

## Ethanol Production from High Solids Loading of Alkali-Pretreated Sugarcane Bagasse with an SSF Process

Yueshu Gao,<sup>a,b</sup> Jingliang Xu,<sup>a</sup> Zhenhong Yuan,<sup>a,\*</sup> Yu Zhang,<sup>a</sup> Cuiyi Liang,<sup>a</sup> and Yunyun Liu<sup>a</sup>

A fed-batch process and high-temperature simultaneous saccharification and fermentation (SSF) process were investigated to obtain high sugar yield and ethanol concentration. Different amounts of alkali-pretreated sugarcane bagasse were added during the first 24 h. For the highest final dry matter (DM) content of 25% (w/v), a maximal glucose and total sugar concentration of 79.53 g/L and 135.39 g/L, respectively, were achieved with 8.3 FPU/g substrate after 120 h of hydrolysis. Based on the hydrolysis experiment, two processes for ethanol production from sugarcane bagasse, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF), were also compared using *S. cerevisiae*. The results indicated that ethanol concentration and yield in the SHF were higher, while ethanol productivity (gram per unit volume and over time) was lower. For 25% substrate loading, the ethanol productivity and ethanol concentration could reach 0.38 g·L<sup>-1</sup>·h<sup>-1</sup> and 36.25 g/L SSF in 96 h, respectively, while that of SHF could reach 0.32 g·L<sup>-1</sup>·h<sup>-1</sup>, with an ethanol concentration of 47.95 g/L in 152 h for SHF. When high-temperature simultaneous saccharification and fermentation (SSF) process was performed by using *Kluyveromyces marxianus* NCYC 587 at 42 °C, 42.21 g/L ethanol (with an ethanol productivity of 0.44 g·L<sup>-1</sup>·h<sup>-1</sup>) was produced with 25% dry matter content and 8.3 FPU cellulase/g substrate, which meant 16.4% more ethanol when compared with SSF of *S. cerevisiae*.

**Keywords:** Sugarcane bagasse; Fed-batch; High temperature simultaneous saccharification and fermentation; Separate hydrolysis and fermentation; Ethanol

**Contact information:** a: Guangzhou Institute of Energy Conversion, Key Laboratory of Renewable Energy and Gas Hydrate, Chinese Academy of Sciences, Guangzhou, 510640 China; b: University of Chinese Academy of Sciences, Beijing, 100039 China; \*Corresponding author: yuanzh@ms.giec.ac.cn

### INTRODUCTION

Because of its potential to help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply, the development of alternative energy sources has attracted wide attention (Goldemberg 2007). The biological conversion of various lignocellulosic feedstocks dedicated to ethanol has potential, but its development is still hampered by economic and technical obstacles (Sanchez and Cardona 2008). Because of the complex chemical structure of the lignocellulosic materials, the direct enzymatic digestion of cellulose is hindered. Therefore, pretreatment is an essential step in obtaining potential sugars in the hydrolysis step. A thermo-chemical pretreatment such as dilute sulfuric acid, steam explosion, or hot-water pretreatment can improve the enzymatic digestibility of lignocellulosic biomass to some extent (Kaar *et al.* 1998; Schell *et al.* 2003; Yu *et al.* 2012). However, these pretreatment methods, which involve a high process temperatures,

require higher energy input and heat-resistant/anti-corrosive pressure cooking equipment. Excessive exposure to high temperatures may also exacerbate the degradation of the useful components and increase the formation of fermentation inhibitors (Wu *et al.* 2011). Preliminary studies show that the lignin present in rice straw, bagasse, and certain grasses was effectively removed after being treated with sodium hydroxide solution at moderate temperatures for a short period of time. Accordingly, the alkali pretreatment was developed to overcome the resistance of lignocellulose to enzymatic hydrolysis, ensuring less energy consumption, and to improve the efficiency of cellulose-to-ethanol conversion.

Ethanol concentration has a major effect on energy demand for subsequent distillation, especially below 4 wt.% (Zacchi and Axelsson 1989). One way to improve ethanol concentration is to increase the concentration of fermentable sugars by increasing substrate loading (Varga *et al.* 2004). However, the high content of lignocellulosic substrate might cause poor mass transfer and high viscosity, which make mixing difficult, lower heat transfer efficiency, and heighten the power consumption in stirred tank reactors (Fan *et al.* 2003; Jørgensen *et al.* 2007b). To decrease these negative effects and maximize end-product concentration, fed-batch hydrolysis has been proposed as a feasible method to improve ethanol production in terms of ethanol concentration as well as product yield (Varga *et al.* 2004; Rudolf *et al.* 2005; Laopaiboon *et al.* 2007).

One option in performing enzymatic hydrolysis and fermentation is separate hydrolysis and fermentation (SHF), which is preceded by various bioconversion conditions. Another option is simultaneous saccharification and fermentation (SSF), in which glucose can be rapidly converted to ethanol by yeast. SSF minimizes the end-product's inhibition to cellulose and contributes to lignocellulosic biomass hydrolysis. This reduces production capital cost and the risk of contamination. A drawback of this option is that each stage has a different optimum pH and temperature (Jørgensen *et al.* 2007a). The optimum temperature for enzymatic hydrolysis (about 50 °C) is higher than that of yeast fermentation, which leads to decreased efficiencies of both hydrolysis and fermentation. Cantarella *et al.* (2004) and Öhgren *et al.* (2007) reported comparisons between SSF and SHF that indicated that the SSF process could lead to significantly increased ethanol productivity and even decrease the negative effect of inhibitors.

Sugarcane bagasse, the main by-product of the sugar industry, contains high amounts of carbohydrates but relatively low lignin levels and could be a particularly appropriate substrate for bioconversion to ethanol (Pandey *et al.* 2000). It was the only substrate used in this study. This work investigated high bioethanol production from high solid loading of alkali-pretreated SCB with a fed-batch SSF process. In addition, an SHF process was compared to SSF in terms of ethanol concentration and productivity using the conventional yeast *Saccharomyces cerevisiae*. To overcome the lower efficiency of enzymatic hydrolysis and further improve ethanol production, the thermo-tolerant yeast *Kluyveromyces marxianus* was employed in SSF.

## EXPERIMENTAL

### Raw Materials

Sugarcane bagasse was provided by Guangxi FengHao Group Co. Ltd. (Chongzuo, China). The fraction between 20 and 80 mesh was screened and collected for hydrolysis experiments.

## Enzyme

Cellulase, mixed with small quantities of other enzymes such as xylanase, was produced from *Penicillium* sp. and obtained from Imperial Jade Bio-technology Co. Ltd. (Yinchuan, China). The filter paper activity (FPA) was 214 FPU/g powder, measured by the method of the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987).

## Alkali Pretreatment

Oven-dried sugar bagasse was placed in a Pyrex glass laboratory bottle and mixed with 0.5 M NaOH at a ratio of 1 g of original raw material to 20 mL of liquid. The slurry was incubated in a water bath at 80 °C for 2 h with agitation. After pretreatment, the solids were separated with a filter and washed with tap water until they achieved a neutral pH. The solids residue was dried in a forced-air oven at 105 °C and kept in desiccation for the subsequent chemical analysis and experiment.

## Enzymatic Hydrolysis

The fed-batch-based enzymatic hydrolysis was conducted at 50 °C with an initial solids loading of 10% (w/v) dry mass (DM) and run for up to 120 h in 250-mL Erlenmeyer flasks at 150 rpm. Each flask contained 100 mL of 0.05 M sodium citrate buffer (pH 5.0). The reaction solution was appended with additional substrates by 8% (w/v) at 12 h and 7% (w/v) at 24 h to increase the substrate loading to 18% and 25% (w/v) DM, respectively. The enzyme loading was 8.3 FPU per g of substrate. To maintain constant enzyme loading, an extra enzyme was added along with a corresponding amount of extra substrate. Samples for sugar analysis were detected at different times (specified in the text) and analyzed with high-performance liquid chromatography (HPLC). Each value is an average of three parallel replicates. The enzymatic hydrolysis rate was calculated as the ratio of glucose in the enzymatic hydrolysis per 100 g of potential glucose in the substrate. The total sugar was calculated as the sum of glucose, cellubiose, and xylose.

## Microorganism and Growth Conditions

The thermo-tolerant yeast used in high-temperature SSF was *K. marxianus* NCYC 587, obtained from the National Collection of Yeast Cultures (UK). *Saccharomyces cerevisiae* NRRL Y-2034, used in SHF and low-temperature SSF, was bought from the National Center for Agricultural Utilization Research (Peoria, IL, USA). Both active cultures for inoculation were obtained from 150-mL Erlenmeyer flasks with 50 mL of growth medium containing glucose, peptone, and yeast extract at concentrations of 20 g/L, 20 g/L, and 10 g/L, respectively. The growth of *K. marxianus* NCYC 587 was carried out on a rotatory shaker at 150 rpm, 42 °C for 12 h, while *S. cerevisiae* NRRL Y-2034 was incubated at 30 °C. At the end of incubation, the contents of these tubes were aseptically centrifuged and used for fermentation. The amount of inoculum added was 0.2 g/L (dry weight/volume).

## Fermentation Process

In the SHF experiments, 10% (v/v) *S. cerevisiae* Y-2034 was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of enzymatic hydrolysate and 2 g/L yeast extract, 5 g/L  $\text{KH}_2\text{PO}_4$ , 2 g/L  $(\text{NH}_4)_2\text{SO}_4$ , and 0.4 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . All of the fermentations were performed at 30 °C, 150 rpm for 48 h, and the pH was initially

adjusted to 5.0 with HAc-NaAc buffer. Samples were taken at 0, 8, 20, 32, and 44 h for ethanol and sugars analysis. In the SSF experiments, 10% (w/v) alkali-pretreated SCB was pre-hydrolyzed for 24 h in a batch process, while 18% and 25% alkali-pretreated SCB were pre-hydrolyzed for 36 h and 48 h, respectively, in a fed-batch process described in the Enzymatic Hydrolysis section. All of the pre-hydrolysis was conducted under the following conditions: pH 5.0, 50 °C, and 150 rpm. The three different concentrations of alkali-pretreated SCB were pre-hydrolyzed with two copies. One copy was used for fermentation at 30 °C, and the other was used at 42 °C. After pre-hydrolysis, both copies were returned to 30 °C for fermentation of hydrolysates with *S. cerevisiae* Y-2034 and 42 °C for fermentation of hydrolysates with *K. marxianus* NCYC 587. Prehydrolysis and fermentation took 120 h. All the Erlenmeyer flasks were sealed with rubber plugs. Samples were collected at 0, 12, 24, 48, 72, 96, and 120 h for ethanol and sugars analysis.

### Analytical Methods

The components of sugarcane bagasse before and after pretreatment were determined according to the standardized methods of the National Renewable Energy Laboratory (NREL, Golden, CO, USA) (Sluiter *et al.* 2004). Hydrolyzed and fermented samples for sugar and ethanol analysis were taken at different times (specified in the text) and centrifuged at 12,000 rpm for 2 min. Sugar concentrations were measured by high-performance liquid chromatography (HPLC, Waters 2695), using a Shodex sugar SH-1011 column coupled with refractive index detector RI 2414. The mobile phase was 5 mmol/L H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.5 mL/min. The analysis was performed with a column temperature of 50 °C. The yield of sugars in the hydrolyzate was calculated based on the amount of sugar polymers in the treated solids. The concentration of total sugar was calculated as the sum of glucose, cellubiose, xylose, and arabinose concentrations. Ethanol concentrations were determined using an Agilent HP 6820 gas chromatograph, with a capillary column (30.0 m×0.25 mm×0.25 μm) and a flame ionization detector (GC-FID, 250 °C). The working conditions were as follows: injector at 250 °C, nitrogen as the carrier gas at a flow rate of 30 mL/min, and a split ratio of 1:50.

## RESULTS AND DISCUSSION

### Compositional Changes Before and After Pretreatment

Dilute NaOH treatment of lignocellulosic biomass causes swelling, which leads to an increase in the internal surface area, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan *et al.* 1987). Lignin acts as a physical barrier, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple 2000), and reducing cellulose hydrolysis by non-productively binding cellulolytic enzymes (Esteghlalian *et al.* 2001). Therefore, the removal of lignin is conducive to enzymatic hydrolysis. Silverstein *et al.* (2007) reported more than 65% lignin reduction in cotton stalk treated with 2% NaOH for 90 min at 394 K with 103.4 kPa. In this study, about 71.86% lignin was removed, and was 28.1% xylan (Table 1). The removal of xylan could increase the mean pore size of the substrate and therefore increase accessibility of the cellulose and its probability of becoming hydrolyzed (Chandra *et al.* 2007). The total sugar and glucan recovery reached 83.6% and 89.6%, respectively.

**Table 1.** Chemical Compositions of Sugarcane Bagasse Before and After Alkali Pretreatment

Condition	Solid remaining (%)	Pretreatment <sup>a</sup>						Total sugar recovery
		Glucan (%)		Xylan (%)		Lignin (%)		
		Content	Recovery <sup>b</sup>	Content	Removal	Content	Delignification	
Untreated SCB	100.00	41.95	—	21.70	—	24.11	—	—
NaOH	61.39	61.24	89.62	25.42	28.09	11.05	71.86	83.54

<sup>a</sup> Based on the initial amount of glucan, xylan, or lignin in the untreated biomass  
<sup>b</sup> Only the residue was considered.

It can be concluded that alkali pretreatment was an effective method to remove lignin and retain the majority of cellulose, which enabled the subsequent enzymatic saccharification at high cellulose content to obtain high sugar concentration.

### Comparison of Fed-Batch Hydrolysis with Different Solid Loadings

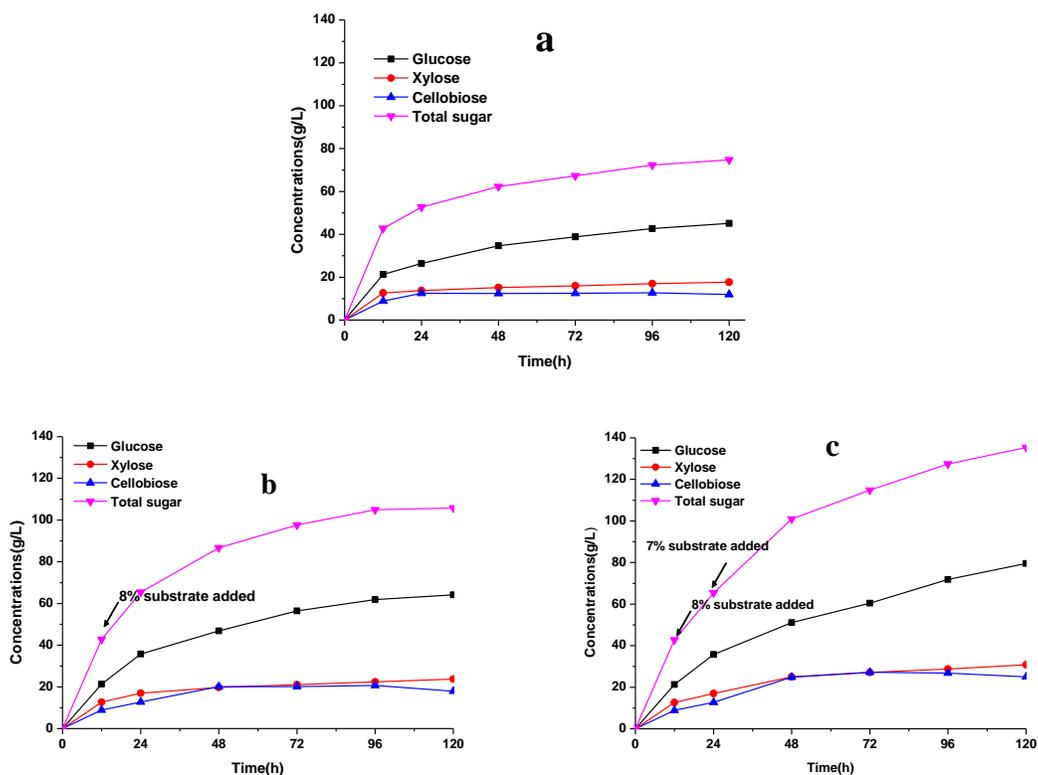
High-solids hydrolysis is a direct and convenient technique for producing high concentrations of sugars. Unfortunately, operating the hydrolysis at solids concentrations above 10% to 15% (w/w) poses technical problems (Jørgensen *et al.* 2007b). In fact, it proved difficult to perform the enzymatic hydrolysis process for batch experiments of 18% and 25% solid loadings in this study. Several groups have used a fed-batch strategy to hydrolyze material with solids concentrations up to 17% (Rudolf *et al.* 2005). In this experiment, three mode experiments were set: an “a” batch process with 10% solids loading; a “b” fed-batch process initiated at 10%, with 8% of fresh substrates fed at 12 h to get a final solids loading of 18%; and a “c” fed-batch process, initiated at 10%, with 8% and 7% of fresh substrates fed twice at 12 and 24 h, respectively, to get a final solids loading of 25%. The detailed operating conditions and results are shown in Table 2.

**Table 2.** Sugars Yield from Batch and Fed-Batch Mode

Run No.	Adding DM (g)			Final DM (%)	Enzyme loading (FPU/g)	Y <sub>xylan</sub> (%)	Y <sub>glucan</sub> (%)
	0 h	12 h	24h				
a	10	—	—	10	8.3	61.26	83.03
b	10	8	—	18	8.3	45.60	66.30
c	10	8	7	25	8.3	42.70	60.73

Figure 1 shows the produced sugar concentration *versus* time in batch and fed-batch mode with final solids loadings of 10%, 18%, and 25%. It can be seen that at the same cellulase loading, higher solids concentration led to higher sugar concentration but lower glucan conversion efficiency (Table 2). About 90% of total glucose was released within the first 96 h, and the total enzymatic hydrolysis process time needed was shorter compared to other groups' reports with high solids levels (*e.g.*, Öhgren *et al.* 2006). A similar tendency was also found in the batch mode with 10% and the fed-batch mode with 18% fed once after 8 h. After 72 h, the batch process with 10% solids loading produced a total sugar content of 67.31 g/L, while the batch processes with 18% and 25% solids loadings yielded 97.61 g/L and 114.82 g/L, respectively. Reducing sugar produced from 18% solids loading was 30.29 g/L more than that from 10% solids loading, but

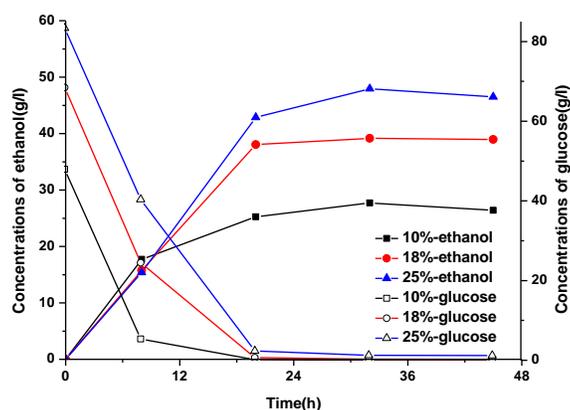
17.21 g/L less than that from 25% solids loading. This demonstrated that the increasing amount of reducing sugar was comparatively reduced with fresh substrate added, which may be caused by the relatively high amount of lignin hindering the accessibility of the enzyme to the substrate (Song *et al.* 2011). Hindrance of enzyme diffusion caused by the lack of free water at higher solids concentrations has also been suggested as a cause for the lower conversion rate (Ingesson *et al.* 2001). High sugar concentration and higher concentration of other inhibitors may impair enzyme activity as well. Finally, the total sugar, cellobiose, glucose, and xylose concentrations of 135.39 g/L, 25.02 g/L, 79.83 g/L, and 30.83 g/L were achieved at 25% final solids loading with 8.3 FPU/g substrate loadings, respectively, after 120 h of enzymatic hydrolysis.



**Fig. 1.** Sugar concentration change curve with batch and fed-batch hydrolysis; "a" has 10% solids added at 0 h; "b" has 10% solids at 0 h and 8% solids added at 12 h; and "c" has 10% solids at 0 h, 8% solids at 12 h, and another 7% solids added at 24 h.

### Batch and Fed-Batch SHF with *S. cerevisiae* Y-2034

To clarify the fermentability of different hydrolyzates obtained from batch and fed-batch hydrolysis, ethanol fermentation experiments were performed with *S. cerevisiae* Y-2034. As shown in Fig. 2, the three ethanol curves in the fermentation process all varied with glucose consumption. At the beginning of fermentation, glucose concentrations decreased rapidly, while ethanol concentrations increased almost linearly. Glucose concentrations stayed below 1 g/L, and ethanol concentrations showed almost no change. Generally, for lignocellulosic ethanol production, the ethanol concentration in broth should be higher than 40 g/L (4%, w/w), which can reduce the cost of industrial-scale distillation greatly (Varga *et al.* 2004).

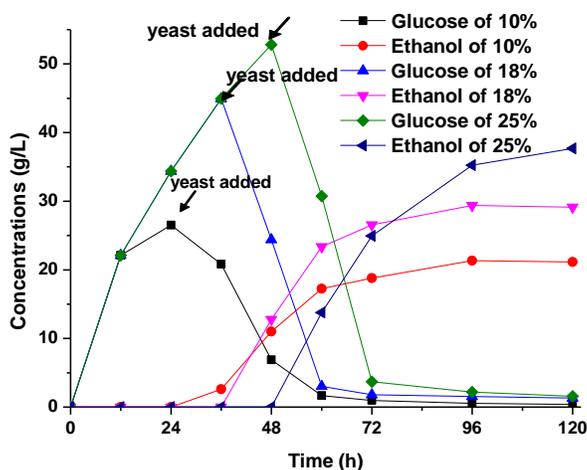


**Fig. 2.** Ethanol and glucose concentration change during the SHF process for *S. cerevisiae*

With the increase of the final substrate loading, more glucose was obtained, which produced a higher concentration of ethanol but a lower percentage of theoretical ethanol yield. However, the times the three groups needed to reach their highest respective ethanol concentrations were equivalent, which implies that the fermentation of Y-2034 is efficient. The highest ethanol concentrations produced from the final substrate loadings of 10%, 18%, and 25% achieved in this work were 27.71 g/L, 39.15 g/L, and 47.95 g/L, respectively, corresponding to overall theoretical ethanol yields (based on the glucan present in the pretreated sugarcane bagasse) of 79.85%, 62.97%, and 55.27%, respectively. This implied that 25% final substrate loading could exceed the benchmark for an economically viable distillation (4% w/v), which could decrease the cost of ethanol separation.

### Batch and Fed-Batch Hybrid SSF with *S. cerevisiae* Y-2034

Figure 3 shows that the glucose and ethanol yield varied with different substrate loadings. It also shows that the glucose concentrations during the batch and fed-batch fermentations decreased rapidly in the first 24 h after yeast was added, after which they decreased slowly.



**Fig. 3.** Ethanol and glucose concentration change during the SSF process for *S. cerevisiae*. When yeasts were added, the temperature dropped from 50 to 30 °C.

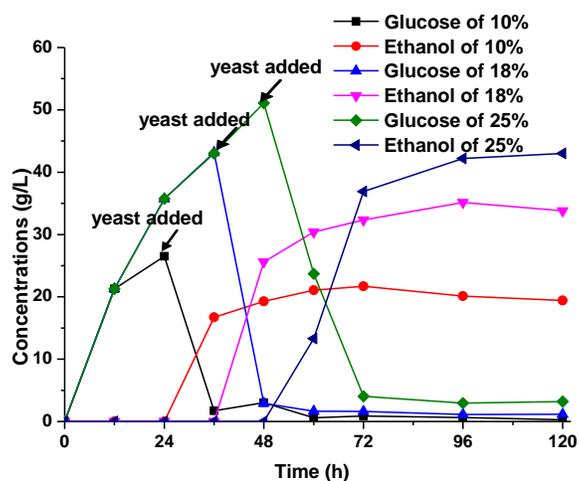
Simultaneously, ethanol concentrations during the batch and fed-batch fermentations increased rapidly at first and then gradually slowed down. The time needed to obtain maximum ethanol concentration was extended with increasing final dry matter concentrations. This was caused by the extension of pre-hydrolysis time, which was needed to balance hydrolysis and fermentation rates. In these three experiments, ethanol concentration reached its maximum at 48 h of fermentation and then decreased slightly afterward. This may be because yeast made use of ethanol as a carbon source when the hydrolysate was low and fermentable sugars were deficient. From a process-economy aspect, it may be desirable to further increase the residence time.

A comparison of SHF and SSF indicated that the bioprocesses were completed in approximately 96 h with SSF and 152 h with SHF. Table 3 shows the ethanol concentrations and productivities for these three different substrate loadings. In the SHF process, significantly higher ethanol concentrations could be obtained. This is why the overall process time for the SHF was much longer, even though the ethanol productivities were lower. For SSF, it was possible for the 25% substrate loading to produce 36.25 g/L ethanol, corresponding to an ethanol yield of 0.19 g/g and volumetric productivity of 0.38 gL<sup>-1</sup>h<sup>-1</sup>; for SHF, this loading produced 47.95 g/L ethanol, which produced an ethanol yield of 0.25 g/g and volumetric productivity of 0.32 gL<sup>-1</sup>h<sup>-1</sup>.

Most reports of SSF processing have considered that the rate of enzymatic hydrolysis was the limiting step compared with the fermentation of yeast (Rudolf *et al.* 2005; Sassner *et al.* 2006, 2008). Simultaneously, temperature was shown to have a significant effect on the enzyme activity and the rate of hydrolysis (Olofsson *et al.* 2008). As shown in Fig. 5, when the *S. cerevisiae* was fed into the hydrolysate and the temperature dropped from 50 to 30 °C, the enzyme activity sharply decreased, which took only 25% of the activity at 50 °C. Compared with the SHF process, by performing the enzymatic hydrolysis simultaneously with the fermentation, end-products of the hydrolysis, which inhibited the cellulase, could be continuously removed; this thereby promoted the hydrolysis process. On the other hand, SSF experiments were run at 30 °C, and lower enzyme activity would lead to a weakened enzyme hydrolysis. Under the impacts of two factors, the ethanol concentration of SSF was finally lower than that of SHF by *S. cerevisiae*.

### Batch and Fed-Batch Hybrid SSF with *K. marxianus* NCYC 587

To further improve ethanol production efficiency, the present study employed the thermo-tolerant yeast *K. marxianus* NCYC 587, which can be cultured at 47 °C, close to the optimum for cellulase hydrolysis (*i.e.*, 50 °C). Though a temperature of 50 °C was found to be optimal, the range of 40 to 50 °C did not affect the yield significantly (Hari Krishna *et al.* 2001); hence, the SSF temperature chosen was 42 °C. Figure 4 shows that the glucose varied at different conditions. It shows that in the 10% and 18% substrate loadings, the glucose concentrations during the batch and fed-batch fermentations decreased rapidly in the first 12 h after yeast was added, and the concentration was less than 1.0 g/L after 48 h. However, as for the 25% substrate loading, only 50% glucose (based on the concentration at 48 h) was consumed from 48 to 60 h. For 10% and 18% substrate loadings, the concentration of cellulose also decreased rapidly, but approximately 1.85 g/L and 4.09 g/L remained, respectively. For 25% substrate loading, the consumption rate of cellulose was slower than it was with the former loadings, and more cellulose (7.56 g/L) remained. Increased substrate loading results in high ethanol concentrations but low ethanol yields.



**Fig. 4.** Ethanol and glucose concentration changed during the SSF process with *K. marxianus* NCYC587. When yeasts were added, the temperature dropped from 50 to 42 °C.

Many researchers have reported the conversion rates (up to 2% w/v ethanol) in 4 to 6 days (Deshpande *et al.* 1983; Saddler *et al.* 1982; Spangler and Emert 1986; Takagi *et al.* 1977). Ballesteros *et al.* (2004) obtained 1.81% ethanol concentration under the condition of 10% w/v, 42 °C, and shake flask operation using wheat straw after a steam pretreatment as substrate. The present experiment achieved ethanol yields of 2.17% (w/v) in 72 h for 10% substrate loading, 3.50% (w/v) in 96 h for 18% substrate loading, and 4.22% (w/v) in 96 h for 25% substrate loading. After an extended time, the hydrolysis process continued to release glucose, but the stability of the yeast cells subjected to high temperature was gradually lost. Thus, the glucose was not used up, but became gradually increased in the final stage.

Though much work on fed-batch simultaneous saccharification and fermentation (SSF) process has been published, there has been little literature in which thermo-tolerant yeast (above 40 °C) was applied into high solids ( $\geq 20\%$ ) ethanol fermentation in SSF process. However, when the solid loading was below 20%, some other thermo-tolerant strains had been used as well. As shown in Table 3, the other two thermo-tolerant yeasts were cited in order to compare with *K. marxianus* NCYC587; these yeasts were both applied into the SSF process under 42 °C. The solid loadings, enzyme dosages, and ethanol concentrations are also presented in Table 3. There was no significant difference in the solid and enzyme loadings among them. *S. cerevisiae* BY4742 gave the least ethanol concentration, though after optimization, the ethanol concentration could get to 37.84 g/L while simultaneously the enzyme loading had to be increased to 30 FPU/g solid, which would greatly increase the production cost (Zhang *et al.* 2013). In comparison with these two reports, the *K. marxianus* NCYC587 showed the best performance in terms of ethanol concentration.

A comparison between SSF with the conventional yeast, *S. cerevisiae*, and the thermo-tolerant yeast, *K. marxianus*, indicated that the consumption of glucose for *K. marxianus* NCYC 587 under this condition was faster than it was for *S. cerevisiae* Y-2034. Table 4 shows that the ethanol concentrations and productivities of *K. marxianus* were higher than those of *S. cerevisiae*. It was possible for the 25% substrate loading to produce 36.25 g/L ethanol, which translated to an ethanol yield of 0.19 g/g and volumetric productivity of 0.38 gL<sup>-1</sup>h<sup>-1</sup> for *S. cerevisiae*. However, the batch with *K.*

*marxianus* NCYC 587, 42.21 g/L ethanol was produced, which translates to an ethanol yield of 0.22 g/g and volumetric productivity of 0.44 gL<sup>-1</sup>h<sup>-1</sup>. To a certain extent, the use of *K. marxianus* NCYC 587 significantly alleviated the conflict of the different optimum temperatures for the enzymatic hydrolysis and yeast fermentation in SSF.

**Table 3** Comparison of Fermentability among Three Thermotolerant Strains under 42 °C

Strains	Substrate	Solid loading	Enzyme loading(FPU/g solid)	Ethanol concentration(g/L)
<i>S. cerevisiae</i> BY4742 (Zhang <i>et al.</i> 2013)	NaOH pretreated wheat straw	16%	10	25.26 <sup>a</sup>
<i>K. marxianus</i> CECT10875 (García-Aparicio <i>et al.</i> 2011)	Stream exploded pretreated barely straw	15%	7.0 <sup>b</sup>	29.4
<i>K. marxianus</i> NCYC587	NaOH pretreated sugarcane bagasse	18%	8.3	35.14

a: The value was calculated according to the literature;

b: Extra xylanase and β-glucosidase were added.

**Table 4.** Ethanol Concentration and Productivity from Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) Results

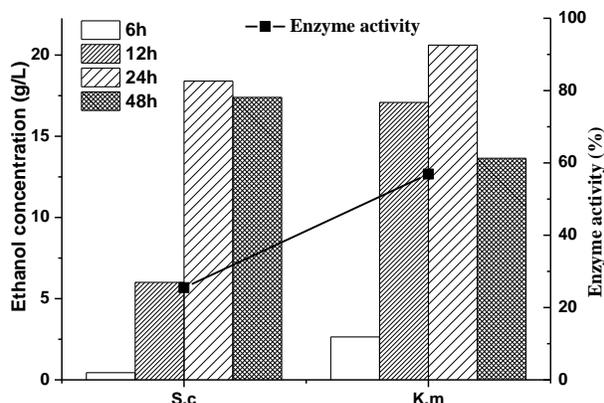
Conditions	10%		18%		25%	
	Maximum [ethanol] (g/L)	Productivity (g/L/h)	Maximum [ethanol] (g/L)	Productivity (g/L/h)	Maximum [ethanol] (g/L)	Productivity (g/L/h)
SHF of <i>S.c</i>	27.71	0.18	39.15	0.26	47.95	0.32
SSF of <i>S.c</i>	21.33	0.22	29.39	0.31	36.25	0.38
SSF of <i>K.m</i>	21.70	0.30	35.14	0.37	42.21	0.44

**Table 5.** Ethanol Yields from Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)

Conditions	Yield (g ethanol/g cellulose)		
	10%	18%	25%
SHF of <i>S. c</i>	0.36	0.29	0.25
SSF of <i>S. c</i>	0.28	0.22	0.19
SSF of <i>K. m</i>	0.29	0.26	0.22

There are many factors that affect the ethanol yield in SSF, such as the pretreatment condition, enzyme dosage, substrate loading, temperature, and yeast strain (Olofsson *et al.* 2008). The main reasons for the result of higher ethanol yields of *K. marxianus* compared to *S. cerevisiae* were the different reaction temperatures and yeast strains, since other three factors were identical. Firstly, the fermentabilities of two yeasts were compared by the fermentations from pure glucose solution with a concentration of 50 g/L to ethanol, and other ingredients were added as mentioned in the section “Fermentation process”. Figure 5 presents the ethanol concentrations of two yeasts at 6 h,

12 h, 24 h, 48 h, as well as the enzyme activities under 30 °C and 42 °C. It could be shown that *K. marxianus* could produce more ethanol than *S. cerevisiae* with the equivalent glucose. Moreover, compared with enzyme activity at 30 °C, the enzyme activity at 42 °C was higher with 56.88% of 50 °C. Therefore it can be inferred that more glucose could be released and then fermented to ethanol at 50 °C compared to 30 °C. Given the advantages of increased ethanol productivity, reduced capital cost, and so on, high-temperature simultaneous saccharification and fermentation (SSF) process with *K. marxianus* was still more favored.



**Fig. 5.** Comparison of fermentability between *S. cerevisiae* and *K. marxianus* and enzyme activity under different temperatures

## CONCLUSIONS

1. To obtain high-concentration fermentable sugars, batch and fed-batch processes were conducted with enzymatic hydrolysis of alkali-pretreated sugarcane bagasse. For the highest final dry matter (DM) content of 25% (w/v), a maximal total sugar concentration of 135.39 g/L (including 79.53 g/L glucose, 30.83 g/L xylose, and other sugars) was obtained after 120 h of hydrolysis with an enzyme loading of 8.33 FPU/(g substrate).
2. Comparison between SHF and SSF using *S. cerevisiae* Y-2034 demonstrated the ethanol productivity (gram per unit volume and time) in the SSF was higher, but the ethanol concentration was lower.
3. To further improve ethanol production and decrease cost, the thermo-tolerant yeast *K. marxianus* NCYC587 was employed. After 96 h of enzymatic hydrolysis and fermentation, ethanol concentration increased to 42.21 g/L and ethanol productivity increased up to 0.44 g·L<sup>-1</sup>·h<sup>-1</sup>. This indicated that the SSF process with *K. marxianus* could alleviate the temperature conflict between enzymatic hydrolysis and fermentation process and increase ethanol concentration and productivity.

## ACKNOWLEDGEMENTS

This work was funded by the National Key Technology R&D Program (2011BAD22B01), the National High-tech R&D Program (2013AA065803), the Program of the National Natural Science Foundation of China (21176237, 21211140237), and the Cooperation Project between the Chinese Academy of Sciences and Guangxi Academy of Sciences and Science & Technology Project of Guangzhou (2013J4300026).

## REFERENCES CITED

- Ballesteros, M., Oliva, J., Negro, M., Manzanares, P., and Ballesteros, I. (2004). "Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875," *Process Biochem.* 39(12), 1843-1848.
- Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F. (2004). "Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF," *Process Biochem.* 39(11), 1533-1542.
- Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., and Saddler, J. N. (2007). "Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?" *Biofuels*, 108, 67-93.
- Chang, V. S., and Holtzapple, M. T. (2000). "Fundamental factors affecting biomass enzymatic reactivity," *Appl. Biochem. Biotechnol.* 84-6, 5-37.
- Deshpande, V., Sivaraman, H., and Rao, M. (1983). "Simultaneous saccharification and fermentation of cellulose to ethanol using penicillium funiculosum cellulase and free or immobilized saccharomyces uvarum cells," *Biotechnol. Bioeng.* 25(6), 1679-1684.
- Esteghlalian, A. R., Srivastava, V., Gilkes, N., Gregg, D. J., and Saddler, J. N. (2001). "An overview of factors influencing the enzymatic hydrolysis of lignocellulosic feedstocks," *ACS Publications*, 769, 100-111.
- Fan, L., Gharpuray, M., and Lee, Y. (1987). *Biotechnology Monographs, Volume 3: Cellulose Hydrolysis*, Springer-Verlag, London.
- Fan, Z., South, C., Lyford, K., Munsie, J., van Walsum, P., and Lynd, L. R. (2003). "Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor," *Bioprocess Biosyst. Eng.* 26(2), 93-101.
- García-Aparicio, M. P., Oliva, J. M., Manzanares, P., Ballesteros, M., Ballesteros, I., González, A., Negro, M. J. (2011). "Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces Marxianus* CECT10875," *Fuel* 90, 1624-1630.
- Ghose, T. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268.
- Goldemberg, J. (2007). "Ethanol for a sustainable energy future," *Science* 315(5813), 808-810.
- Ingesson, H., Zacchi, G., Yang, B., Esteghlalian, A. R., and Saddler, J. N. (2001). "The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose," *J. Biotechnol.* 88(2), 177-182.

- Jørgensen, H., Kristensen, J. B., and Felby, C. (2007a). "Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities," *Biofuels, Bioprod. Biorefin.* 1(2), 119-134.
- Jørgensen, H., Vibe-Pedersen, J., Larsen, J., and Felby, C. (2007b). "Liquefaction of lignocellulose at high-solids concentrations," *Biotechnol. Bioeng.* 96(5), 862-870.
- Kaar, W., Gutierrez, C., and Kinoshita, C. (1998). "Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol," *Biomass Bioenerg.* 14(3), 277-287.
- Krishna, S. H., Reddy, T. J., and Chowdary, G. V. (2001). "Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast," *Bioresour. Technol.* 77(2), 193-196.
- Laopaiboon, L., Thanonkeo, P., Jaisil, P., and Laopaiboon, P. (2007). "Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*," *World J. Microbiol. Biotechnol.* 23(10), 1497-1501.
- Öhgren, K., Rudolf, A., Galbe, M., and Zacchi, G. (2006). "Fuel ethanol production from steam-pretreated corn stover using ssf at higher dry matter content," *Biomass Bioenerg.* 30(10), 863-869.
- Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., and Zacchi, G. (2007). "A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover," *Process Biochem.* 42(5), 834-839.
- Olofsson, K., Rudolf, A., and Liden, G. (2008), "Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*," *J. Biotechnol.* 134, 112-120.
- Olofsson, K., Bertilsson, M., and Liden, G. (2008), "A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks," *Biotechnol. Biofuels*, 1.
- Pandey, A., Soccol, C. R., Nigam, P., and Soccol, V. T. (2000). "Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse," *Bioresour. Technol.* 74(1), 69-80.
- Rudolf, A., Alkasrawi, M., Zacchi, G., and Liden, G. (2005). "A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce," *Enzyme Microb. Technol.* 37(2), 195-204.
- Saddler, J., Hogan, C., Chan, M. K. H., and Louis-Seize, G. (1982). "Ethanol fermentation of enzymatically hydrolysed pretreated wood fractions using trichoderma cellulases, *Zymomonas mobilis*, and *Saccharomyces cerevisiae*," *Can. J. Microbiol.* 28(12), 1311-1319.
- Sanchez, O. J., and Cardona, C. A. (2008). "Trends in biotechnological production of fuel ethanol from different feedstocks," *Bioresour. Technol.* 99(13), 5270-5295.
- Sassner, P., Galbe, M., and Zacchi, G. (2006), "Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated salix at high dry-matter content," *Enzyme Microb. Technol.* 39, 756-762.
- Sassner, P., Galbe, M., and Zacchi, G. (2008), "Techno-economic evaluation of bioethanol production from three different lignocellulosic materials," *Biomass and Bioenergy* 32, 422-430.
- Schell, D. J., Farmer, J., Newman, M., and McMillan, J. D. (2003). "Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor," in: *Biotechnology for Fuels and Chemicals*, Springer, London, pp. 69-85.

- Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., and Osborne, J. (2007). "A comparison of chemical pretreatment methods for improving saccharification of cotton stalks," *Bioresour. Technol.* 98(16), 3000-3011.
- Sluiter, A., Hames, B., Ruiz, R., Scarlatta, C., Sluiter, J., Templeton, D., and Crockeret, D. (2004). "Determination of structural carbohydrates and lignin in biomass," NREL Technical Report #TP-510-42618, National Renewable Energy Laboratory, Golden, CO.
- Song, A., Ren, T., Zhang, L., Wang, F., and Xie, H. (2011). "Optimization of corn stover hydrolysis by fed-batch process," *Chin. J. Agric. Biotechnol.* 27, 393.
- Spangler, D. J., and Emert, G. H. (1986). "Simultaneous saccharification/fermentation with *Zymomonas mobilis*," *Biotechnol. Bioeng.* 28(1), 115-118.
- Varga, E., Klinke, H. B., Réczey, K., and Thomsen, A. B. (2004). "High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol," *Biotechnol. Bioeng.* 88(5), 567-574.
- Wu, L., Arakane, M., Ike, M., Wada, M., Takai, T., Gau, M., and Tokuyasu, K. (2011). "Low temperature alkali pretreatment for improving enzymatic digestibility of sweet sorghum bagasse for ethanol production," *Bioresour. Technol.* 102(7), 4793-4799.
- Yu, Q., Zhuang, X., Lv, S., He, M., Zhang, Y., Yuan, Z., Qi, W., Wang, Q., Wang, W., and Tan, X. (2012). "Liquid hot water pretreatment of sugarcane bagasse and its comparison with chemical pretreatment methods for the sugar recovery and structural changes," *Bioresour. Technol.* 129, 592-598.
- Zacchi, G., and Axelsson, A. (1989). "Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions," *Biotechnol. Bioeng.* 34(2), 223-233.
- Zhang, W., Lin, Y., Zhang, Q., Wang, X. Y., Wu, D. Y., and Kong, H. N. (2013). "Optimisation of simultaneous saccharification and fermentation of wheat straw for ethanol production," *Fuel* 112, 331-337.

Article submitted: October 17, 2013; Peer review completed: January 9, 2014; Revised version received: February 25, 2014; Accepted: March 5, 2014; Published: April 28, 2014.