such as physical comminution, solvent fractionation-based, acid-based, alkali-based, oxide-based, and biological and ionic liquid treatments need to be performed before enzymatic hydrolysis and fermentation (Chundawat *et al.* 2010). The chemical-involved pretreatments always lead to a loss of carbohydrates and the formation of inhibitors. Size reduction of lignocellulose through milling may overcome these issues. Meanwhile, high viscosity is thought to be one of the main obstacles for ethanol production at high solid loading. Size reduction has also been shown to reduce the viscosity of the slurry (Viamajala *et al.* 2009). However, the effect of particle size reduction on the digestibility of the lignocellulose is controversial. Rivers and Emert (1988a, 1988b) reported that particle size was not the sole or major factor determining accessibility and susceptibility of lignocellulose to cellulase. Harun *et al.* (2013) reported that the relationship between particle size and

sugar conversion was affected by the severity of ammonia fiber expansion. However, other studies have shown that reduction of particle size could enhance the digestibility of lignocellulose. Reduced particle size can lead to a higher glucose yield from both wet oxidized and dry oxidized wheat straw (Pedersen and Meyer 2009). Smaller particle size also leads to higher glucose yield when microcrystalline cotton cellulose is used as a substrate (Yeh *et al*. 2010). A particle size reduction from 5 to 20 to 0.075 to 5 mm can significantly enhance the final methane yield from corn stover with alkaline pretreatment (Xiao *et al.* 2013). It seems that pretreatment method and substrate type may affect the relationship between particle size and digestibility.

An enzyme cocktail of cellulase, xylanase, arabinofuranosidase, esterase, feruloyl esterase, ferulic acid esterase, and mannanase has been used to extensively enhance the convertibility of the pretreated lignocellulose (Hu *et al*. 2011; Kumar and Wyman 2009a, 2009b; Tabka *et al*. 2006; Várnai *et al*. 2011; Yu *et al*. 2003; Zhang *et al*. 2011, 2013). Synergistic effects of cellulase, xylanase, and pectinase were investigated by using different pretreated SCBs based on an enzyme replacement system. The results showed that replacement of 20% of cellulase by xylanase could enhance the cellulose convertibility. Replacement of 20% of cellulase by pectinase did not depress the cellulose convertibility (Li *et al.* 2014c). However, limited work has focused on the hydrolysis of superfine, ground lignocellulose through the use of an enzyme cocktail.

Based on this information, the effect of particle size on enzymatic hydrolysis was investigated. The enzymatic hydrolysis of superfine, ground sugarcane bagasse (SGP400) was studied by using enzymes and their cocktails. Bioethanol production from SGP400 by employing two fermentation modes using an enzyme cocktail was also investigated. Synergistic effects between cellulase, xylanase, and pectinase were also considered.

EXPERIMENTAL

Milling Treatment of Sugarcane Bagasse (SCB)

SCB was acquired from the Qianwu Sugar Refinery, Zhuhai, Guangdong, China. It was air-dried and then stored at room temperature in woven bags. SCB was ground using a hammer mill (HM) (FY130, Tianjin Taisitie Instrument Co., Ltd, China) and a jet mill (superfine grinding) (NADA Superfine Tech. Co., Ltd, Nanjing, China). The sieve used in the jet mill was 400-mesh. Very small amount of P400 could be obtained by HM. Superfine grinding was effective and it could produce large amount of P400 sample. Therefore, it was selected to prepare SGP400.

Enzymes

Celluclast 1.5 L from *Trichoderma reesei* and Novozym 188 from *Aspergillus niger* were provided by Novozymes. Novozym 188 is not a cellulase, but it is essential for digesting lignocellulose to monosaccharides. Therefore, in the following context, cellulase indicates the blend of Celluclast 1.5 L and Novozym 188 for easy description. Endo-1,4-β-xylanase from *Trichoderma longibrachiatum* (Sigma-Aldrich). Pectinase, derived from *A. niger*, was provided by Genencor.

Particle Size Fractionation

Particle size fractionation was carried out using a power-driven sieve shaker (8411, Shangyu Screen Factory, Zhejiang, China) equipped with 100-, 200-, 300-, and

400-mesh sieves, corresponding to sieve pores of 150, 75, 48, and 38 μm, respectively. In HMPy, the HM refers to hammer milling, the P refers to passing through a specific mesh, and the "y" refers to the mesh sieve (*i.e.*, the fraction that passed through a 400-mesh sieve was P400). SGP400 indicates superfine ground SCB.

Enzymatic Hydrolysis

To investigate the effect of particle size on the enzymatic hydrolysis, the loadings of substrate, Celluclast 1.5 L, and Novozym 188 of 2%, 24 FPU/g, and 45.8 IU/g SCB, respectively, were added by following the method described previously (Li *et al.* 2013). One gram of sample was mixed with 50 mL of 0.1 M citrate buffer (pH 4.8), 24 FPU of Celluclast 1.5 L, and 45.8 IU of β-glucosidase. After 48 h hydrolysis, products were measured. The mixtures were placed in an incubator at 45 °C and 160 rpm.

For investigating the synergistic effects between cellulase (Celluclast 1.5 L, Novozym 188), xylanase, and pectinase, the loadings of cellulase and β-glucosidase were reduced. A mixture of Celluclast 1.5 L, Novozym 188, xylanase, and pectinase with loadings of 10 FPU, 17.5 IU, 10 U, and 5 U/g substrate, respectively, were added.

The degree of synergism (DS) was calculated as (Hu *et al*. 2011),

$$DS = \frac{GC_{mixture}}{\sum GC_{individual}} \quad (1)$$

where GC_{mixture} is the substrate hydrolysis achieved with an enzyme cocktail and $\sum GC_{\text{individual}}$ is the sum of substrate hydrolyses achieved with the individual enzymes.

Fermentation

The microorganism strain of *Saccharomyces cerevisiae* As 2.489 was cultured in a YPD medium at 30 °C and 200 rpm in an incubator shaker for 36 h. Then, 1 mL of *S. cerevisiae* As 2.489 was aseptically transferred to 50 mL of sterile YPD medium. The flask was incubated at 30 °C and 200 rpm for 48 h. The *S. cerevisiae* As 2.489 cells were obtained by centrifugation for 10 min at 5000 rpm and 4 °C using a centrifuge (Anke, Shanghai, China), and the cell pellets were washed twice with sterile, deionized water. The cells were then re-suspended by sterile, deionized water with a loading of 1 g dry cell weight (DCW)/L and used to initiate fermentation. The whole process was completed within 2 h to maintain the activity of the yeast.

Simultaneous saccharification and fermentation (SSF) and semi-SSF were conducted following the method described by Li *et al.* (2014b). Briefly, the serum bottles were sealed with rubber stoppers equipped with a water trap, which permitted CO_2 removal without air injection. The bottles were placed on an incubator at 200 rpm and 30 °C. The composition of fermentation medium was 10% SGP400 (as sole carbon source), 1% yeast extract, and 2% peptone. In semi-SSF, prehydrolysis of SGP400 was carried out at 45 °C and 160 rpm for 48 h. Subsequently, yeast was added directly into the hydrolysate to initiate the fermentation. The enzyme cocktail loading was the same as for the synergistic experiments since the enzyme cocktail behaved better than the combination of Celluclast 1.5 L and Novozym 188.

Analytical Methods

All reagents were of analytical grade. The chemical composition of samples was determined by following the NREL method (Sluiter *et al.* 2008). The concentrations of glucose, xylose, and ethanol were quantified by HPLC (LC-15, Shimadzu, Japan) equipped with an Aminex HPX-87H column and a Cation H+ Cartridge Micro-Guard column (Bio-Rad, Hercules, CA, USA) at 55 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Each sample was centrifuged at 12,000 rpm at room temperature for 5 min, and the supernatant was filtered through 0.45-μm membrane filters (Sartorius, Gottingen, Germany) prior to analyses (Li *et al.* 2014a).

RESULTS AND DISCUSSION

Chemical Composition of the Fractionated Samples

The chemical compositions of all samples were analyzed, and the results are shown in Table 1. The cellulose content of the SCB was approximately 38%, while the hemicellulose and lignin contents were approximately 26% and 24%, respectively. The chemical composition of all the samples showed no significant difference, suggesting that no carbohydrates were lost during the milling process. Thermal or physic-chemical pretreatments always degraded hemicellulose and lignin to generate inhibitors. Inhibitors, including organic acids (acetic, formic, and levulinic acids), furan derivatives (furfural and 5-hydroxymethylfurfural), and phenolic compounds from lignin polymers could inhibit cellulase as well as microorganisms. Theses inhibitors should be removed before hydrolysis and/or fermentation (Zhuang *et al.* 2009). However, the milling process could eliminate the generation of such inhibitors because no chemicals or thermo treatments were involved in the milling process. Therefore, SCB from milling process could avoid inhibitor removal steps.

Table 1. Chemical Composition of the Fractionated Samples

Samples	Particle size (μm)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
HMP100	75 to 150	37.74 ± 0.48	25.32 ± 0.17	27.92 ±5.49
HMP200	48 to 75	39.05 ± 0.38	27.02 ± 0.89	23.95 ±1.73
HMP300	38 to 48	39.06 ± 0.92	27.40 ± 1.41	21.86 ±3.79
HMP400	≤ 38	37.72 ± 0.58	26.77 ± 1.22	25.48 ±1.92
SGP400	≤ 38	36.99 ± 0.32	26.91 ± 0.67	22.85 ±1.19

Each point in the table is a mean value ± standard deviation from three independent experiments. HMP was obtained from hammer milled, fractionated, and passed a specific sieve.

Effect of Particle Size on Enzymatic Hydrolysis

The effect of particle size on sugar yields is shown in Fig. 1. The sugar yield increased with decreasing particle size. The glucose yield from HMP100 reached 21% of the theoretical maximum. The highest glucose yield reached 44.75% of the theoretical maximum, which was obtained from SGP400.

Previous work by the authors showed that the glucose yield was 68.75% from steam exploded SCB (Li *et al.* 2014b). However, the glucose yield from wet disk milling-treated SCB was 49.3% (Da Silva *et al*. 2010), which was comparable to the current work. Compared to glucose from HMP100, that from SGP400 increased more than twofold. The obtained sugar yields from HMP400 and SGP400 did not show significant difference ($p > 0.05$). Since SG could obtain much more product than HM, SGP400 was selected for the following experiments. The xylose yield from SGP400 was approximately 16% of the theoretical maximum. Superfine grinding may be an option to pretreat lignocellulose.

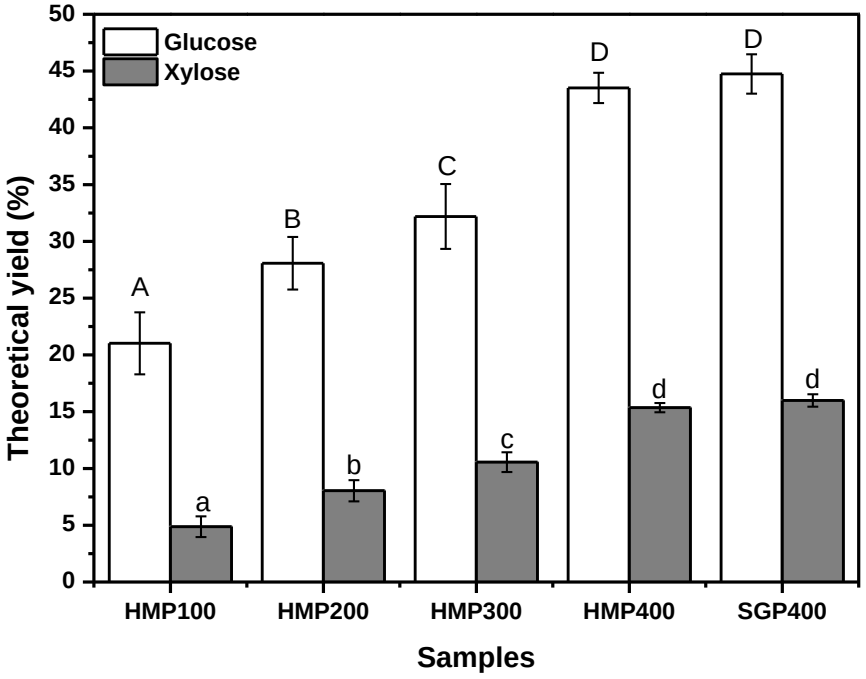

Fig. 1. Sugar yields from SCB with different particle sizes. HM and SG were obtained from hammer-milled and superfine ground SCB, respectively. Values presented in figures are means ± standard deviations from two independent runs. Different letters (capital letters for glucose and lower case letters for xylose only) indicate significant difference ($p < 0.05$).

Reducing sugar from superfine ground, steam-exploded rice straw was 2.3-fold more than that of standard steam-exploded rice straw (Jin and Chen 2006). It has also been suggested that superfine grinding is a good way to fractionate steam-exploded rice straw into easily converted parts and resistant parts.

Effect of Enzyme Cocktail on Enzymatic Hydrolysis

Lignocellulosic biomass contains components such as cellulose, xylan, lignin, and pectin. These components are organized together in a complex matrix. The existence of xylan, lignin, and pectin make the cellulose more recalcitrant to cellulase (Chundawat *et al*. 2010). The enzyme cocktail strategy may benefit the degradation of native lignocellulose. The enzymatic hydrolysis of SGP400, using individual enzymes and enzyme cocktails, was carried out and is shown in Fig. 2. Cellulase contributed much more than xylanase or pectinase in glucose production (Fig. 2A). Pectinase exhibited

xylanase activity (Fig. 2A). Approximately 3 g/L of glucose was formed using individual cellulase after 24 h. The sugar formation rate using individual enzymes was fast in the initial stage (10 h) and thereafter slowed down (Fig. 2A).

The enzymatic hydrolysis of SGP400 using the combination of two enzymes is shown in Fig. 2B. The combination of cellulase and xylanase obtained the highest glucose yield, followed by the combination of cellulase and pectinase and then the combination of xylanase and pectinase. Synergism between cellulase and accessory enzymes such as xylanase and pectinase may enhance the digestibility of the lignocellulose (Hu *et al*. 2011; Kumar and Wyman, 2009a, 2009b). The DS was calculated and is shown in Table 2. Synergism between xylanase and pectinase was the most obvious, followed by cellulase and pectinase, for both glucose and xylose, regardless of enzymatic hydrolysis time. Synergistic effect was observed in all the enzyme cocktails for glucose formation when the saccharification time was 2 h. However, the synergistic effect disappeared at the end of the saccharification (48 h). This suggested that synergism could accelerate the formation of glucose at the initial stage but did not last long. In another report (Li *et al.* 2014c), DS_g was also found to be decreased with the prolonged hydrolysis time. Considering the yield of glucose, the enzyme cocktail of cellulase, xylanase, and pectinase resulted in the highest yield, followed by the enzyme cocktail of cellulase and xylanase.

Table 2. Sugar Concentration at Different Times in Enzymatic Hydrolysates and the Degree of Synergism

Enzyme	2 h				48 h			
	Glucose (g/L)	DS_{g2}	Xylose (g/L)	DS_{x2}	Glucose (g/L)	DS_{g48}	Xylose (g/L)	DS_{x48}
C	1.31 ± 0.05	NA	0.19 ± 0.00	NA	2.93 ± 0.02	NA	0.62 ± 0.00	NA
X	N/D	NA	0.24 ± 0.01	NA	2.10 ± 0.00	NA	0.71 ± 0.10	NA
P	N/D	NA	0.04 ± 0.00	NA	N/D	NA	0.17 ± 0.00	NA
X+P	0.66 ± 0.01	∞	0.30 ± 0.06	1.07	3.24 ± 0.02	1.54	0.98 ± 0.02	1.11
C+X	1.97 ± 0.01	1.50	0.35 ± 0.01	0.81	4.26 ± 0.04	0.85	1.04 ± 0.00	0.78
C+P	1.61 ± 0.03	1.23	0.24 ± 0.01	1.04	3.50 ± 0.22	1.20	0.84 ± 0.03	1.06
C+X+P	2.24 ± 0.06	1.71	0.44 ± 0.08	0.94	4.52 ± 0.09	0.90	1.16 ± 0.02	0.77

NA: not applicable. N/D: not detected. DS_{g2}: degree of synergism for glucose at 2 h. DS_{x2}: degree of synergism for xylose at 2 h. DS_{g48}: degree of synergism for glucose at 48 h. DS_{x48}: degree of synergism for xylose at 48 h. DS>1 meant there was a synergism between the enzymes while DS<1 meant there was no synergism. Values presented in table are means ± standard deviations from two independent runs.

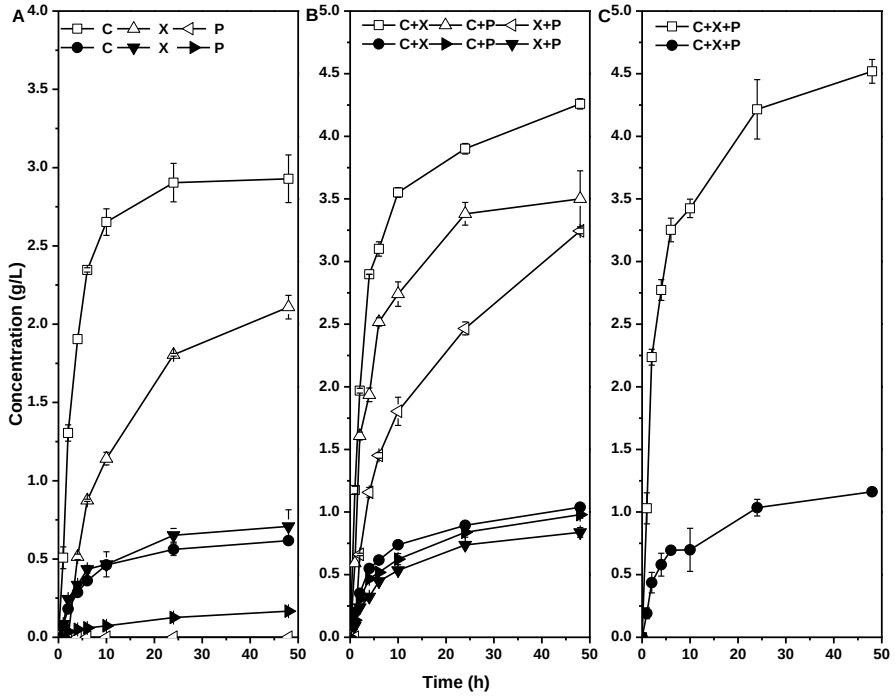

Fig. 2. Glucose (open symbols) and xylose (closed symbols) of different hydrolysates from individual enzyme (A) and different enzyme cocktails (B and C). Values presented in figures are means ± standard deviations from two independent runs.

A glucose yield of 3.5 g/L was obtained when the loading of Celluclast 1.5 L and Novozym 188 was 24 FPU and 45.8 IU/g SGP400 (Fig. 1). In contrast, a glucose yield of 4.52 g/L, equivalent to 56% of the theoretical maximum, was obtained using the enzyme cocktail of Celluclast 1.5 L, Novozym 188, xylanase, and pectinase at loadings of 10 FPU, 17.5 IU, 10 U, and 5 U/g SGP400, respectively (Fig. 2C). This suggests that a replacement of part of the cellulase could enhance the glucose yield. Our previous study also showed that replacing 20% of cellulase by xylanase could enhance the glucose yield when using NaOH, H_2O_2-pretreated, and steam exploded SCB (Li *et al.* 2014c).

Compared to the glucose yield using individual cellulase, the glucose yield using the enzyme cocktail of cellulase, xylanase, and pectinase was 1.54 times more. Xylanase improved the glucose yield at 48 h at approximately 1.45-fold, but no synergism was observed between cellulase and xylanase. On the contrary, pectinase improved the glucose yield at 48 h by only 1.19-fold but showed great synergistic effect. Monosaccharides production from SGP400 using an enzyme cocktail appeared to be very effective.

Wyman pointed out that enzyme cocktails are important for achieving the high yields needed for large-scale competitiveness because the enzyme cocktails can effectively release the remaining hemicellulose in pretreated biomass (Wyman 2007). The enzyme cocktail of cellulase, xylanase, and pectinase has been used to hydrolyze fresh, steam-exploded, and ensiled hemp. The results showed that sugar formation was significantly reduced (Zhang *et al*. 2013). Supplementation of endoxylanase and α-L-arabinofuranosidase enhances the enzymatic hydrolysis of steam-exploded wheat straw (Alvira *et al*. 2011). The chemical composition of the substrate may affect the

effectiveness of accessory enzymes such as xylanase, arabinofuranosidase, esterase, laccase, and mannanase.

Bioethanol Production using Enzyme Cocktail

Bioethanol production from SGP400 through the use of enzyme cocktails was also investigated by employing SSF and semi-SSF, and the results are shown in Figs. 3 and 4. In general, semi-SSF resulted in higher ethanol yield than SSF.

For SSF, when individual cellulase was employed, the maximum ethanol yield reached 6.12 g/L after 72 h of fermentation (Fig. 3A). Xylose formation increased with fermentation time, while glucose was not detected from the start to the end. Figure 3B shows ethanol and xylose profiles by employing cellulase and pectinase. The same as in Fig. 3A, glucose was not detected throughout the process. The ethanol yield reached 7.00 g/L, which was increased by 14.4% compared to that from individual cellulase. When the enzyme cocktail of cellulase and xylanase was employed, ethanol formation was improved further and reaching a value of 7.22 g/L (Fig. 3C). An ethanol yield of 7.30 g/L, which was 35.11% of the theoretical maximum, was obtained using the enzyme cocktail of cellulase, xylanase, and pectinase. This yeast strain could produce 82% of the theoretical maximum of ethanol when pure glucose was used as substrate (Li *et al.* 2014a). Xylanase supplementation enhanced the xylose formation greatly.

For semi-SSF, the ethanol reached its maximum value only after 12 h of fermentation. Thereafter, ethanol concentration was slightly decreased (Fig. 4). In line with the results shown in Fig. 2, prehydrolysis for 48 h at 45 °C gave rise to maximal glucose yield of 18.71 g/L and xylose yield of 7.4 g/L, which was obtained from the enzyme cocktail of cellulase, xylanase, and pectinase. When using cellulase alone, 15.90 and 5.66 g/L of glucose and xylose were obtained respectively. Xylanase supplementation enhanced the glucose and xylose yields significantly by 16.5% and 27.2%, respectively. However, supplementation of pectinase increased the glucose and xylose yields by 1.4% and 5.5%, respectively. Xylanase and pectinase supplementation increased ethanol yield by 24.5% and 11.5%, respectively. The highest ethanol yield, 7.81 g/L corresponding to 37.5% of the theoretical maximum, was obtained by using enzyme cocktail of cellulase, xylanase, and pectinase. The results clearly showed that supplementation of accessory enzymes could improve the ethanol yield by both fermentation modes.

Comparing the above-mentioned fermentation modes, semi-SSF was better than SSF for higher ethanol production and shorter time consumed. The glucose yield reached 56% of the theoretical maximum during enzymatic hydrolysis using the enzyme cocktail of cellulase, xylanase, and pectinase (Fig. 2). In the semi-SSF, the glucose yield reached 41.6% of the theoretical maximum (Fig. 4D). The possible reason is that higher initial solid loading could result in lower enzymatic digestibility (Kristensen *et al.* 2009).

An ethanol yield of 6.61 g/L was obtained from un-detoxified steam-exploded SCB with cellulase and solid loadings of 10 FPU/g substrate and 5%, respectively (Li *et al.* 2014b). Compared with those results, the final ethanol concentration in the present work was higher. However, the solid loading in this work was two times more than our previous one. It should be noted that ethanol yield could not be increased linearly with the solid loading by using un-detoxified steam-exploded SCB because inhibitors accumulation could depress cellulase and yeast. In another study (Li *et al.* 2014a), the jet milled SCB was used as feedstock to produce ethanol by a newly built fermentation mode named one-pot SSF. In that study, 0.5% of NaOH solution was used to pretreat SGP400

at 121 °C for 1 h. H_3PO_4 was adopted to neutralize the whole slurry followed by cellulase and nitrogen source injection and inoculum.

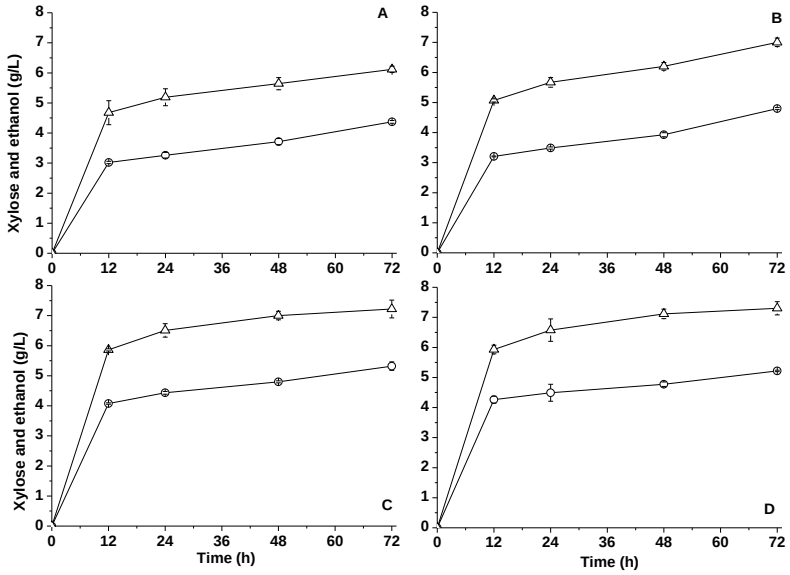

Fig. 3. Time course of simultaneous saccharification and fermentation with *S. cerevisiae* As 2.489. A: cellulase. B: cellulase plus pectinase. C: cellulase plus xylanase. D: cellulase, pectinase, plus xylanase. ∆ indicates ethanol and ○ indicates xylose. The inoculum size was 1 g DCW/L and the solid loading was 10% in all the runs. Values presented in figures are means ± standard deviations from two independent runs.

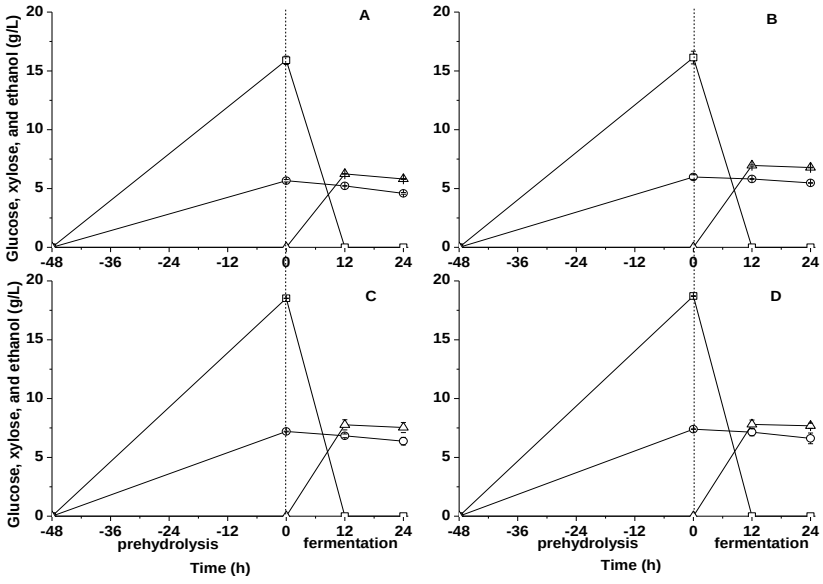

Fig. 4. Time course of semi-simultaneous saccharification and fermentation with *S. cerevisiae* As 2.489. A: cellulase. B: cellulase plus pectinase. C: cellulase plus xylanase. D: cellulase, pectinase, plus xylanase. □ indicates glucose, ∆ indicates ethanol, and ○ indicates xylose. The inoculum size was 1 g DCW/L and the solid loading was 10% in all the runs. Values presented in figures are means ± standard deviations from two independent runs.

The final ethanol reached 12.64 g/L, which corresponds to 60.73% of the theoretical maximum without the aid of other accessory enzymes. This suggests that chemical pretreatment is still necessary for higher convertibility. But with the aid of the superfine grinding, chemical pretreatment conditions could be mild, which results in lower generation of inhibitors. The combination of superfine grinding with a mild chemical pretreatment is promising since a new fermentation configuration, one-pot SSF (Li *et al*. 2014a), has been built.

CONCLUSIONS

1. A positive relationship was observed between size reduction and saccharification efficiency.
2. Enzyme cocktails improved the sugar yield further. Xylanase was more important than pectinase. Supplementation of xylanase and pectinase increased the glucose yield by 54%.
3. Semi-SSF was found to be better than SSF for ethanol production from SGP400 by using the enzyme cocktail. The enzyme cocktail increased the ethanol yield by 19.4% and 25.1% for SSF and semi-SSF, respectively, compared with that from cellulase alone.
4. Superfine grinding could be merged with mild chemical pretreatment to establish integrated processes for cellulosic ethanol production.

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