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Industrial cellulase performance in the simultaneous saccharification and co-fermentation (SSCF) of corn stover for high-titer ethanol production

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Abstract

Background: Cellulase enzymes contribute to the largest portion of operation cost on production of cellulosic ethanol. The industrial cellulases available on the industrial enzyme market from different makers and sources vary significantly in hydrolysis and ethanol, and finally lead to the changes of enzyme cost. Therefore, the selection of the proper industrial cellulase enzymes for commercial-scale production of cellulosic ethanol is crucially important in terms of high performance and cost reduction.

Results: In this study, three major cellulase enzyme products available on the Chinese industrial enzyme market were selected and evaluated as the biocatalysts for the biorefining process of lignocellulose biomass into high-titer ethanol. The cellulase enzymes included Cellic CTec 2.0 from Novozymes (Beijing), and LLC 4 from Vland (Qingdao), as well as # 7 from an industrial enzyme maker. The detailed assays on the filter paper activity, the cellobiase activity, and the total protein contents of the enzymes were conducted according to the standard protocols. When the cellulase enzymes were applied to the practical hydrolysis and ethanol-fermentation operation under the conditions of high solids loading and low range of cellulase dosage, the hydrolysis yield shows the significant difference, and the difference was narrowed in the final ethanol yield.

Conclusions: The commercially available cellulase enzymes showed different performances in the activities, the cellulose hydrolysis yield, and the ethanol fermentation yields based on the protein dosage per gram of cellulose of corn stover. In general, the industrial cellulase products give satisfactory performance and can be applied for the practical cellulosic ethanol production on commercial scale.

Keywords: Industrial cellulase enzyme, Activity, Ethanol, Hydrolysis, Lignocellulose

Background

Currently cellulosic ethanol is on the way to its large scale commercialization in USA, Europe, and China. Beta renewables first launched the first commercial cellulosic ethanol plant with the annual ethanol production of 40,000 metric tons from corn stover in Oct 2013, Italy (Ramesh 2013). DuPont biofuels solution started the plant for production of 89,600 metric tons of ethanol annually from corn stover and switchgrass. Abengoa Bioenergy produced 74,900 metric tons of ethanol annually from corn stover and other non-feed energy crops. Poet-DSM produced 59,700 metric tons of ethanol annually from corn stover (Chiaramonti et al. 2013). In China, Shandong Longlive Co. used corncob residue from xylitol industry and produced 50,000 metric tons of ethanol (Lei et al. 2014). However, due to the reasons of low petroleum price and relatively high cost, the factories are not operated in full scale and further modifications on the plants are still going on.

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In the trend of commercialization of cellulosic ethanol, a proper cellulase enzyme with high hydrolysis performance and low cost is crucially important because cellulase enzyme contributes to the largest portion of the cost on lignocellulose biorefining process (Klein-Marcuschamer et al. 2012; Gang et al. 2016). In the past several decades, great efforts were made by the enzyme industry worldwide, and several high-performance cellulase enzymes had been developed and introduced into the market for practical use in cellulosic ethanol production. The latest cellulase enzyme products include the CTec series from Novozymes, such as the recent products of Cellic CTec 2, Cellic CTec 3, and HTec 3 (Chen et al. 2016); and the Accellerase series from the former Genencor (now part of DuPont), such as Accellerase 1500 (Marcos et al. 2013). On the Chinese industrial enzyme market, several homemade cellulase enzymes are available for use in cellulosic ethanol production (Zhang et al. 2015, 2016; Liu and Wang 2014).

In this study, we selected three major cellulase enzyme products available on the China industrial market including Cellic CTec 2.0 from Novozyme (Beijing), LLC 4 from Vland (Qingdao), and # 7 from an industrial enzyme maker as the saccharification biocatalyst of corn stover. The lignocellulose feedstock, corn stover, was pretreated by dry dilute acid pretreatment (DDAP) and biologically detoxified to remove the inhibitors, then hydrolyzed at the high solids loading (30%, w/w) of the pretreated and detoxified corn stover. The ethanol fermentation was performed under the simultaneous saccharification and co-fermentation (SSCF) by a xylose utilizing yeast strain to achieve the high-titer ethanol and yield. The results indicate that the industrial enzymes available as cellulase products in the market give the satisfactory performance in general and can be applied for the practical cellulosic ethanol production on commercial scale.

Methods

Corn stover feedstock

Corn stover (CS) was harvested from Bayan Nur League, Inner Mongolia Autonomous Region, China in fall 2015. The collected corn stover was milled coarsely and screened through a mesh with the circle diameter of 10 mm. Then the milled corn stover was water washed to remove the field dirts, stones and metal pieces, and air dried. The composition of corn stover was determined by the two-step acid hydrolysis method according to National Renewable Energy Laboratory (NREL) protocols (Sluiter et al. 2008, 2012). On dry weight base (w/w), corn stover contained 35.4% of cellulose, 24.6% of hemicellulose, 16.1% of lignin, and 3.5% of ash.

Cellulase enzymes

We selected three representative commercial cellulases from the Chinese industrial enzyme market, by the availability, the production capacity, and the representativeness in activity and cost, including Cellic CTec 2.0 [kindly donated by Novozymes (China), Beijing, China], Vland LLC 4 (purchased Vland Biotech, Qingdao, China), and # 7 (purchased from an industrial enzyme maker). CTec 2.0 and LLC 4 are liquid enzymes, and # 7 is solid enzyme. The liquid enzyme was taken as water and the mass of the total liquid of the saccharification process was calculated. The # 7 enzyme is the solid enzyme produced by the adsorption of the liquid enzyme onto the solid bran particles and dried for the purpose of long-term storage. There is no need for the extraction step because the bran is hydrolyzed very quickly when the enzyme is put into the hydrolysis system and the cellulase enzyme adsorbed on the bran is releases into the hydrolysate. The filter paper activity was determined according to the NREL protocol LAP-006 (Adney and Baker 1996). The cellobiase activity was determined using the method of Ghose (1987). The total protein concentration was determined by Bradford method using bovine serum albumin (BSA) as protein standard (Bradford 1976). The cellulase enzyme was used based on the total protein weight per gram of cellulose substrate in the biomass feedstock.

The reagents KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, and H_2SO_4 were purchased from Lingfeng chemical reagent, Shanghai, China. Yeast extract was procured from Angel Yeast Co., Yichang, Hubei, China. Agar was purchased from Aladdin BioChem, Shanghai, China.

Strains and medium

Biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated in our previous works and stored in China General Microorganism Collection Center (CGMCC), Beijing, China with the registration number 7452 (Zhang et al. 2010). *A. resinae* ZN1 was maintained on a potato dextrose agar medium (PDA) slant. The PDA medium was prepared by boiling 200 g of peeled and sliced potatoes in 1 L deionized water for 30 min. 15 g/L of agar was added for preparation of PDA slant for *A. resinae* ZN1 growth.

Ethanol fermentation strain S*accharomyces cerevisiae* XH7 was an engineered strain from the wild diploid *Saccharomyces cerevisiae* BSIF (Li et al. 2015). The strain was cultured in YPD medium containing 20 g/L of glucose, 20 g/L of peptone, and 10 g/L of yeast extract. The culture vial was stored in the YPD medium containing 30% of glycerol at −80 °C freezer.

The media used included (1) activation medium, 20 g/L of glucose, 20 g/L of peptone, 10 g/L of yeast extract; (2) seed culture medium, 5% (w/w) of the pretreated and

biodetoxified corn stover, the cellulase dosage of 10 mg protein per gram of cellulose, 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract; (3) adaptation seed medium, 10% (w/w) of the pretreated and biodetoxified corn stover, the cellulase dosage of 10 mg protein per gram of cellulose, 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract. The seed culture medium contained only low concentration of xylose from the pretreated corn stover feedstock and no glucose or pre-hydrolysate in it at the beginning of the culture. As the seed culture proceeds, the added pretreated and detoxified corn stover is hydrolyzed into soluble glucose by the cellulase, and the glucose is acting as the carbon source for the seed cell growth. In this way, the pure glucose sugar is saved by corn stover feedstock; and (4) SSCF medium, 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract.

Pretreatment, biodetoxification, and SSCF

Corn stover was pretreated using the dry dilute acid pretreatment (DDAP) method (Zhang et al. 2011; He et al. 2014). The major components' contents and the inhibitors' contents in the pretreated biomass feedstocks were determined according to NREL protocols (Sluiter et al. 2008, 2012). The pretreated biomass was briefly disk milled to remove the long cellulose fibers to avoid the blockage of the slurry flow of the downstream hydrolysate and broth.

The pretreated biomass solids were biodetoxified in a 15-L bioreactor at 28 °C and aeration for 36 h to remove the inhibitors generated during the dry dilute acid pretreatment operation (Zhang et al. 2010; He et al. 2016).

The pretreated solids were firstly converted into liquid hydrolysate slurry containing both monosaccharides (glucose and xylose) and oligomer sugars (oligo-glucan and oligo-xylan) in the specially designed 5 L bioreactors equipped with helical ribbon impeller (Zhang et al. 2011) at 50 °C, pH 4.8 for 12 h. Then glucose and xylose were co-fermented into ethanol simultaneously with the further hydrolysis of cellulose and oligomer sugars (simultaneous saccharification and co-fermentation, SSCF) by the engineered *Saccharomyces cerevisiae* XH7 at the high solids loading (30%, w/w) in the same bioreactor at 30 °C for 96 h by inoculating the shortly adapted yeast seed cells into the hydrolysate at 10% (v/v). The nutrients added included 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract. Samples were periodically withdrawn and for analysis of glucose, xylose, ethanol, glycerol, acetic acid, furfural, and 5-hydroxymethylfurfural (HMF).

The *S. cerevisiae* XH7 seed broth was prepared in a two-step adaption procedure using the pretreated biomass feedstock as the carbon source instead of glucose sugar (Qureshi et al. 2015).

Calculation of ethanol yield

The ethanol yield (%) in SSCF and the xylose utilization (%) were calculated based on the method proposed by Zhang and Bao (2012):

$$\text{Ethanol yield (\%)} = \frac{[\text{Ethanol}] \times W}{976.9 - 0.804 \times [\text{Ethanol}]} \cdot \frac{1}{0.511 \times ([\text{Cellulose}] \times 1.111 + [\text{Xylose}]) \times [\text{Solids}] \times M} \times 100\%$$

where [Ethanol] is the concentration of ethanol in the fermentation broth at the end of the SSCF (g/L); W is the total water input into the hydrolysis or the SSCF system (g); M is the total weight of the hydrolysis or the SSCF system at the beginning of the operation (g); [Cellulose] is the cellulose content in the dry pretreated solids (g/g); [Xylose] is the xylose content in the dry pretreated solids (g/g); [Solids] is the pretreated solids loading of the hydrolysis and SSCF system on the dry-weight base (g/g); 976.9 is the ethanol correction factor (g/L) between the mass concentration (g/g) and the volumetric concentration (g/L); 0.804 is the dimensionless factor in calculating water loss in SSCF; 1.111 is the dimensionless conversion factor for cellulose to equivalent glucose; 0.511 is the dimensionless conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast.

Xylose conversion is calculated by measuring the percentage ratio of the decreased xylose concentrations in the hydrolysate at the beginning and the end of the SSCF operation over the total xylose concentration.

Analysis

Sugars, ethanol, acetic acid, 5-HMF, Furfural, and Glycerol were analyzed on HPLC (LC-20AD, Shimazu, Kyoto, Japan) equipped with a Bio-rad Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) and RID-10A detector (Shimadzu, Kyoto, Japan). 5 mM H_2SO_4 solution was used as flow phase at the flow rate of 0.6 mL/min. Furans were analyzed on HPLC (LC-20AT, Shimazu, Kyoto, Japan) equipped with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan).

The yeast cell viability in the simultaneous saccharification and co-fermentation (SSCF) was assayed by counting the colony-forming units (CFU) on the YPD (Gu et al. 2015) petri dish when the 100 μL of the 10^{-5} or 10^{-6} diluted fermentation broth withdrawn at different time points were stretched and cultured for 48 h at 30 °C.

Results and discussion

Enzyme assays of filter paper unit, cellobiase activity, and total protein content

The activities and protein contents of the three industrial cellulase enzymes from different makers were assayed. The filter paper unit (FPU/mL), the cellobiase activity or β-glucosidase activity (CBU/mL), and the total protein content (mg/mL) of Cellic CTec 2.0, # 7, and LLC 4 were determined as shown in Table 1. The results show that the filter paper activity and the cellobiase activity of CTec 2.0 and Vland LLC 4 were similar based on the volumetric basis, while # 7 is relatively low (only 30% of the filter paper activity and 2% in the cellobiase activity of CTec 2.0). The total protein concentrations of CTec 2.0 and LLC 4 were also similar at 75–90 mg/mL, while that of # 7 was less than half of the two. The specific filter paper activities of CTec 2.0 and LLC 4 were also close, and that of # 7 was about half of the first two enzymes. # 7 was absorbed on wheat bran solid particles; thus, its specific activity was relatively lower than that of the other two enzymes.

SSCF assay under the same cellulase protein additions

The hydrolysis performances of the three industrial cellulase enzymes were evaluated under the high solids loading of pretreated corn stover and the simultaneous saccharification and co-fermentation (SSCF). The corn stover feedstock was pretreated by dry dilute acid pretreatment (DDAP) and then biologically detoxified to remove the inhibitors. The moderate cellulase dosages of the pretreated corn stover and a xylose utilizing *S. cerevisiae* XH7 strain were used. The pre-hydrolysis lasted for 12 h at 50 °C, and then the SSCF was started and performed for 96 h (Fig. 1; Table 2).

The 12-h pre-hydrolysis of 30% (w/w) solid content system generated 82.4, 74.3, and 63.6 g/L of glucose by CTec 2.0, LLC 4, and # 7, respectively. The hydrolysis result indicates that the hydrolysis capacity of CTec 2.0 is advantage to LLC 4 and # 7. The 96-h SSCF generated 76.3, 73.0, 76.0 g/L of ethanol and 73.8, 73.7, 76.4% of conversion yields by CTec 2.0, LLC 4, and # 7, respectively. Similar ethanol titer and yield were obtained in the given cellulase dosage (10 mg protein per gram of cellulose), although the considerable difference in hydrolysis capacity existed. The SSCF results indicate that the all the three cellulase worked well in the SSCF to achieve the high ethanol titer and yield, but certain differences existed. The xylose conversions achieved were 83.4, 85.6, and 89.5%, respectively. when CTec 2.0, LLC 4, and # 7 were used. The cell viability result shows that the lower cellulose conversion and glucose accumulation by # 7 led to the better cell growth of the fermenting yeast cells, while CTec 2.0 and LLC 4 with the high hydrolysis yields

Table 1 Activity assays of the industrial cellulase enzymes

Enzyme type	Cellic CTec 2.0	Vland LLC 4	#7
Total protein content (mg/mL or mg/g)	87.3	75.8	46.7
Filter paper unit (FPU/mL or FPU/g)	203.2	199.4	63.0
β-glucosidase unit (CBU/mL or CBU/g)	4900	5500	99.9
FPU and CBU ratio (FPU/CBU)	1:24.1	1:27.6	1:1.6
Specific filter paper unit (FPU/mg protein)	2.33	2.62	1.35

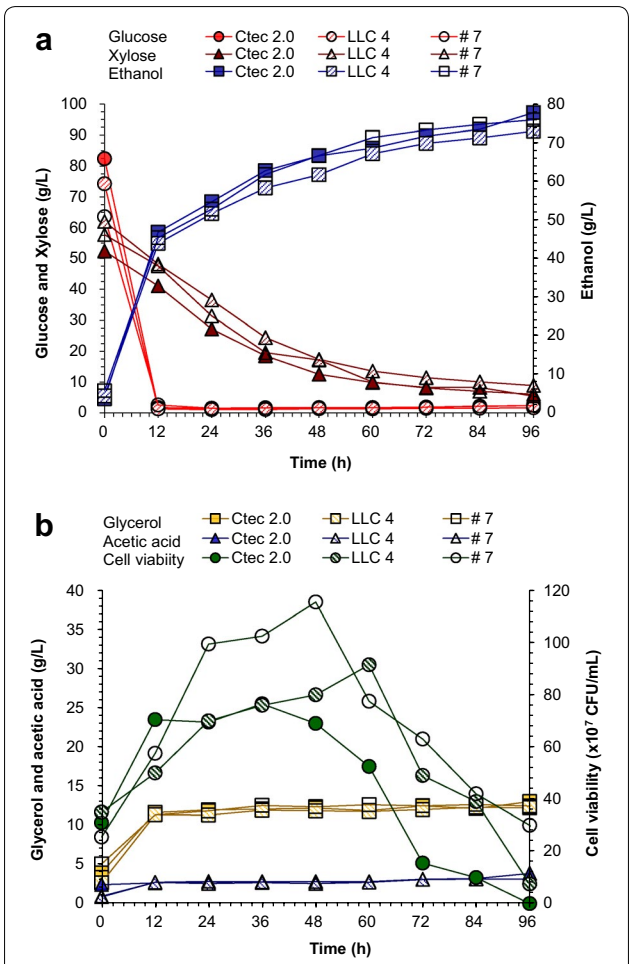

Fig. 1 Ethanol fermentability evaluation of the selected industrial cellulase enzymes by SSCF at the same cellulase dosage based on the cellulose content. **a** Glucose, xylose and ethanol concentrations about SSCF of dry dilute acid pretreated and biodetoxified corn stover, **b** cell viability, glycerol and acetic acid concentrations about SSCF. Conditions: 30% solids loading, cellulase dosage of 10 mg total protein/g cellulose. 10% (v/v) inoculum ratio of *S. cerevisiae* XH7 seed. In pre-hydrolysis step, 50 °C, pH 4.8, 12 h; In SSCF step, 30 °C, pH 5.5, 96 h

Table 2 SSCF of corn stover using different industrial cellulase enzymes

	Pre-hydrolysis		SSCF			
	Glucose (g/L)	Xylose (g/L)	Ethanol titer (g/L)	Ethanol yield (%)[a]	Xylose conversion (%)	Glycerol (g/L)
Cellic CTec 2.0	82.39	52.38	78.50	77.72	90.83	12.79
Valnd LLC 4	74.26	62.00	77.25	78.32	86.29	12.84
# 7	63.58	57.70	77.09	77.62	90.08	12.87

[a] The sugars in the enzyme solution or solids were not taken into account

did not help in making the xylose conversion. Glycerol formation accumulated to about 12 g/L and considerably reduced the ethanol yield, revealing that the fermenting yeast was under the stress of high glucose concentration then easily led to the glycerol formation.

Cellulase enzymes may also contain some soluble carbohydrates (Zhang et al. 2007), which can be directly used for ethanol production in SSCF. Three industrial cellulases, CTec 2.0, LLC 4, and # 7, contained 277.1, 13.4, and 12.9 mg/g of glucose, respectively, as well as 36.2, 4.4, and 0.9 mg/g of xylose. At 30% solids loading, and with the cellulase dosage of 10 mg protein per gram of cellulose, the three enzymes would provide 4.88, 0.32, and 0.40 g/L of carbohydrates (including glucose and xylose) which theoretically generated 2.5, 0.16, and 0.20 g/L of ethanol, individually. Compared with the other two enzymes, CTec 2.0 supplied more fermentable sugars for SSCF. However, the ethanol obtained from the sugars was quite lower, and it was difficult to calculate it accurately in practice. As a result, the effect of the carbohydrates from the cellulase on the ethanol production was not considered in this study.

SSCF assays under varying cellulase enzyme dosage

Following the same enzyme dosages for use in SSCF, different cellulase dosages of each industrial enzyme on the SSCF performance were tested in the ranges of 5, 10, 15, and 25 mg total protein per gram cellulose of the pretreated corn stover as shown in Fig. 2.

The 12-h pre-hydrolysis assay shows the similar tendency at varying cellulase dosages. The hydrolysis capacity of CTec 2.0 showed the maximum hydrolysis yield at each cellulase dosage from 5 to 25 mg/g, followed by the LLC 4 and then # 7. The hydrolysis yield increased with the increasing cellulase dosage but the increase almost ceased or slowed down due to the increasing glucose inhibition on the cellulase enzyme activity. When the cellulase dosage was in the range of 5–10 mg protein per gram of cellulose, the glucose concentrations increased by 66, 21, and 17% with the use of # 7, CTec 2.0, and LLC 4, respectively. When the cellulase dosage was in the range of 10–15 mg protein per gram of cellulase, the glucose concentration increased by 14, 11, and 12%, respectively. When the cellulase dosage was in the range of 15–25 mg protein per gram of cellulose, the glucose concentrations only increased by 7 and 10% by CTec 2.0 and LLC 4, respectively, while # 7 did not show the increase in glucose concentration. The xylose utilization by # 7 was the optimal because of the low glucose concentration provided.

The SSCF assay shows that the ethanol titer and yield from the SSCF by CTec 2.0, LLC 4, and # 7 were close (about 80 g/L) when the cellulase dosage was in the higher range of 10, 15, 25 mg protein per gram of cellulose, indicating the higher overdose of cellulase enzyme did not help in further improvement of ethanol titer and yield. High cellulase dosage led to the high glucose concentration in the pre-hydrolysis step, and the high glucose inhibited the cell growth and conversion rate of glucose and xylose to ethanol. However, the minimum dosage of cellulase (5 mg protein per gram of cellulose) showed the differences in the ethanol titer and yield by CTec 2.0, LLC 4, and # 7 were significantly different: the 96-h SSCF generated 68.1, 68.7, 56.1 g/L of ethanol and 66.6, 69.0, 49.8% of conversion yields by CTec 2.0, LLC 4, and # 7, respectively. The ethanol titer and yield by # 7 were relatively lower at the minimum dosage of cellulose. As shown in Fig. 2c, d, the activities of filter paper unit (FPU) and cellobiose (CBU) of # 7 were much lower than those of CTec 2.0 and LLC 4 at the same cellulase dosage, especially in the minimum range, which inevitably led to lower hydrolysis yield, ethanol titer, and ethanol yield by # 7.

Based on the SSCF results at the 10 mg protein per gram of cellulose, for producing 1 kg of cellulosic ethanol, 21.6 g of cellulase protein of CTec 2.0, equivalent to 247 g of the liquid enzyme; or 20.9 g cellulase protein of LLC 4, equivalent to 275 g of the liquid enzyme; or 21.3 g of cellulase protein of # 7, equivalent to 457 g of the solid enzyme, is needed. If the cellulase enzyme is based on the same price per kilogram enzyme by volume or weight, then CTec 2.0 is the least expensive. However, if the enzyme is sold based on the total proteins of the enzyme product, the cost of the enzymes is similar for the three enzymes.

Conclusion

The industrial cellulase enzymes shows significantly different performances in activity and cellulose hydrolysis yield, and less significant ethanol titer and yield based

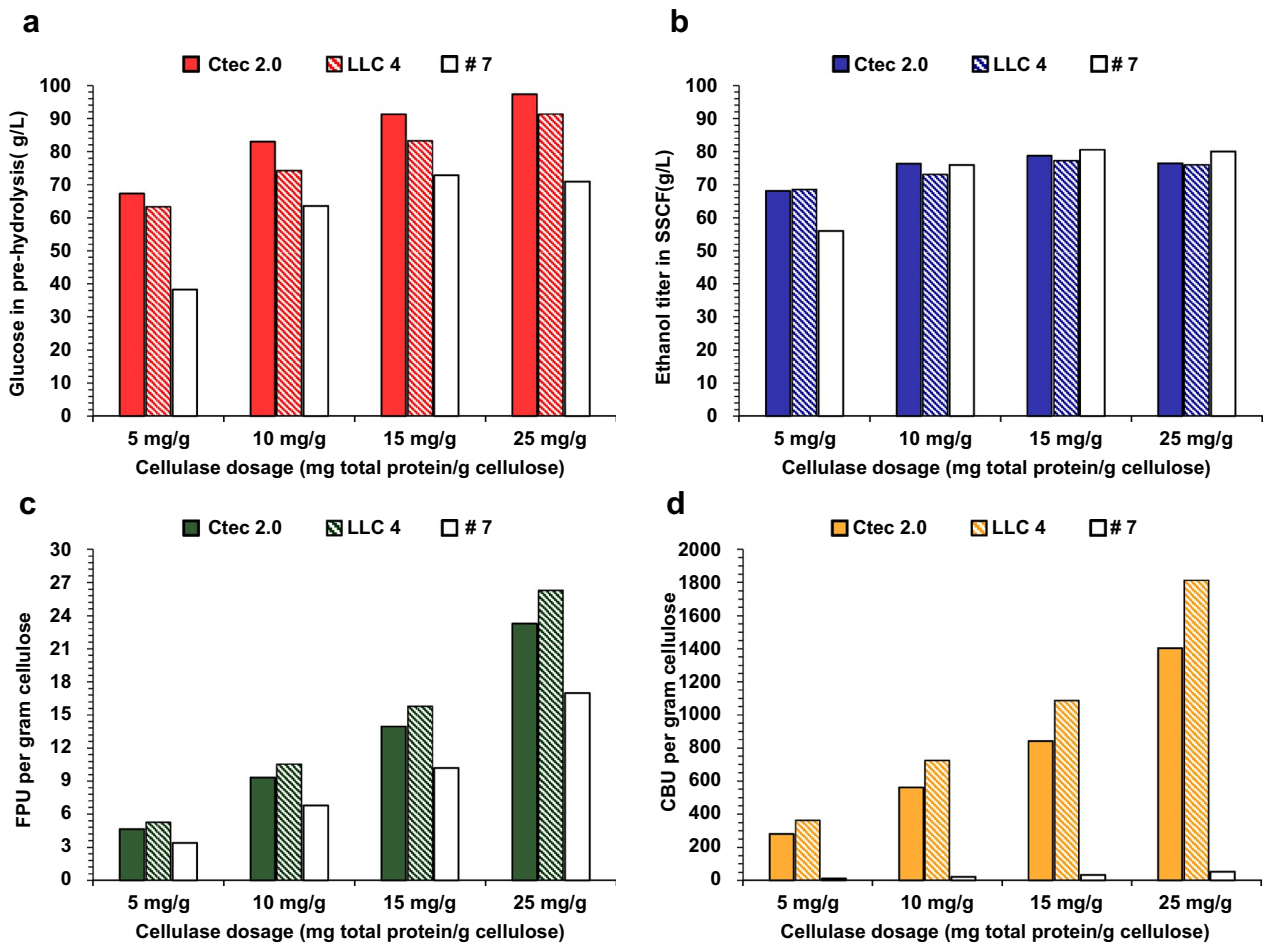

Fig. 2 Ethanol fermentability evaluation of the selected industrial cellulase enzymes by SSCF at the varying cellulase dosage based on the cellulose content. **a** Glucose concentrations after 12 h pre-hydrolysis, **b** final ethanol concentrations about SSCF, **c** filter paper unit (FPU) per gram cellulose about SSCF, **d** β-glucosidase unit (CBU) per gram celluose about SSCF. Conditions: cellulase dosage of 5, 10, 15, 25 mg total protein/g cellulose; 30% solids loading; 10% (v/v) inoculum ratio of *S. cerevisiae* XH7 seed. In pre-hydrolysis step, 50 °C, pH 4.8, 12 h; In SSCF step, 30 °C, pH 5.5, 96 h

on the total protein dosage per gram of cellulose of corn stover. In general, the industrial enzymes available as cellulase products in the market give satisfactory performance and can be applied for the practical cellulosic ethanol production on a commercial scale.

Authors' contributions

QZ and JB designed and wrote the experiment; QZ conducted the experiment; JB conceived the research. Both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included in the main manuscript file.

Ethics approval, consent to participate, and consent for publication

All authors have read and approved the manuscript before submitting it to bioresources and bioprocessing. There is no competing interests to declare by any author in relation to the submission.

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