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# Simultaneous saccharification and fermentation of dilute alkaline-pretreated corn stover for enhanced butanol production by *Clostridium saccharobutylicum* DSM 13864

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**One sentence summary:** Simultaneous saccharification and fermentation of dilute alkaline-pretreated corn stover for enhanced butanol production by *Clostridium saccharobutylicum* DSM 13864.

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## ABSTRACT

Simultaneous saccharification and fermentation (SSF) process was applied for biobutanol production by *Clostridium saccharobutylicum* DSM 13864 from corn stover (CS). The key influential factors in SSF process, including corn steep liquor concentration, dry biomass and enzyme loading, SSF temperature, inoculation size and pre-hydrolysis time were optimized. In 5-L bioreactor with SSF process, butanol titer and productivity of 12.3 g/L and 0.257 g/L/h were achieved at 48 h, which were 20.6% and 21.2% higher than those in separate hydrolysis and fermentation (SHF), respectively. The butanol yield reached 0.175 g/g pretreated CS in SSF, representing 50.9% increase than that in SHF (0.116 g/g pretreated CS). This study proves the feasibility of efficient and economic production of biobutanol from CS by SSF.

**Keywords:** simultaneous saccharification and fermentation; butanol; *Clostridium saccharobutylicum*; corn stover

## INTRODUCTION

Recent attention in air pollution and energy security has aroused increased interests on the utilization of lignocellulosic materials (such as corn stover) for biofuel production including butanol (Ding *et al.* 2016), ethanol (Zhu *et al.* 2014), biodiesel (Kim *et al.* 2015), hydrogen (Datar *et al.* 2007). Butanol possesses excellent merits such as higher energy density (29.2 MJ/L), blending ability, hydrophobicity and compatibility to combustion engines, lower viscosity, less corrosive for certain motor parts and octane rating, and is being regarded as a renewable source of energy (Durre 2007; Pfromm *et al.* 2010; Ni, Wang and Sun 2012). The economics of the biobutanol production is largely dependent on the cost of conversion of biomass into fermentable sugars. The biomass feedstock should be widely available at low cost (Lynd, Wyman and Gerngross 1999). In USA, more than 216 million tons of corn stover (CS) is produced every year. They are mostly used for animal feeding and bedding, besides a portion of them used for producing ethanol and other industrial products (Sokhansanj *et al.* 2002; Kadam and McMillan 2003). In China, the annual production of CS is around 220 million tons, and about

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90% remains unused or is burnt resulting in severe atmosphere pollution (Zheng *et al.* 2010). As a result, the efficient utilization of CS as a potential feedstock for butanol production is of significant importance.

Lignocellulosic biomass, as a widely distributed renewable carbon source on the earth, is mainly composed of 40%–60% cellulose, 20%–40% hemicellulose and 10%–24% lignin (Abdeshahian *et al.* 2010). Cellulose has been proved to be applicable in conversion into fermentable glucose for biofuel production (Abdeshahian *et al.* 2010). Hemicellulose is a polysaccharide consisted mainly of pentoses which are rarely consumed by biofuel-producing microorganisms (Van Vleet and Jeffries 2009). *Clostridium saccharobutylicum* has been reported in utilizing xylose and arabinose for butanol fermentation (Ni *et al.* 2013). The adoption of *C. saccharobutylicum* in biofuel fermentation could therefore improve the sugar utilization of lignocellulosic biomass. Additionally, 50∼60 g/L of total sugar concentration is demanded in butanol fermentation, which is much lower than that in the production of ethanol (Wang *et al.* 2012), succinic acid (Yan *et al.* 2014), lactic acid (Hu *et al.* 2015) and so on. However, higher loading of dry CS (> 9%) in the fermentation always leads to high viscosity and mass transferring hindrance in CS pretreatment and fermentation. Thus, CS is especially suitable for butanol production by *Clostridial* strains.

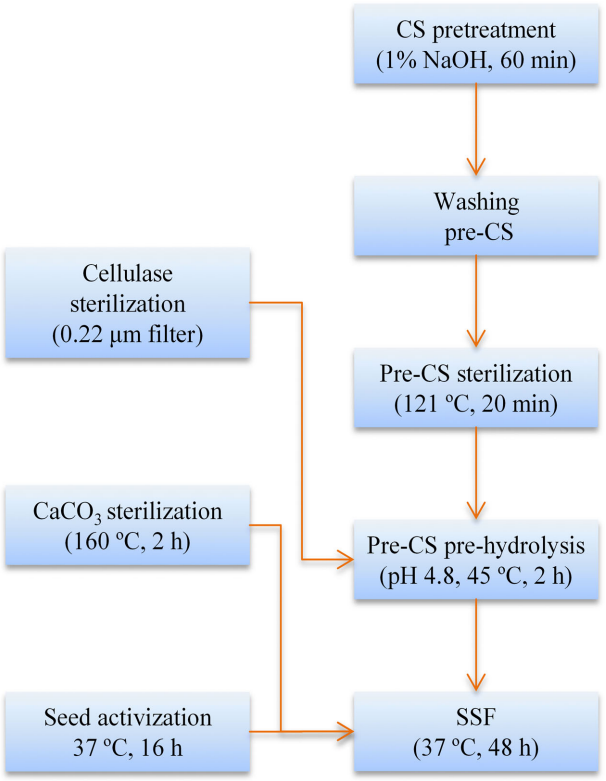
Separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are two main processes in biofuel fermentation from lignocellulosic biomass. Both processes use pretreated lignocellulosic biomass as substrate. In SHF process, the pretreated lignocellulosic biomass is first converted into fermentable sugar by cellulase, and then the fermentable sugar is sterilized and used as carbon source in microbial fermentation. In SSF process, the pretreated lignocellulosic biomass is converted into biofuel in the presence of both cellulase and microorganism in a bioreactor. In SHF process, certain nutrients could be destroyed, and furfural and 5-hydroxymethylfurfural (two of the Maillard reaction products) generated during medium sterilization at high temperature are deleterious for cell growth and solvent production. (Qureshi *et al.* 2010a). In SSF process, however, less nutrient ingredients are lost and none Maillard reaction product is generated since sterilization is unnecessary. Furthermore, SSF process encompasses hydrolysis and fermentation in the same reactor simultaneously. Consequently, the SSF process is more facile, simple and efficient than SHF (Zhu *et al.* 2014).

In our previous work, diluted alkaline and ionic liquid (recycled for 10 times) were adopted in CS pretreatment and butanol fermentation using *C. saccharobutylicum* DSM 13864, in which high temperature (130°C) is required for ionic liquid pretreatment (Ding *et al.* 2016). Here, the feasibility of SSF in the butanol fermentation with *C. saccharobutylicum* from CS was investigated (Scheme 1). The operation of this SSF process was optimized and carried out in a 5-L stirred bioreactor.

## MATERIALS AND METHODS

### Raw materials, strain and enzyme

CS was chopped and stored at room temperature. Before pretreatment, it was milled, screened (fractions between 20 and 80 meshes were collected) and dried at 60°C for 24 h. *Clostridium saccharobutylicum* DSM 13864 was purchased from DSMZ and manipulated as previously described (Ding *et al.* 2016). ACCELLERASE® 1500 cellulase (50 FPU/mL) was provided by Genencor (Wuxi) Bio-Products Co.




**Scheme 1.** SSF process employed for butanol fermentation using *C. saccharobutylicum* DSM 13864.

### Pretreatment of CS with diluted alkaline

Untreated CS was soaked in 1 wt% NaOH at 120°C for 1 h. Then, the CS was filtered and washed with tap water until pH reached 7.0–8.0. The pretreated CS (Pre-CS) was dried at 80°C for 24 h in an oven and stocked in sealed plastic bags for further use. Lignin content of untreated CS is 17.2%. Lignin content of Pre-CS is 8.2%, and the delignification rate was 75% (Zheng *et al.* 2010).

### Analytical methods

The total reducing sugar and monosaccharides hydrolyzed from CS were measured as reported (Ding *et al.* 2016). The concentrations of butanol, acetone and ethanol were determined by gas chromatography (Ni *et al.* 2013). The concentration of crude protein was calculated by determining nitrogen using Kjeldahl method. Furfural and 5-hydroxymethylfurfural were determined by high-performance liquid chromatography as previously described (Dong *et al.* 2013).

### Simultaneous saccharification and fermentation

SSF were performed anaerobically in duplicates using 150-mL anaerobic bottles containing 50 mL of SSF medium (Pre-CS, 7% (w/v); $CaCO_3$, 4 g/L; $(NH_4)_2SO_4$, 2 g/L; $K_2HPO_4$, 0.5 g/L; $MnSO_4{\cdot}H_2O$, 0.01 g/L). General fermentation conditions were 5% (v/v) inoculation size, 20 FPU/g Pre-CS, 2 h pre-hydrolysis, 7% (w/v) dry biomass at 37°C unless otherwise stated. The effects of various influential factors on the butanol production and reducing sugar consuming were investigated, including SSF temperature (33, 35, 37, 39 and 40°C), inoculation size (0, 2, 5, 10 and 15%), enzyme loading (0, 10, 20, 30 and 40 FPU/g Pre-CS), corn steep liquor (CSL;

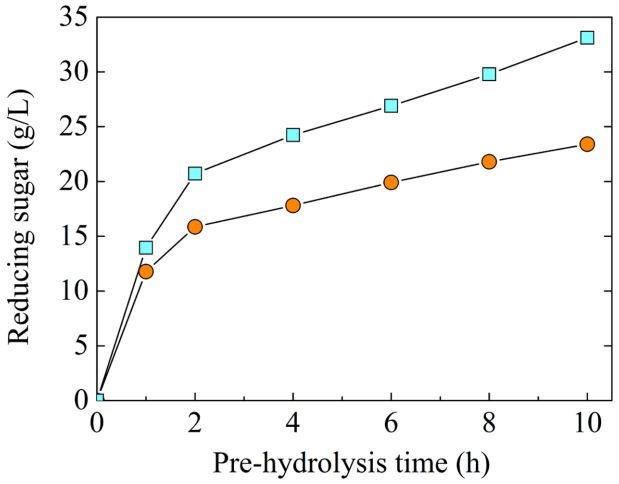



**Figure 1.** Saccharification of diluted alkaline Pre-CS hydrolyzed by ACCELLERASE® 1500 cellulase at 37°C (orange solid circle) and 45°C (blue solid square).

0, 2, 5, 10 and 20 g/L), pre-hydrolysis time (0, 1, 2, 4 and 8 h) and dry mass loading (5, 7, 9, 11 and 13%). SSF was also carried out in a 5-L bioreactor filled with 3 L of medium as mentioned above.

## RESULTS

### Optimization of butanol production in SSF process

Various influential factors in the SSF with *C. saccharobutylicum* DSM 13864 were studied to achieve economic butanol production. Pre-hydrolysis process has been reported to be capable of reducing the viscosity of fermentation medium and providing initial sugar for strain growth (Jin *et al.* 2010). The CS was pretreated with diluted NaOH at 37 and 45°C, and further hydrolyzed by cellulase (Fig. 1). Reducing sugar was fast released in the initial 2 h (15.8 g/L for 37°C, 20.7 g/L for 45°C), and then increased slowly. The Pre-CS of different pre-hydrolysis time was then applied in the SSF (Fig. 2E). As the increase of pre-hydrolysis time from 0 to 8 h, the initial reducing sugars were increased from 0 to 29.8 g/L. Studies showed that lower reducing sugar could limit the cell growth and butanol production, while higher reducing sugar could impose higher osmotic stress (Zhu *et al.* 2014). The highest butanol titer of 12.8 g/L was achieved at 48 h using Pre-CS of 2 h pre-hydrolysis.

Since the optimum temperatures for butanol fermentation with *C. saccharobutylicum* and Pre-CS saccharification are 37°C and 50°C, respectively (Ni *et al.* 2013; Ding *et al.* 2016), the operational temperature of SSF process should therefore optimized (Fig. 2A). At 33°C, 35°C, 39°C and 41°C, reducing sugars were accumulated as high as 30 g/L in the initial 24 h, whereas the butanol titers were lower than 0.5 g/L, ascribing to the lower growth rate of *C. saccharobutylicum*. The highest butanol titer and productivity of 11.6 g/L (48 h) and 0.39 g/L/h (24 h) were achieved at 37°C. Thus, 37°C was selected as the optimal temperature for the SSF process.

Effect of inoculum size was investigated in SSF process at 37°C (Fig. 2B). In control with no inoculation, 27.7 g/L reducing sugar were accumulated at 24 h, while 30.5 and 27.8 g/L reducing sugar were accumulated at 24 h with 2% and 5% (v/v) inoculation size, indicating the SSF process could promote the enzymatic hydrolysis of Pre-CS (Qureshi *et al.* 2014). Also, the lag phase of SSF process with 2%–5% inocula was longer than that with higher inocula (10%–15%). Nevertheless, higher butanol titers were obtained at 2% (10.38 g/L) and 5% (10.96 g/L) inocula at 48 h than those of 10% and 15% inocula, likely due to the fast senescence. Hence, the optimum inoculation size of SSF process was 5%.

Enzyme loading was critical for the saccharification in SSF (Fig. 2C). At 10 FPU/g Pre-CS, the butanol titer was only 2.0 g/L at 24 h, much lower than that at 20–40 FPU/g Pre-CS, which was due to the low reducing sugar released. The highest butanol titer of 11.60 g/L was achieved at 20 FPU/g Pre-CS. However, slightly lower butanol titers (11.10 and 10.25 g/L) were attained at enzyme loadings of 30–40 FPU/g Pre-CS, possibly due to the inhibitory effect of high sugar concentration accumulated. About 0.41 g/L butanol was produced with no cellulase added,

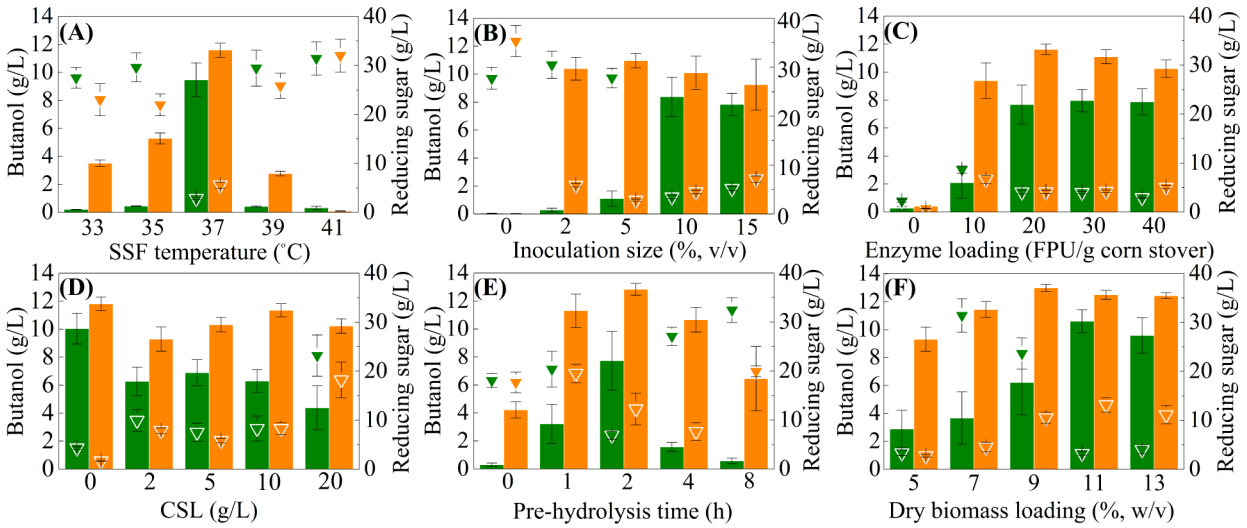



**Figure 2.** Effect of different conditions on the butanol production by *C. saccharobutylicum* DSM 13864 in SSF process. Experiments were carried out in 150-mL anaerobic bottles in duplicates, under following conditions: initial pH 5.0, 37°C (except A), 10% (v/v) inoculation size (except B), enzyme loading of 30 FPU/g pretreated CS (except C), 10 g/L (A, B, C) or 0 g/L CSL (E, F), 6 h (A, B, C, D) or 2 h pre-hydrolysis (F), and 7% (w/v) dry biomass loading (except F). Bar: butanol produced at 24 h (green) and 48 h (orange); inverted triangle: reducing sugar at 24 h (green) and 48 h (orange).

**Table 1.** Summary of butanol fermentation from CS by *C. saccharobutylicum* DSM 13864 in SSF and SHF.

| | SHF | SSF |
|---|---|---|
| Process time (h) | 120 (72[a]+48[b]) | 50 (2[a]+48[b]) |
| Input pretreated corn sover (%) | 8.8 | 7.0 |
| Butanol production (g/L)[c] | 10.2 | 12.3 |
| Butanol productivity (g/L/h)[c] | 0.212 | 0.257 |
| Butanol yield (g/g Pre-CS)[c] | 0.116 | 0.175 |
| ABE production (g/L)[d] | 15.7 | 19.2 |

[a]Pre-hydrolysis time.
[b]Fermentation time.
[c]Fermentation time is 48 h.
[d]ABE stands for acetone, butanol and ethanol.

suggesting Pre-CS could be slightly hydrolyzed under weak acidic condition (about pH 5.0).

CSL is an important nutrient for fermentation and was tested to improve the fermentation medium in SSF process (Fig. 2D). The result shows that Pre-CS could provide enough nitrogen source for butanol production. With the addition of 2–10 g/L CSL, the butanol titer increased gradually from 9.29 to 11.35 g/L in 48 h, while only 10.22 g/L butanol was attained with 20 g/L CSL. The maximum butanol titer of 11.81 g/L was obtained without addition of CSL in SSF. Based on nitrogen determination, Pre-CS contains around 2.2% of crude protein, and could therefore serve enough nitrogen source for butanol fermentation. Our results showed that the concentrations of furfural and 5-hydroxymethylfurfural were 0.4 and 6.54 mg/L in 10 g/L sterilized CSL, both of which were reported to inhibit cell growth and butanol production of *C. saccharobutylicum* (Qureshi *et al.* 2010a).

Effect of different dry biomass loading was investigated in SSF (Fig. 2F). The butanol titers increased along with the increasing dry biomass loading from 5% to 9% (w/v). Although the highest butanol titer (12.99 g/L) was achieved at 9% dry biomass loading, the residual sugar is higher than that of 7%. Considering the economics of SSF process, 7% was regarded as the appropriate dry biomass loading.

Above results provide guidance for the feasibility of SSF process in butanol fermentation with *C. saccharobutylicum* DSM 13864. And the optimum conditions for the SSF process are determined to be 37°C, 5% inoculum, 20 FPU/g Pre-CS, no CSL addition, 2 h pre-hydrolysis and 7% dry biomass loading. To further prove its feasibility, SSF butanol fermentation was conducted in a 5-L bioreactor, and compared with SHF process.

### SSF and SHF butanol production

SSF and SHF processes were evaluated in butanol fermentation with *C. saccharobutylicum* DSM 13864 in 5-L bioreactor and summarized in Table 1. Dry biomass of 8.8% and 7.0% were loaded for SHF and SSF, respectively. The production of acetone, butanol and ethanol (ABE) and consumption of glucose, xylose and arabinose were determined (Fig. 3). For SHF, the pretreatment and fermentation time was 120 h in total, while the time is 50 h for SSF process. The highest butanol titer of 12.85 g/L (ABE 19.9 g/L) was reached at 64 h in SSF, which was 27% higher than that in SHF (10.10 g/L). The highest butanol productivity was 0.257 g/L/h for SSF (at 48 h), which was 21.2% higher than that for SHF (0.212 g/L/h). To achieve high efficiency in butanol fermentation by *C. saccharobutylicum*, 55 g/L initial reducing sugar is required in SHF process (Ding *et al.* 2016), corresponding to 8.8% dry biomass loading. However, 7% dry biomass loading is the best in SSF. Considering the Pre-CS loading, the butanol yield of SSF is 0.175 g/g Pre-CS in SSF, which is 50.9% higher than that in SHF (0.116 g/g Pre-CS). Consequently, the SSF process was proved to be much more economic and efficient in butanol fermentation from CS with *C. saccharobutylicum*.

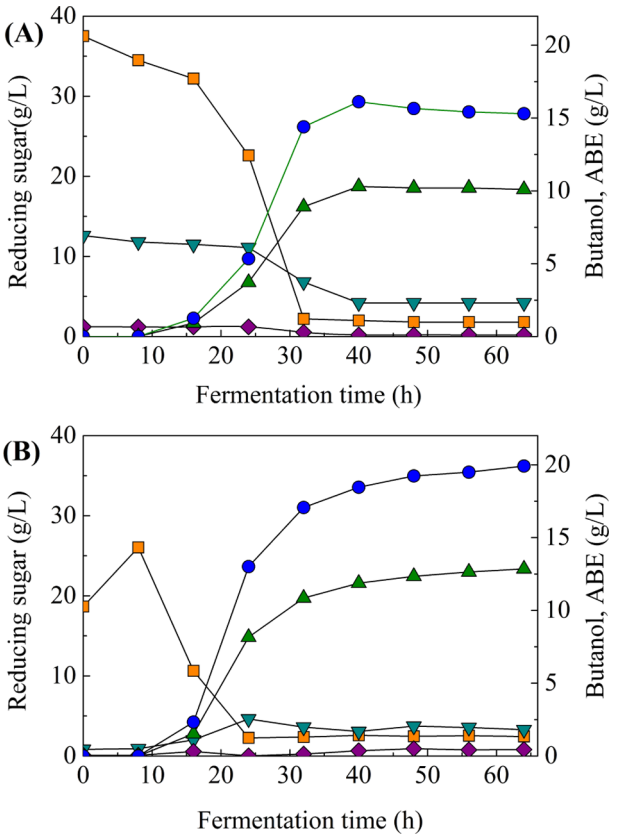



**Figure 3.** Time courses of butanol fermentation in SHF (**A**) and SSF (**B**) processes using CS pretreated with diluted alkaline by *C. saccharobutylicum* DSM 13864 in 5-L bioreactor. Blue filled circle: ABE; green triangle: butanol; orange solid square: glucose; cyan inverted triangle: xylose; purple diamond: arabinose.

## DISCUSSION

The SSF process is facile and efficient for butanol fermentation with *C. saccharobutylicum* DSM 13864. Compared with SHF, SSF process reduces the time and energy consumption required for enzymatic saccharification. Only 50 h (2 h pre-hydrolysis and 48 h SSF) is needed for SSF butanol production, saving 70 h than SHF (Table 1). The butanol yield of Pre-CS in SSF is 50.9% higher than that in SHF, which might be ascribed to the following reasons. (1) High concentration of reducing sugar and other components might inhibit the butanol production. In SSF process, the reducing sugar concentration was kept lower than 30 g/L (Fig. 3B). (2) Less reducing sugars are lost in SSF than that in SHF which requires autoclaving. High loading of Pre-CS (8.8%) is required in SHF butanol production, while only 7% of Pre-CS is demanded and the sugar conversion in SSF is 14.2% higher than in SHF. (3) SSF process reduces the generation of poisonous compounds during butanol fermentation. It has been reported that Pre-CS hydrolysate contains sodium citrate, cellulose and hemicellulose, and reducing sugar (especially pentose) will react with amide (Maillard reaction) to form furfural and 5-hydroxymethylfurfural during autoclaving, which are toxic

**Table 2.** Comparison of butanol production from various lignocellulosic biomass using *Clostridium* strains.

| Strain | Fermentation process | Biomass | Maximum butanol titer (g/L) | References |
|---|---|---|---|---|
| *C. saccharobutylicum* DSM 13864 | SHF | CS | 7.90 | Ding *et al.* (2016) |
| | SHF | Corncobs | 12.27 | Gao and Rehmann (2014) |
| | SSF | CS | 12.85 | This study |
| *C. saccharobutylicum* BAA-117 | Batch fermentation | Poplar wood | 7.28 | Wang *et al.* (2015) |
| *C. beijerinckii* P260 | SHF | CS | 14.50 | Qureshi *et al.* (2010b) |
| | SHF | Switchgrass | 5.79 | |
| | SSF | CS | 8.98 | Qureshi *et al.* (2014) |
| | SSF recovery | CS | 11.58 | |
| *C. acetobutylicum* ATCC824 | SSF | Wheat straw | 5.05 | Wang *et al.* (2013) |

to microbial cells (Banerjee, Bhatnagar and Viswanathan 1981; Qureshi *et al.* 2010a).

In our previous work, 15 g/L CSL was used in butanol fermentation from CS hydrolysate using SHF process (Ni *et al.* 2013). However, no improvement in butanol titer was observed when CSL was added in the SSF process (Fig. 2D). On one hand, 2.2% of crude protein in Pre-CS could act as nitrogen source in SSF; on the other hand, CSL contains Maillard reaction products (furfural and 5-hydroxymethylfurfural) generated during its preparation, which have inhibitory effect on the SSF butanol fermentation process. In this study, there was a slight increase in butanol titer as the increase of CSL from 2 to 10 g/L at 48 h in SSF process, which await further clarification. One major difference between SSF and SHF is the autoclaving step, which is conducted before saccharification and fermentation in SSF, while after saccharification in SHF. In SHF process, the crude protein in Pre-CS could react with reducing sugars produced in saccharification during autoclaving, leading to the loss of nitrogen. In SSF process, the crude protein in Pre-CS could be retained after autoclaving, since no reducing sugars exists. As a result, CSL (15 g/L) supplementation might provide main source of nitrogen source and promote butanol production in SHF, although CSL (15 g/L) contains furfural (0.6 mg/L) and 5-hydroxymethylfurfural (9.81 mg/L).

Butanol production from various lignocellulosic biomass using *Clostridium* strains were summarized in Table 2. Except for butanol titer of 14.50 g/L achieved in SHF process (Qureshi *et al.* 2010b), butanol titer in this report (12.85 g/L) is higher compared with other SHF, SSF and batch fermentation processes utilizing various lignocellulosic biomass such as corncobs (Gao and Rehmann 2014), CS (Qureshi *et al.* 2014), poplar wood (Wang *et al.* 2015), switchgrass (Qureshi *et al.* 2010b) and wheat straw (Wang *et al.* 2013).

In this study, an SSF process for butanol production from CS with *C. saccharobutylicum* DSM 13864 was established to achieve high butanol titer of 12.85 g/L, which is higher than our previous work (Ni, Wang and Sun 2012; Ni *et al.* 2013). Furthermore, 58% of process time and 34% of CS input were saved compared with than SHF, and no addition of CSL is necessary. This is the first report on SSF butanol production using *C. saccharobutylicum*. The substitution of expensive commercial cellulase with cellulase-producing microorganisms is being carried out to further reduce the cost of butanol fermentation.

## FUNDING

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***Conflict of interest.*** None declared.

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