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BioEnergy Research

ISSN 1939-1234
Volume 5
Number 4

Bioenerg. Res. (2012) 5:1020-1026
DOI 10.1007/s12155-012-9219-x

Springer

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Simultaneous Saccharification and Ethanol Fermentation of Corn Stover at High Temperature and High Solids Loading by a Thermotolerant Strain *Saccharomyces cerevisiae* DQ1

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Published online: 16 May 2012

Abstract The thermotolerant strain *Saccharomyces cerevisiae* DQ1 was applied to the simultaneous saccharification and fermentation (SSF) at high temperature and high solids loading of the dilute acid-pretreated corn stover in the present study. The SSF using *S. cerevisiae* DQ1 was operated at 30 % solids loading of the pretreated corn stover with three-step SSF mode and achieved up to ethanol titer of 48 g/L and yield of 65.6 %. *S. cerevisiae* DQ1 showed strong thermotolerance in both the regular one-step SSF and the three-step SSF with changing temperature in each step. The three-step SSF at 40°C using *S. cerevisiae* DQ1 tolerated the greater cellulase dosage and solids loading of the pretreated corn stover and resulted in increased ethanol production. The present study provided a practical potential for the future SSF of lignocellulose feedstock at high temperature to reach high ethanol titer.

Keywords Simultaneous saccharification and ethanol fermentation (SSF) · Thermotolerant yeast · *Saccharomyces cerevisiae* DQ1 · High solids loading · Corn stover

Introduction

Currently, one of the important process options for ethanol production when lignocellulose feedstock was used is simultaneous saccharification and ethanol fermentation (SSF) for the purpose of lessening product (sugars) inhibition on the cellulase enzymes and reducing capital cost [1]. However, the match of the optimal temperatures between the ethanologenic microorganisms (generally below 37°C) and the cellulase enzymes (50°C or above) is a great challenge [2, 3]. Many studies focused on the thermotolerant ethanologenic strains for the SSF of lignocelluloses: Edgardo et al. [4] developed a mutant *Saccharomyces cerevisiae* IR2-9a for the SSF of the organosolv-pretreated *Pinus radiata* chips at 40°C with 10 % solids loading in the flask, and the ethanol titer reached 22 g/L; Kadar et al. [5] used a *S. cerevisiae* strain for the SSF of the old corrugated cardboard at 40°C at 6 % substrate loadings in the flask, and the maximum ethanol titer was 14.2 g/L; Hari Krishna et al. [6] used *S. cerevisiae* NRRL-Y-132 for the SSF of the 10 % alkaline H_2O_2-pretreated sugar cane leaves at 40°C, and the ethanol titer was 18 g/L. Although various thermotolerant *S. cerevisiae* strains were tested at 40°C, none of them was employed in the SSF at both the elevated temperature (40°C and above) and the high solids loading of the pretreated lignocellulose to produce high ethanol concentration, which is required by the downstream product purification and reduction in the distillation energy cost [7, 8]. On the other hand, the accumulated toxic inhibitors and the ethanol concentration by increased solids loading impaired the fermentation performance of *S. cerevisiae* strains [9] and, as a result, the thermotolerant behaviors of the strains may be down-regulated. Furthermore, as the substrate loadings in SSF increase, cellulose saccharification and ethanol production generally tend to decrease [1, 7].

S. cerevisiae DQ1 has demonstrated its stress resistance and robustness for the SSF of the stream explosion-pretreated corn stover (CS) [7, 10] and the dilute acid-pretreated corn stover [11, 12] at high solids loading (up to 30 % *w*/*w*) at moderate temperature (37°C). In the present study, the thermotolerance of *S. cerevisiae* DQ1 was tested

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in the SSF of the dilute acid-pretreated corn stover at high solids loading. Various operation modes and fermentation parameters were conducted and the results showed that, using the present *S. cerevisiae* DQ1 strain, the SSF of the dilute acid-pretreated corn stover could tolerate up to 40°C and reach a high ethanol titer of 48 g/L and the ethanol yield of 65.6 %, demonstrated the better fermentation performance than the previous reports on *S. cerevisiae* when the actual lignocellulose was applied under practical operation conditions [4, 5, 13]. The present study provided a practical potential for the future SSF of lignocellulose feedstock at high temperature to reach high ethanol titer.

Materials and Methods

Raw Material and Pretreatment Operation

Corn stover was grown in Jilin province, China, and harvested in Fall 2007. The corn stover was milled and screened through a mesh with a circle diameter of 5 mm. The milled materials were washed, dried, and stored in sealed plastic bags for use. The CS composition was determined using Foss 2021 Cellulose Analyzer (Foss A/S, Hillerod, Denmark). The composition analysis was the two-step sulfuric acid hydrolysis. First, the CS sample was hydrolyzed using 4.9 % (*w*/*w*) sulfuric acid solution supplemented with 1.96 % hexadecyl trimethyl ammonium bromide, in which step the hemicellulose was removed from the sample; then, the CS residues were subsequently hydrolyzed by 72 % (*w*/*w*) sulfuric acid solution, where the cellulose was removed. The raw material contained 32.1 % glucan, 26.4 % xylan, and 8.1 % lignin (*w*/*w*, dry weight base). The pretreatment was performed at 190°C for 3 min using 2.5 g sulfuric acid per 100 g dry CS using a 10-L reactor at the full filling ratio of the presoaked corn stover with the solids/liquid presoaking ratio of 2.0, and the liquid content of the pretreated corn stover was 50 % (*w*/*w*) after the hot steam was absorbed onto the pretreated corn stover material. Therefore, no free liquid fraction was generated and the mass loss of the corn stover could be safely ignored. The details of this "dry" pretreatment were described in Zhang et al. [12]. The pretreated CS without washing could give an 85.1 % glucose yield at 5 % solids loading [12]. The dilute acid-pretreated CS was washed with tap water at threefolds of the pretreated CS weight and stirred for 1 h. Then, the liquid was squeezed with a hydraulic press machine at 15 MPa (P-204, Dazhang Filter Equipment, Shanghai, China) until the solid content closed to around 50 % (*w*/*w*). After pretreatment, the substrate contained 33.7 % glucan and 3.7 % xylan. The pretreated corn stover was washed for only one time before use in the SSF experiments. The yeast used could not ferment the sugars from the hemicellulose part of the corn stover. This fraction could be used for biogas production or fermented to ethanol by other proper microorganisms. The washed pretreated CS was neutralized to around pH 5.0 by adding 5 M NaOH before use.

Microorganism and Enzymes

A thermo- and inhibitor-tolerant mutant strain *S. cerevisiae* DQ1 was developed and stored in China General Microbial Collection Center with registration number 2528. The culture solution was aliquoted into 2-mL vials containing 30 % (*w*/*w*) glycerol and stored in a freezer at −80°C. One vial of *S. cerevisiae* DQ1 from the freezer was directly inoculated in the seeding culture. The inoculum used for SSF of the dilute acid-pretreated corn stover was cultured through a three-step hydrolysate adaptation procedure as described in the previous work [7]. A vial of *S. cerevisiae* DQ1 was firstly inoculated into a 100-ml Erlenmeyer flask containing 20.0 ml of sterilized synthetic medium, cultured in a shaking incubator at 30°C, 150 rpm, for 18 h; then, the culture was inoculated into a 100-ml flask containing 20.0 ml of sterilized medium containing 50 % of corn stover hydrolysate and 50 % of synthetic medium (pH 5.0), cultured at 30°C, 150 rpm for 15 h; finally, the culture was inoculated into a 500-ml flask containing 200.0 ml of sterilized hydrolysate medium at pH 5.0, cultured at 30°C, 150 rpm for 15 h. The synthetic medium contained (per liter) 20.0 g glucose, 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, and 1.0 g yeast extract. The optical density of yeast cell in the inoculum at 600 nm was approximately 11. An OD_{600} of 1 equaled to 0.5 g/L dry cell mass (DCW). Thus, the initial yeast concentration of the SSF was equivalent to 0.55 g/L DCW at the inoculation ratio of 10 % (*v*/*v*).

The fermentation of *S. cerevisiae* DQ1 in the YPD medium (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L) was carried out in a 100-mL Erlenmeyer flask with a working volume of 20 mL at pH 5.0 and 150 rpm. The fermentation temperatures were set to 30, 35, 37, 40, 42, and 44°C, respectively. All the experiments were done in triplicate.

The cellulase enzyme used in this study was Accellerase 1000 from Genencor International (Rochester, NY, USA). The filter paper activity and the cellobiase activity were determined to be 65.8 FPU/ml and 152.0 IU/ml, respectively.

SSF Operation

The SSF operation was carried out in a 5-L bioreactor equipped with a helical stirrer as described previously [7] using washed pretreated corn stover as the substrate. The pH and stirring rate were maintained at 5.0 and 150 rpm, respectively. Two SSF modes at elevated temperatures and high solids loading proposed in this study are shown in

Fig. 1. Figure 1a indicates the regular one-step SSF; the *S. cerevisiae* DQ1 culture was inoculated at the beginning of the SSF at different temperatures (30, 35, 40, or 42°C). Figure 1b indicates the three-step SSF which was a hybrid process encompassing an enzymatic prehydrolysis step and a two-stage SSF at different temperatures. First, the enzymatic prehydrolysis of the pretreated corn stover was conducted for 12 h at 50°C (step 1), then the inoculum was added and the fermentation was conducted at 35°C for 24 h (step 2), and in the final step the temperature was increased to 40°C for 36 h (step 3). The pretreated corn stover was gradually fed into the bioreactor. The samples were withdrawn at regular intervals and the first sample was taken after adding the total substrate. The initial concentration of furfural, 5- hydroxymethylfurfural, and acetic acid was 0.6, 0.25, and 4.0 g/L, respectively, in the SSF at 30 % (*w*/*w*) solids loading. Unless otherwise stated, nutrients were added to the SSF experiments including 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4{\cdot}7H_2O$, and 1 g/L yeast extract.

Analytical Methods

Glucose, ethanol, and lignocellulose degradation, such as furfural, 5-hydroxymethylfurfural, and acetic acid, were measured using an HPLC (LC-20 AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with a Bio-rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at the column temperature of 65°C with 5 mM H_2SO_4 as eluent and with a flow rate of 0.6 mL/min. Samples periodically taken from the fermentations were centrifuged at 13,000 rpm for 5 min and then filtered through a 0.22-μm filter before injection.

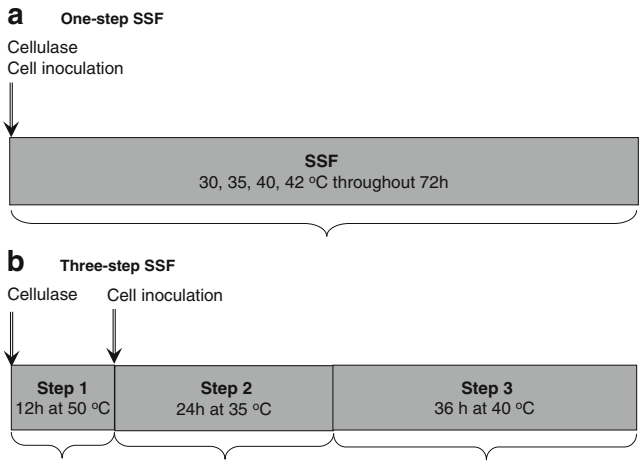

Fig. 1 Schematic diagram of the one-step SSF (**a**) and the three-step SSF (**b**) operation modes. *Step 1* enzymatic prehydrolysis, *Step 2* and *Step 3* SSF

Results and Discussion

Ethanol Fermentation in the YPD Medium and the SSF of the Pretreated Corn Stover

The thermotolerance and the growth of *S. cerevisiae* DQ1 were examined in the YPD medium at 30, 35, 37, 40, 42, and 44°C, respectively, as shown in Fig. 2a. Cell growth by optical density measurement at 600 nm (OD_{600}) decreased with increasing temperature and a sharp decrease occurred when the temperature was above 40°C. The glucose in the YPD medium was consumed completely at 30, 35, 37, and 40°C within 24 h, while at 42 and 44°C only 35 and 13 % of the original glucose was consumed, respectively. Ethanol production followed a similar tendency with the cell growth: it decreased with increasing temperature with a sharp decrease above 40°C. The results indicate that *S. cerevisiae*

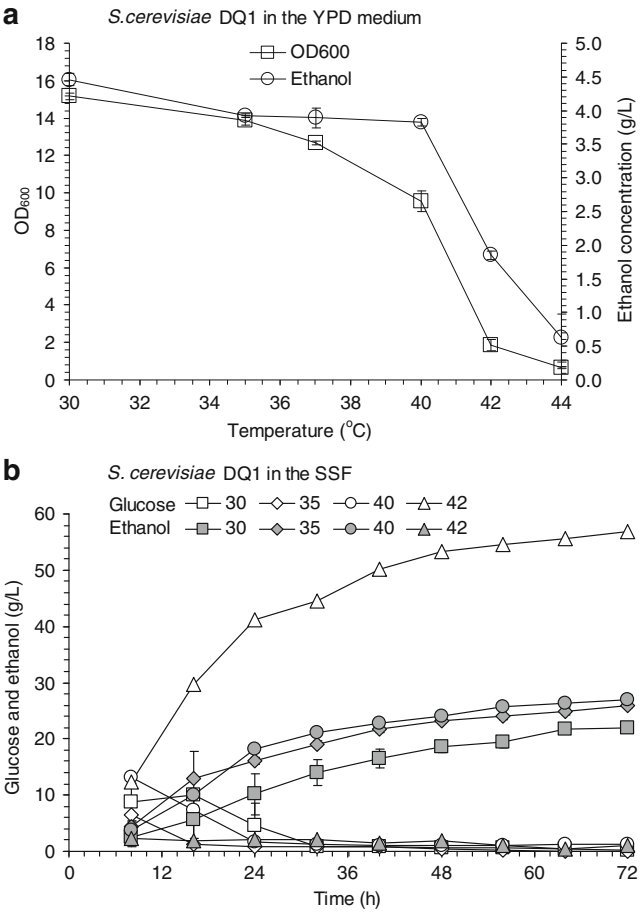

Fig. 2 Comparison of ethanol fermentation of *S. cerevisiae* DQ1 at different temperatures in the YPD medium and the SSF operation using the dilute acid-pretreated corn stover. **a** The experiment was carried out in a 100-mL flask with 20 mL YPD medium with shaking of 150 rpm at 30, 35, 37, 40, 42, and 44°C. **b** The experiment was carried out in a 5-L helical stirring reactor at 20 % (*w*/*w*) solids loading, 5 FPU/g DM according to the one-step SSF procedure (Fig. 1a), pH 5.0, and 150 rpm

DQ1 cells could grow and produce ethanol at elevated temperatures below 40°C; when above 40°C, the cells could still survive but with significantly low ethanol titer.

Figure 2b shows the SSF of *S. cerevisiae* DQ1 at 20 % (*w*/*w*) solids loading of the dilute acid-pretreated CS with a relatively low cellulase loading (5 FPU/g DM) at different temperatures of 30, 35, 40, and 42°C. Since the determination of cell growth (OD_{600}) was not applicable for yeast in the SSF operation, glucose consumption and ethanol formation were measured. The results showed that ethanol fermentation performance in the SSF was similar to that in the YPD medium. When the temperature is below or at 40°C, both glucose consumption and ethanol production were carried out normally. The final ethanol titer increased with increasing temperatures from 30, 35, to 40°C, indicating that the elevated temperature could improve SSF performance in terms of ethanol titer. The improvement of ethanol titer should come from the enhanced hydrolysis of cellulose by cellulase at the elevated temperatures because ethanol formation from glucose showed no increase at the elevated temperatures (Fig. 2a). When the SSF temperature was 42°C, only hydrolysis of the pretreated CS occurred and the released glucose accumulated to 58 g/L at 72 h, while almost no ethanol was formed. The result indicates that cell growth of *S. cerevisiae* DQ1 in the SSF of the pretreated CS decreased sharply above 40°C, and the phenomenon was in agreement with that in the YPD medium.

Effects of Nutrients, Cellulase Dosage, and Solids Loading of the Pretreated CS on SSF

Nutrients in the medium are important for the cell growth of microorganisms in general [14]; thus, various nutrients were supplemented into the SSF broths, aiming to enhance cell survival at elevated temperatures and high solids loading. Table 1 shows the SSF performance of the pretreated CS at 40°C and the 20 % solids loading with different nutrients supplementation, including $(NH_4)_2SO_4$, yeast extract, and distillers' dried grains with solubles (DDGS). Unfortunately, no significant improvements on ethanol fermentation were found by the addition of nutrients to the SSF of the pretreated CS. This result indicates that the addition of nutrients was not the prominent factor on the SSF of the pretreated CS at elevated temperatures and high solids loading. The result was also in agreement with previous studies using *Kluyveromyces marxianus* strain [15, 16].

The effect of cellulase dosage on the ethanol production of the SSF of the pretreated CS at elevated temperatures and high solids loading was investigated and the results were shown in Fig. 3. Figure 3 suggests that, at 40°C and the 20 % (*w*/*w*) solids loading, there was no significant increase of the ethanol titer observed with the increasing cellulase dosages from 5 to 15 FPU/g DM. At the cellulase loading of 15 FPU/g DM, the glucose released was accumulated and the ethanol production decreased compared with that at the cellulase dosage of 5 and 10 FPU/g DM. Although evidences have shown that cellulase may harm the cell walls of the fermentation strains [17, 18], the SSF performance of the pretreated CS at moderate temperatures such as 30–37°C was always improved with the increased cellulase dosage, even at high solids loading [7]. However, the results in Fig. 3 indicate that, at an elevated temperature close to or above 40°C, the fermentation cell viability may become more sensitive to cellulase enzymes. This occurrence might be explained by the accelerated wall decomposition and the reduction of cell growth by cellulase as indicated in the ordinary cultivations [17, 18]. The results here demonstrated that this phenomenon might exist in the SSF of the dilute acid-pretreated corn stover due to the increase of substrate or cellulase loadings at the elevated temperatures.

The SSF of higher solids loading is required to its maximum point because the higher feedstock concentration is the prerequisite for the higher ethanol titer and then consequently leads to the reduced energy consumption in the downstream distillation [7, 15]. The effect of the solids (the pretreated CS) loading of the SSF on ethanol production was carried out at

Table 1 Effect of various nutrients addition on SSF of the dilute acid-pretreated corn stover at 40°C

	Control[a]	$(NH4)_2SO_4$ (g/L)[b]				Yeast extract (g/L)[c]		DDGS (g/L)[d]			DDGS (g/L)[e]	
	–	5	2	1	0	1	0	12.5	10	5	10	5
Final ethanol (g/L)	24.9	25.9	26.0	26.2	26.0	26.2	24.5	25.5	25.8	24.9	24.5	25.6
Residual glucose (g/L)	1.47	2.37	1.30	1.27	1.03	1.27	1.48	0.93	1.35	1.67	1.08	1.12

SSF was conducted in a 5-L helical stirring reactor at 20 % (*w*/*w*) solids loading with 5 FPU/g DM, 40°C, 150 rpm, and pH 5.0 according to the procedure in Fig. 1a. The parameters were measured at 72 h of SSF

[a] Without any external nutrients addition

[b] With variable $(NH_4)_2SO_4$ while other nutrients were added as described in "Materials and Methods"

[c] With/without yeast extract while other nutrients were added as described in "Materials and Methods"

[d] DDGS to replace yeast extract while other nutrients were added as described in "Materials and Methods"

[e] Supplemented with DDGS alone as nutrients

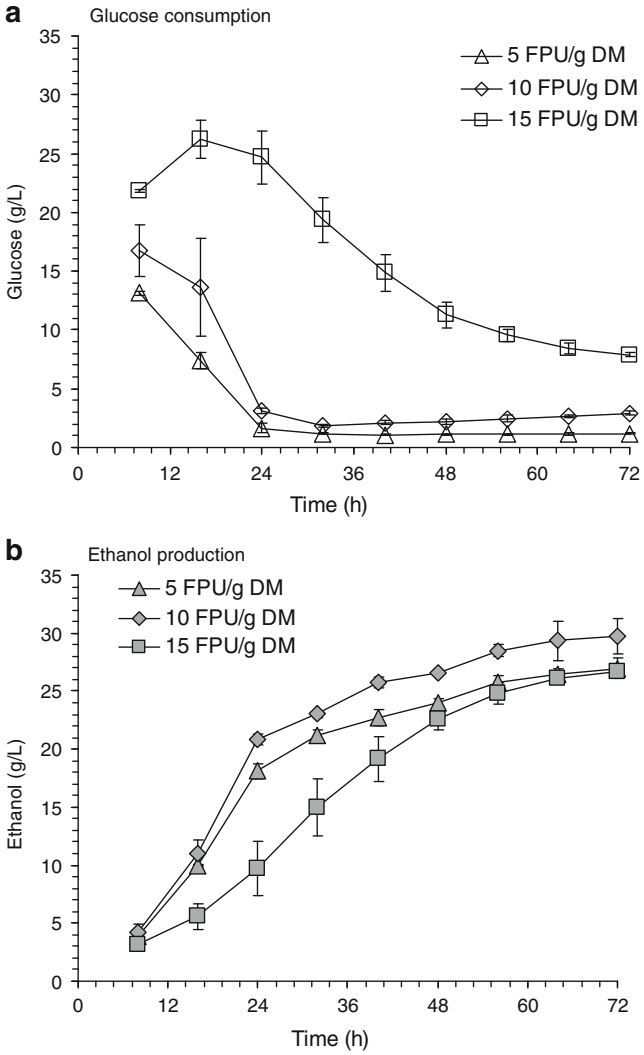

Fig. 3 **a**, **b** SSF of the diluted acid pretreated corn stover at different cellulase loadings. 40°C and 20 % (*w*/*w*) solids loading in a 5-L helical stirring reactor according to the one-step SSF procedure (Fig. 1a)

40°C and 5 FPU/g DM and the results are shown in Fig. 4. Again, the elevated temperature of the SSF showed a strong negative effect on ethanol production at high solids loading. Figure 4 indicates that the ethanol concentration decreased with increasing solids loading from 20, 25, and 30 % (*w*/*w*). The glucose released accumulated to a considerably high level at 25 and 30 % (*w*/*w*); ethanol fermentation was significantly poor at 25 % (*w*/*w*) and almost halted at 30 % (*w*/*w*). As a comparison, ethanol concentration increased with the solids loading at 37°C [7, 8]; the elevated temperatures negatively affected the cell growth of *S. cerevisiae* DQ1 and the SSF performance at high solids loading. In the SSF operation, the glucose or cellobiose from cellulose hydrolysis was almost consumed completely. The effect of end-product inhibition may not be the reason for the decreasing degree of hydrolysis. However, as our previous study has shown, the ethanol formed demonstrated a significant inhibitory effect on the hydrolysis performance of the cellulase with an increase of the solids loading [19]. As the solids loading increased, the difficulty of the substrate mixing and the increased possibility of non-productive bind between celulase and the substrate might also account for the decrease in the degree of hydrolysis.

Operation Mode on the SSF of the Dilute Acid-Pretreated Corn Stover

As stated above, the SSF of the pretreated CS at the elevated temperatures (40°C or above) showed different fermentation behaviors compared to that at moderate temperatures (30–37°C). At such conditions, the SSF could not proceed at higher solids and cellulase loadings. To improve the performance, a temperature swing operation was proposed as shown in Fig. 1b. In this three-step SSF (Fig. 1b), first, the

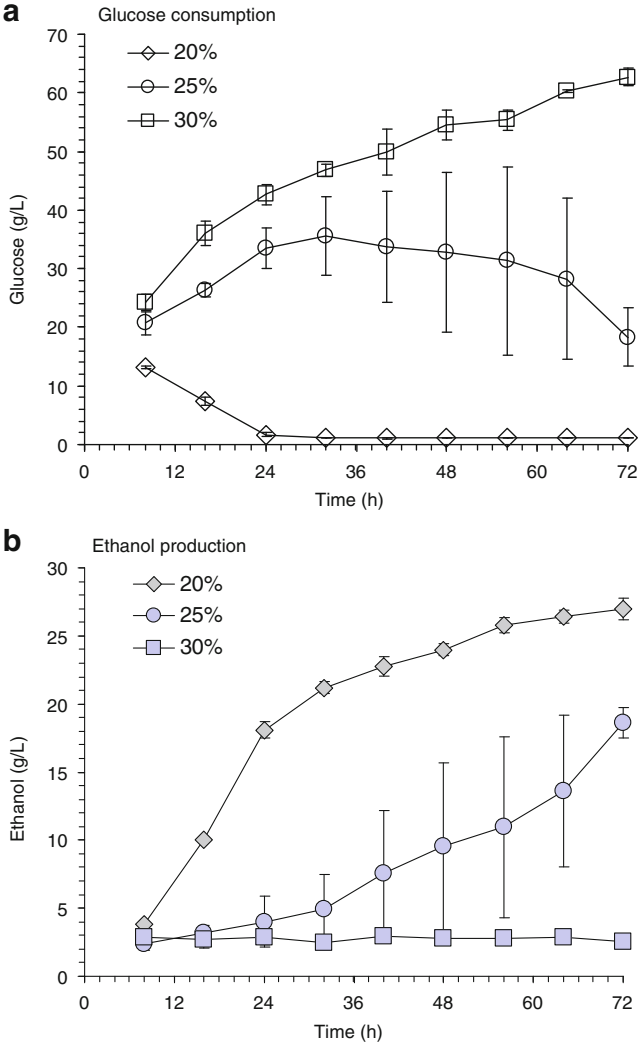

Fig. 4 **a**, **b** SSF of the dilute acid pretreated corn stover at different solids loading. 40°C and 5 FPU/g DM in a 5-L helical stirring reactor according to the one-step SSF procedure (Fig. 1a)

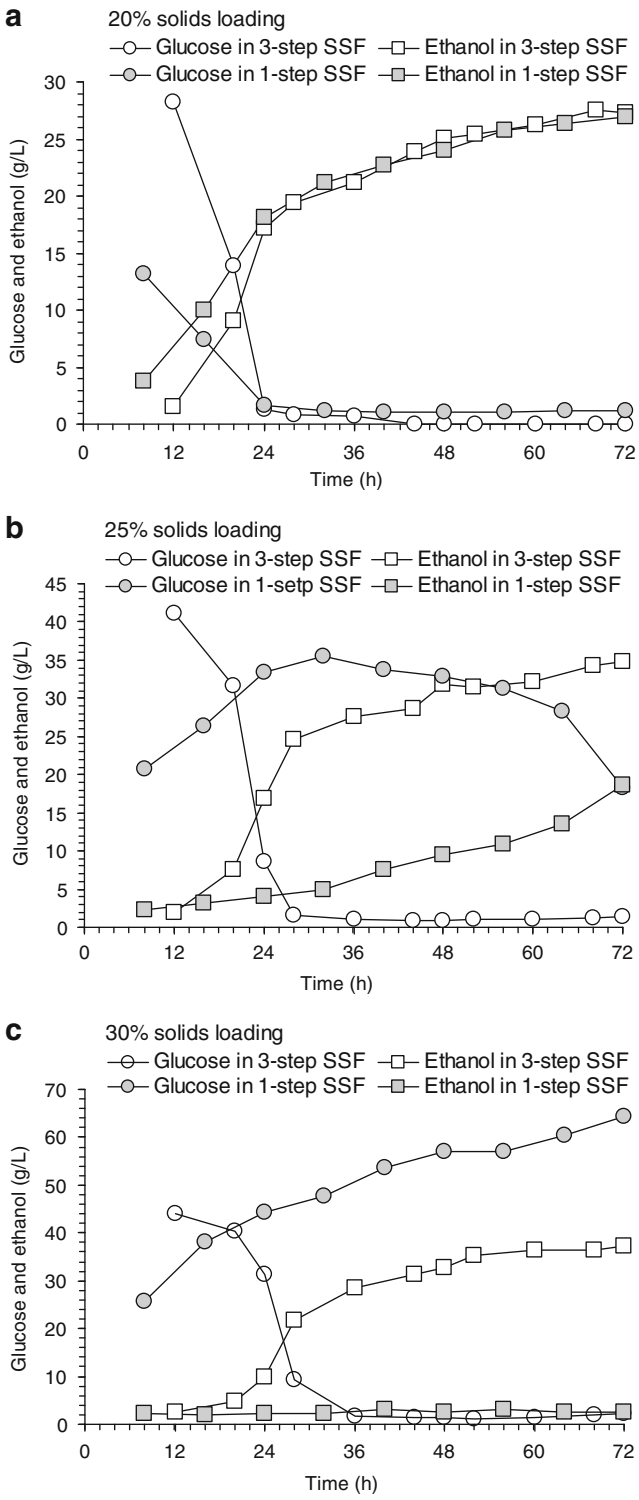

Fig. 5 Comparison of the one-step SSF and the three-step SSF using the dilute acid-pretreated corn stover at different solids loading of **a** 20 % (*w*/*w*), **b** 25 % (*w*/*w*), and **c** 30 % (*w*/*w*). 5 FPU/g DM in a 5-L helical stirring reactor. The operation procedure was shown in Fig. 1a, b. The one-step SSF was conducted at 40°C throughout the whole process. The three-step SSF was conducted at 50°C for 12 h, then 35°C for 24 h, and 40°C for 36 h

quick liquefaction and saccharification of the pretreated CS at a maximum temperature (50°C) for 12 h (step 1) was carried out, followed by acceleration of the cell growth of *S. cerevisiae* DQ1 at a mild temperature (35°C) for 24 h in the second step (step 2) and elevated-temperature SSF (40°C) for 36 h in the third step (Step 3). Figure 5 shows the comparison of the regular one-step SSF operation with the three-step temperature swing operation at the solids loading of 20, 25, and 30 % (*w*/*w*) with the cellulase dosage of 5 FPU/g DM. Figure 5 indicates that two operations gave similar results at 20 % (*w*/*w*) of the solids loading (Fig. 5a); at the solids loading of 25 % (*w*/*w*), the three-step SSF worked with no glucose accumulation and satisfying ethanol titer, while the one-step SSF gave poor ethanol fermentation performance (Fig. 5b); at the solids loading of 30 % (*w*/*w*), the three-step SSF gave a relatively fair ethanol

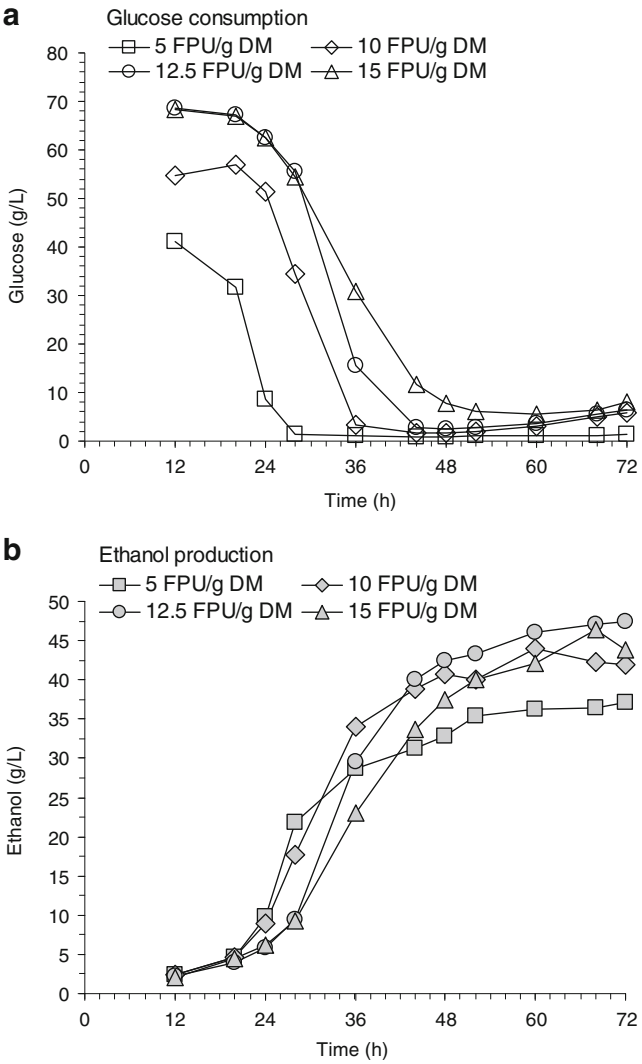

Fig. 6 Three-step SSF of the dilute acid-pretreated corn stover at different cellulase loadings. 5-L helical stirring reactor and 30 % (*w*/*w*) solids loading followed the procedure in Fig. 1b

fermentation, while the one-step SSF operation showed no ethanol fermentation (Fig. 5c).

To achieve the maximum ethanol titer, the three-step SSF at 30 % (*w*/*w*) solids loading was performed at different cellulase dosages. Figure 6 shows that the ethanol concentration increased with the increasing cellulase dosages from 5 to 12.5 FPU/g DM then decreased with the further increase to 15 FPU/g DM, similar to that in the one-step SSF operation. The result indicates that, at the present SSF conditions, the cellulase dosage up to 15 FPU/g DM might be a harmful dosage to cell viability. The results in Figs. 5 and 6 indicate that the three-step temperature swing operation improved the SSF performance at high cellulase dosage and solids loading compared to the one-step operation of SSF. The strain used at present, *S. cerevisiae* DQ1, could not utilize xylose from hemicellulose. It would result in higher ethanol yield if xylose was converted to ethanol using the proper xylose-utilizing strains.

Conclusions

Thermotolerant strain *S. cerevisiae* DQ1 was applied to the simultaneous saccharification and fermentation at high temperature and high solids loading of the dilute acid-pretreated corn stover in the present study. The SSF using *S. cerevisiae* DQ1 was operated at three-step SSF with changing temperature and the 30 % solids loading of the pretreated corn stover, and the ethanol titer and ethanol yield reached 48 g/L and 65.6 %, respectively. *S. cerevisiae* DQ1 showed a strong thermotolerance in both the regular one-step SSF and the three-step SSF with changing temperature in each step. The three-step SSF at 40°C using *S. cerevisiae* DQ1 tolerated the greater cellulase dosage and solids loading of the pretreated corn stover and resulted in increased ethanol production.

Acknowledgments This research was supported by National Basic Research Program of China (2011CB707406), Natural Science Foundation of China (20976051), and China Postdoctoral Science Foundation (2011M500742).

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