Effect of β-glycosidase Supplementation on Vinasse Saccharification and L-lactic Acid Fermentation

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Efficient pretreatment and enzymatic hydrolysis is critical to achieve effective utilization of lignocellulosic biomass. In this study, the cellulase composition for lignocellulosic biomass hydrolysis was strategically optimized to improve the efficiency of vinasse saccharification and thus enhance L-lactic acid production. The results showed that the supplementation of β-glycosidase (BG) increased sugar production, and the glucose concentration exceeded cellobiose concentration after 48 h of hydrolysis. These results suggested that the addition of BG aided the hydrolysis of cellobiose and reduced the inhibitory effects caused by sugar accumulation. After 72 h to 96 h of hydrolysis, the BG supplementation improved cellobiose and glucose production by 25.7% and 27.4%, respectively. The effect of BG supplementation on L-lactic acid production during the fermentation of microwave-alkali pretreated vinasse was also investigated. Here, the L-lactic acid production from simultaneous saccharification and fermentation (SSF) with the addition of BG was 20.8% higher than that without BG addition, and was also 37.0% higher than production from separate hydrolysis and fermentation with BG addition. These results indicated the utilization efficiency of lignocellulosic biomass for L-lactic acid production could be enhanced by supplementation of BG in SSF.

Keywords: Coupled microwave-alkali pretreatment; β-glycosidase; Product inhibition; Cellobiose; L-lactic acid; Vinasse.

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INTRODUCTION

Lignocellulosic materials represent the most abundant type of biomass on earth, and their efficient utilization has attracted widespread attention globally (Kumar *et al.* 2017). Vinasse is a by-product of wine production, and approximately 6 million tons to 8 million tons of solid vinasse is produced in China every year (Shi 2006). Wine vinasse has a high content of lignocellulose, with cellulose/hemicellulose content greater than 48% by mass (Liu *et al.* 2013). Most vinasse is stored or disposed untreated, which occupies land and causes serious environmental problems due to its high biological oxygen demand and acidic nature.

A common treatment of the lignocellulosic material involves pretreatment, enzymatic hydrolysis, and then fermentation of hydrolysate to produce high value-added

products, including ethanol, L-lactic acid, hydrogen, and biogas (Antonopoulou *et al.* 2016; Du *et al.* 2016; Wang *et al.* 2017). L-lactic acid is a raw material that is used for the production of polylactic acid, a biodegradable plastic that has wide application prospects and great market demand. Therefore, production of L-lactic acid with high optical purity from lignocellulosic materials has attracted broad interest (Abdel-Rahman *et al.* 2011; Jin *et al.* 2015). However, the complex fiber structure and low enzymatic hydrolysis efficiency of lignocellulosic materials limits their commercial utilization (Berlin *et al.* 2006).

There have been numerous studies on the pretreatment and enzymolysis of cellulosic biomass (Taherzadeh and Karimi 2008; Hendriks and Zeeman 2009; Tomáspejó *et al.* 2010). The main enzymes used to hydrolyse cellulose in lignocellulosic materials include endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BG) (Jalak *et al.* 2012; Kostylev and Wilson 2012). During cellulose hydrolysis, EG randomly attacks β-1,4-glycosidic bonds within the polymer chains to generate cello-oligosaccharides of varying lengths. Cello-oligosaccharides are then cleaved by CBH either from reducing ends (by CBH I) or from non-reducing ends (by CBH II), thus resulting in the generation of cellobiose. Finally, BG is used to hydrolyse cellobiose into glucose. Unbalanced cellulase enzyme composition may lead to low enzymatic hydrolysis efficiency and product inhibition, which can adversely affect glucose production and further L-lactic acid production. Cellobiose, the intermediate product of hydrolysis, and glucose, the end-product, have strong inhibiting effects on CBH and BG, respectively (Holtzapple *et al.* 1990; Gruno *et al.* 2004). Supplementation of BG can effectively reduce the inhibition effect of cellobiose on CBH and BG (Subramaniyan and Prema 2002; Andriä *et al.* 2010;). Shi *et al.* (2013) optimized cellulase composition by co-fermentation of *Trichoderma reesei* and *Aspergillus niger* to obtain higher sugar production. However, the role of cellulase optimization on promoting the cellulose hydrolysis and subsequent L-lactic acid fermentation has not yet been reported.

In a previous study microwave-assisted alkali pretreatment was found to be the most suitable pretreatment strategy for vinasse. The optimal pretreatment conditions were microwave power 523 W, a pretreatment time of 8 min, and NaOH dosage 0.06 g/g vinasse (Liu *et al.* 2012; 2013). Based on this result, cellulase composition was analysed and optimized to build a cellulase preparation with increased enzyme activity, the synergistic effect between the enzyme components was determined, and the effect and mechanism of enzyme composition optimization on L-lactic acid production was investigated.

EXPERIMENTAL

Materials

Vinasse was obtained from a distillery in Beijing, which used sorghum for brewing and rice husk as an auxiliary material to adjust porosity. The characteristics of the vinasse are shown in Table 1. Cellulose and hemicellulose represented 48.4% of the dry matter (DM). The vinasse contained only a small amount of crude protein and crude fat (< 5% DM each).

Industrial cellulase (270 U/g) was purchased from Beijing Donghua Qiangsheng Biotechnology Co., Ltd. (Beijing, China) and β-glycosidase (BG) was purchased from Sigma G4511 (CAS# 9001-22-3; 20 U/mg to 40 U/mg activity; St. Louis, MO, USA). The fermentative bacteria *Lactobacillus casei* (CICC 6106) was acquired from China Centre of Industrial Culture Collection. Chromatography-grade reagents were used for liquid

chromatography analysis, and analytical grade reagents were used for other analysis.

Table 1. Characteristics of the Vinasse

Parameters	Content* (%)	Parameters	Content* (%)
Soluble reducing sugar	1.00±0.30	Cellulose	27.23±0.04
Soluble total sugar	2.12±0.34	Hemicellulose	21.17±0.69
L-lactic acid	3.26±0.80	Lignin	14.95±0.52
Crude protein	0.78±0.02	Water	66.67±0.40
Crude fat	4.90±0.10	pH	3.45

*Components of the vinasse (except water content) were based on dry matter

Pretreatment of Vinasse and Enzymatic Hydrolysis

The microwave-assisted alkali pretreatment was performed in 500 mL Erlenmeyer flasks containing 20 g of vinasse (DM) and 160 mL of water. The NaOH dosage was 0.06 g/g vinasse (DM). The flask was placed into a microwave and heated at 523 W for 8 min. The flask was then allowed to cool at room temperature for 2 h, and water was added to the samples to supplement the evaporative losses (Liu *et al.* 2012). After pretreatment, the cellulose, hemicellulose, and lignin content of the vinasse was 36.2%, 17.2%, and 12.8%, respectively.

Enzymatic hydrolysis of the pretreated vinasse was performed in 200 mL Erlenmeyer flasks containing 5 g of vinasse (DM) and 75 mL of 50 mM sodium citrate buffer (pH 6.0) with a specific dosage of cellulase at 40 °C for 96 h. The hydrolysis reactions were terminated by heating the mixtures at 95 °C for 5 min. The hydrolysis efficiency was calculated with Eq. 1,

$$\text{Hydrolysis efficiency } (\%) = \frac{(C_{Cellobiose} \times \frac{360}{342} + C_{Glucose}) \times V_{\text{Hydrolysate}} \times 0.9}{W_{Vinasse} \times \eta_{Glucan}} \times 100 \quad \textbf{(1)}$$

where $C_{\text{Cellobiose}}$ and C_{Glucose} denote the cellobiose and glucose concentration after hydrolysis, respectively, $V_{\text{Hydrolysate}}$ denotes the volume of the hydrolysate, and W_{Vinasse} and η_{Glucan} denote the weight and glucan content of the vinasse used for enzymatic hydrdysis, respectively.

Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)

To carry out SHF, pretreated vinasse was placed in Erlenmeyer flasks for enzymatic hydrolysis. The cellulase dosage was 60.6 U/g vinasse (DM). After hydrolysis for 72 h, the mixture was centrifuged at 4000 × *g* for 10 min, and 150 mL of supernatant was placed in a 250 mL fermentation bottle for fermentation. Exactly 10 mL of inoculum was centrifuged at 4000 × *g* for 15 min, and the settled cells were inoculated to the fermentation medium. The fermentation was performed at 35 °C and 50 rpm under anaerobic conditions.

The SSF was performed in a 250 mL fermentation bottle that contained 10 g of pretreated vinasse (DM) and 150 mL of 50 mM sodium citrate buffer (pH 6.0). The cellulase dosage was 60.6 U/g vinasse (DM). Exactly 10 mL of inoculum was centrifuged at 4000 × *g* for 15 min, and the settled cells were inoculated to the fermentation medium. SSF was performed at 35 °C and 50 rpm under anaerobic conditions. The L-lactic acid yield (g/g) was defined as amount of L-lactic acid (g) divided by total reducing sugar (g).

Analytical Methods

The activities of the industrial cellulase (carboxymethyl cellulase activity (CMCase), filter paper activity (FPA) and BG activity) were measured according to the standard (IUPAC 1987). The sugar concentration of the hydrolysate was determined by a high-performance liquid chromatography (HPLC) system (LC-20AT, Shimadzu, Tokyo, Japan) equipped with an Inertsil NH_2 Column (4.6 mm × 250 mm, 5 μm) and a refractive index detector (RID-10A, Shimadzu) at 40 °C, using 25% water-75% acetonitrile at a flow rate of 1.0 mL/min as the mobile phase. The injection volume was 5 μL.

L-lactic acid was detected by HPLC (LC-20AT, Shimadzu) using a Chiral column (CHIRALPAK MAt, 4.6 mm × 50 mm, 5 μm) and an ultraviolet absorption detector at 254 nm. A 2 mmol/L copper sulphate solution at a flow rate of 1.0 mL/min was used as the mobile phase. The injection volume was 5 μL.

RESULTS AND DISCUSSION

Component Characteristics of the Industrial Cellulase

The degradation of crystalline cellulose was not possible with a single enzyme but required the synergistic actions of at least three groups of enzymes: EG, CBH, and BG (Tsai *et al.* 2014). Among them, EG and CBH catalyze the hydrolysis of cellulose-to-polysaccharide and polysaccharide-to-cellobiose, respectively, and BG is the key enzyme that catalyzes the hydrolysis of cellobiose to glucose (Zhang and Lynd 2004). The three enzymes synergistically hydrolyze the cellulose to glucose. Commercial cellulase usually contains all three groups of enzymes. The comprehensive hydrolysis ability of the three enzymes can be represented by FPA. Filter paper is a fibrous material with medium polymerization degree and crystallinity. When measuring the FPA of the cellulose, filter paper is used as the substrate for enzymatic hydrolysis and the amount of reducing sugar produced after hydrolysis can represent the saccharifying ability of the cellulase. This method has been widely used, and can reflect the synergy of three kinds of enzyme components. However, the BG activity in industrial cellulase is usually low, resulting in cellobiose accumulation and consequently reducing the hydrolysis efficiency; thus, an increased cellulase dosage and higher production costs are required (Gusakov and Sinitsyn 1992; Bozeman and Murdock 1995; Kruus *et al.* 1995; Medve *et al.* 1998).

The industrial cellulase used in this study was food-grade and had the highest activity among commercial cellulases. The activities of the three groups of enzyme and FPA were shown in Fig. 1. Among the three enzymes, the activities of EG and CBH reached 34300 U/g and 15900 U/g, respectively, while BG had a much lower activity (5540 U/g). The average activity of the three enzymes was 18700 U/g, which was 5.4 times greater than FPA (3450 U/g). This result suggested that although the individual activities of the three enzymes were relatively high, the overall hydrolysis ability (*i.e.*, FPA) was well below the lowest of activity of the three components (*i.e.*, BG, 5540 U/g). This was likely due to the cellobiose inhibition (product inhibition) caused by the unbalanced enzyme activity.

The comprehensive hydrolysis ability relies not only on enzyme dosage, but also on the proportion of the enzyme components (Schwarz 2001). Zhu *et al.* (2006) showed that adding BG to the commercial cellulase caused enhancement in both the initial rates and degree of solubilization of cellulose. Therefore, the current study investigated the hydrolysis efficiency of cellulase after supplementing three individual enzymes at a dosage

of 2000 U/g. As shown in Fig. 1, supplementation of EG and CBH did not improve FPA, while the addition of BG increased FPA by 51.6%. This indicated that the FPA was limited by the BG activity. As the FPA represents the hydrolysis ability of the cellulose to glucose, the BG activity was the factor that restricted enhancement of enzymatic hydrolysis efficiency. Similar results have been observed in other studies (Lamed *et al.* 1991; Kim *et al.* 2015).

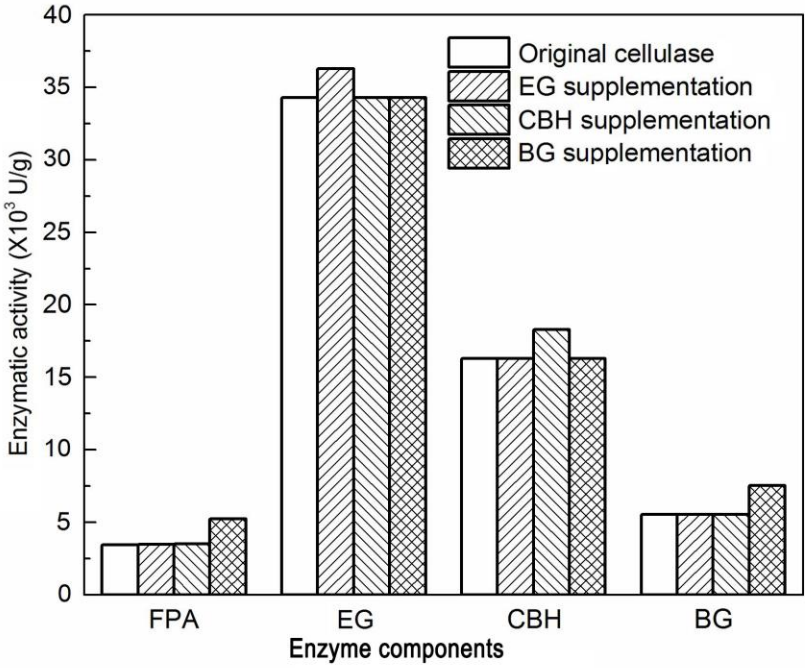

Fig. 1. Enzyme activities of cellulase before and after supplementation of 2000 U/g with three different components of the cellulase

Effect of BG Supplementation on FPA

To improve the activity of the cellulase, BG was incrementally supplemented to optimise cellulase performance. As shown in Fig. 2, the FPA of the enzyme mixture increased with increased dosages of BG. However, at BG supplementation levels less than 4000 U/g, the FPA of the enzyme mixture remained less than the total BG activity (existing combined with supplemented). The maximum FPA activity (12400 U/g) was achieved when BG supplementation was 5000 U/g. No further FPA improvement occurred at BG supplementations of less than 5000 U/g. The correlation analysis indicated that the total BG activity directly related with FPA performance ($r^2 = 0.97$).

Enhanced Vinasse Hydrolysis by BG Supplementation

The effect of BG supplementation on the enzymatic hydrolysis of the alkali-microwave-pretreated vinasse was studied. The FPA of the enzyme mixture with and without BG supplementation was 12400 U/g and 3950 U/g, respectively. As shown in Fig. 3, during the hydrolysis with BG addition, the glucose and cellobiose concentration reached 11.04 g/L and 9.33 g/L, respectively, which was 27.4% and 25.7% higher than that without BG addition, respectively. During hydrolysis of the mixture with added BG, the concentration exceeded the cellobiose concentration 12 h sooner than the mixture without BG addition, suggesting that adding BG enhanced the conversion of cellobiose to glucose.

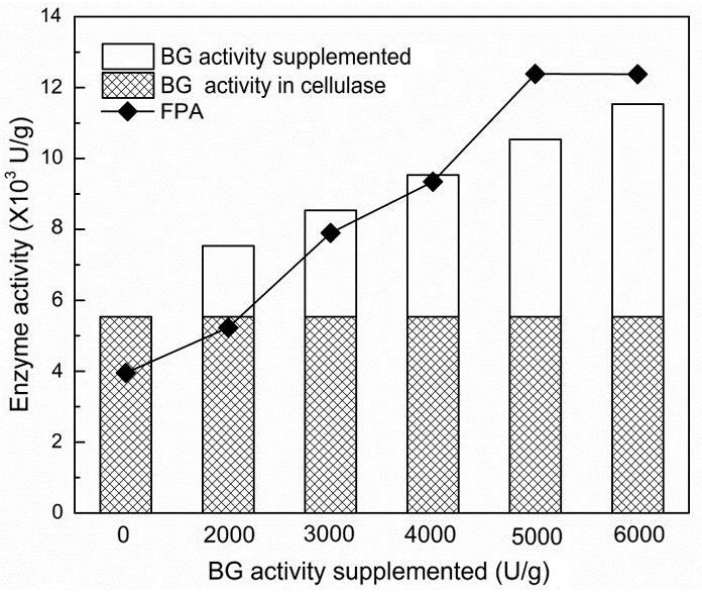

Fig. 2. Effect of BG supplementation on FPA of the enzyme mixture

As shown in Fig. 3b, the cellobiose and glucose concentration continued to increase at the end of the hydrolysis (72 h to 96 h). This indicated that the addition of BG had improved the hydrolysis of cellobiose to glucose, which relieved the product inhibition caused by cellobiose and promoted the overall hydrolysis efficiency of the enzyme mixture.

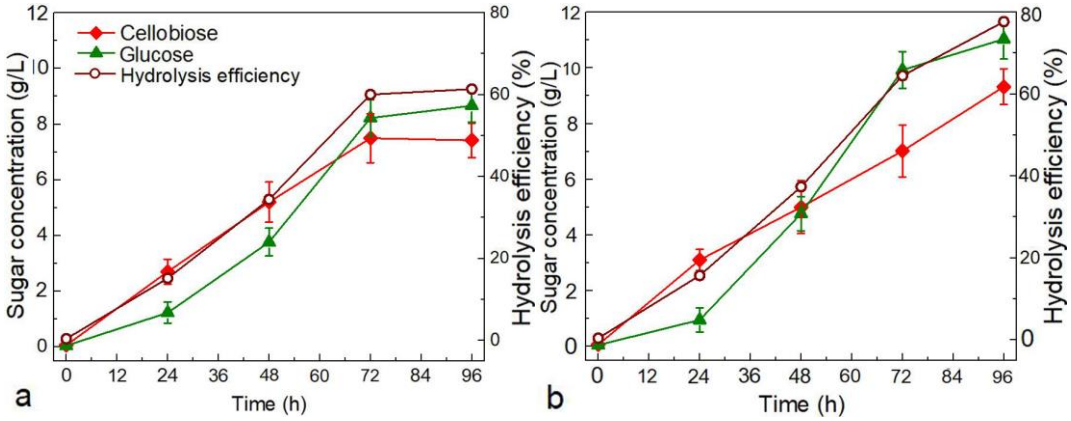

Fig. 3. Changes of sugar concentration and hydrolysis efficiency during the hydrolysis of microwave-alkali pretreated vinasse without (a) and with (b) BG supplementation

After 96 h of hydrolysis, the hydrolysis efficiency of vinasse with BG supplementation was 77.7%, which was 26.6% higher than the efficiency of vinasse without BG supplementation. This result suggested that, besides increasing the cellulase dosage, optimizing the proportion of the cellulase components can also obtain a high FPA, thus improving the overall hydrolysis efficiency. Chen *et al.* (2007) supplemented the cellulase solution from *Trichoderma reesei* with BG obtained from *Aspergillus niger*, and the hydrolysis efficiency reached 83.9% with a dosage of 6.5 IU/g substrate. Long *et al.* (2012) found that the hydrolysis efficiency increased by 13.82% with the addition of BG (6 IU/g.). These results were consistent with the current study.

Effect of BG Supplementation on L-lactic Acid Production

SHF and SSF are the two most common fermentation methods for L-lactic acid production. Figure 4 shows the changes of L-lactic acid, cellobiose, and glucose concentrations during the fermentation of microwave-alkali pretreated vinasse.

For SHF with BG addition (Fig. 4b), the initial cellobiose and glucose concentrations were 9.33 g/L and 11.04 g/L, respectively, which were higher than the concentrations obtained for vinasse without BG addition (7.42 g/L and 8.66 g/L, respectively, Fig. 4a). However, the maximum L-lactic acid concentration was 17.45 g/L, which was only 2.81 g/L higher than the concentration of vinasse without BG addition, and the cellobiose was not completely consumed after 96 h of fermentation. This indicated that the addition of BG in SHF increased the glucose and cellobiose concentration during the hydrolysis process but did not significantly improve the conversion of sugar to L-lactic acid.

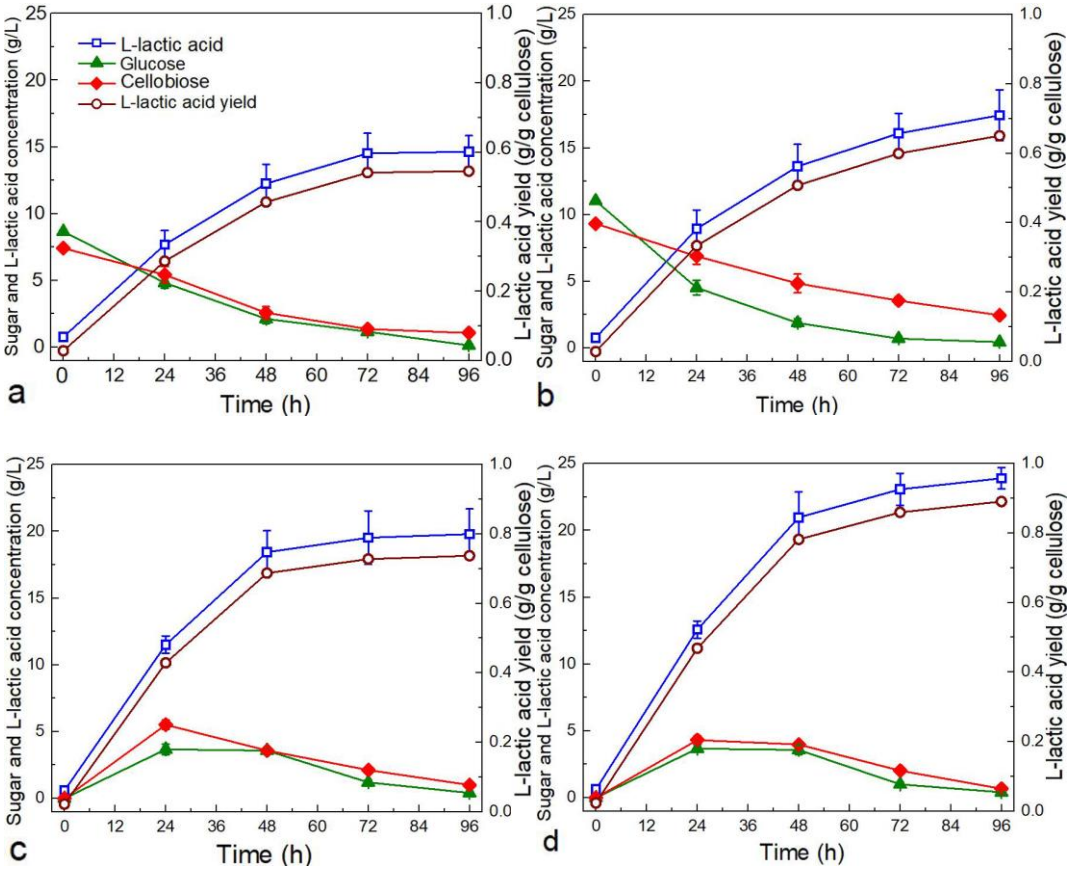

Fig. 4. L-lactic acid production by SHF and SSF: (a) SHF without BG supplementation, (b) SHF with BG supplementation, (c) SSF without BG supplementation, (d) SSF with BG supplementation

During the SSF process, the maximum cellobiose and glucose concentrations were observed at 24 h, but the concentration never exceeded 6.0 g/L. Thus, inhibition caused by sugar accumulation was avoided. The L-lactic acid concentration of SSF with BG addition reached 23.90 g/L (Fig. 4d), which was 20.8%, 37.0%, and 63.2% greater than the concentrations of SSF without BG addition, SHF with BG addition, and SHF without BG addition, respectively. Moreover, the glucose and cellobiose were completely consumed. This result suggested that addition of BG in SSF not only improved the cellulose hydrolysis

efficiency, but also promoted the overall utilization efficiency of the substrate.

Moreover, the L-lactic acid concentration in SSF with BG addition (23.90 g/L) was higher than the total concentration of cellobiose and glucose during hydrolysis (20.36 g/L). Though the hydrolysis was not performed at its optimal conditions during SSF, more cellulose was hydrolyzed in SSF than in SHF. This was likely because the inhibition of glucose and cellobiose on cellulose hydrolysis was relieved (Berlin *et al.* 2006; Zheng *et al.* 2013). Compared to SHF, the SSF with BG addition showed a distinct advantage in (1) high substrate hydrolysis efficiency and product yield; (2) shortened process time to complete the hydrolysis and fermentation.

CONCLUSIONS

1. The supplementation of BG significantly improved the hydrolysis efficiency of the microwave-assisted alkali pretreated vinasse.
2. The SSF fermentation mode avoided the inhibition effect caused by sugar accumulation, which improved the continuity of the hydrolysis and fermentation process and improved the L-lactic acid production.
3. Further research is needed to study the synergistic reaction mechanisms of the cellulase components to lay a good foundation for the industrialized application of lignocellulosic materials.

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