Appl Biochem Biotechnol (2010) 160:360–369
DOI 10.1007/s12010-008-8306-0

Influence of High Solid Concentration on Enzymatic Hydrolysis and Fermentation of Steam-Exploded Corn Stover Biomass

**Yifeng Lu · Yonghong Wang · Guoqian Xu · Ju Chu ·
Yingping Zhuang · Siliang Zhang**

Received: 26 January 2008 / Accepted: 24 June 2008 /

Abstract Steam-exploded corn stover biomass was used as the substrate for fed-batch separate enzymatic hydrolysis and fermentation (SHF) to investigate the solid concentration ranging from 10% to 30% (*w*/*w*) on the lignocellulose enzymatic hydrolysis and fermentation. The treatment of washing the steam-exploded material was also evaluated by experiments. The results showed that cellulose conversion changed little with increasing solid concentration, and fermentation by *Saccharomyces cerevisiae* revealed a nearly same ethanol yield with the water-washed steam-exploded corn stover. For the washed material at 30% substrate concentration, i.e., 30% water insoluble solids (WIS), enzymatic hydrolysis yielded 103.3 g/l glucose solution and a cellulose conversion of 72.5%, thus a high ethanol level up to 49.5 g/l. With the unwashed steam-exploded corn stover, though a cellulose conversion of 70.9% was obtained in hydrolysis at 30% solid concentration (27.9% WIS), its hydrolysate did not ferment at all, and the hydrolysate of 20% solid loading containing 3.3 g/l acetic acid and 145 mg/l furfural already exerted a strong inhibition on the fermentation and ethanol production.

Keywords Bioethanol · Corn stover · High solid concentration · Enzymatic hydrolysis · Fermentation

Introduction

Replacement of petroleum by biofuel becomes an inevitable trend in many countries worldwide [1]. It is driven by the aims of a secure and sustainable energy supply and an expectation to decrease the greenhouse gas emission. Renewable biofuel, including bioethanol, becomes a topic issue all over the world [2]. The bioethanol used today is mainly produced from sugar or starch. However, it is clear that the large-scale use of bioethanol will require lignocellulosic biomass to be used as raw material [3].

Y. Lu · Y. Wang (✉) · G. Xu · J. Chu · Y. Zhuang · S. Zhang
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology,
Shanghai 200237, China
e-mail: yhwang@ecust.edu.cn

Several schemes to use lignocellulosic biomass including corn stover for the bioethanol production have been indicated [4, 5]. The general concept includes a pretreatment stage that increases the digestibility of the material followed by enzymatic hydrolysis. In fermentation stage, the soluble sugar transforms into ethanol. And finally, the product ethanol is produced after distillation process. Corn stover is an abundant agricultural by-product with low cost [6]. But now, most of the corn stover is wasted mainly because sugars in corn stover exist as macromolecules, like glucan and xylan, and these macromolecules combined with lignin to form a complex structure. In order to recover sugars in the corn stover for fermentation and ethanol production, a complicated process is necessary. It was verified that steam-explosion pretreatment and subsequent enzymatic hydrolysis was an effective method to recover monomeric sugars at high yield [7–11].

In bioethanol production process, the commercial feasibility becomes the most important factor. Very high solid content (30% and above) is widely used in fuel ethanol industry based on starch [12]. Operation with high substrate loading will increase the product concentration and facilitate the downstream process and product recovery, which may have a significant effect on capital cost and operating cost due to reduce energy expenditure for distillation and decrease the number of operation to reach the same ethanol output. A material from Energy Efficiency and Renewable Energy shows that about 10% overall operation cost may be reduced when the solid level was increased from 20% to 30% [13]. Several studies also have demonstrated that the ethanol concentration should reach about 4% to 5% (*w*/*v*) to make the process economically feasible. It is reported that the energy demand reduced about two thirds by increasing the initial ethanol concentration from 1% to 5% in a single distillation unit for final concentration of 94.5% (*w*/*w*) [14]. For corn stover material, 4% (*w*/*v*) final ethanol concentration requires a solid level above 20% (assuming 80% glucose theoretical yield is achieved and an ethanol yield of 0.5 g/g glucose according to the composition of our material).

Unfortunately, hydrolysis process at solid concentration above 15% may face technical problem. The high solid concentration causes corresponding high viscosity, which makes mixing difficult and additional power consumption in the ethanol production process [15]. So, reported results by batch operation at solid concentrations above 15% are scarce. Fed-batch mode hydrolysis has been suggested as a possibility to solve some of the problems. Several groups have used a fed-batch strategy to hydrolyze material with solid concentration up to 17% [16, 17]. On the other hand, simultaneous saccharification and fermentation (SSF) of steam-exploded corn stover up to 30% (*w*/*w*) solid level and wheat straw solid concentration up to 40% have been reported [18, 19]. In pilot scale plants, 15% to 20% solid level has been reported as maximum that can be operated [20]. But they all reported that the glucose yield gradually decreased with the increasing solid concentration, and the cellulose conversion is reduced to about 40% at 30% (*w*/*w*) solid level. Apparently, the result is unacceptable in large-scale industrial bioethanol production.

Another problem in the whole bioethanol production process is the by-product produced in the pretreatment stage [21, 22]. The crude sugar solution from the pretreatment process contains various inhibitors such as formic acid, acetic acid, furfural, and 5-hydroxymethyl furfural. These components are toxic to microorganisms, showing negative effects on the subsequent fermentation process. This is a well-known problem associated with the hydrolysis process yet to be resolved. The situation becomes more severe under high solid loading and may result in a non-fermentable hydrolysate [23, 24].

In this study, steam explosion was carried out as a pretreatment method to prehydrolyze the corn stover. The steam-exploded stover was washed by water before enzymatic hydrolysis. The concentration of toxic by-products in the steam-exploded corn stover decreased after being washed by water because most of those harmful components were water soluble. Fed-batch separate enzymatic hydrolysis and fermentation (SHF) at 10%, 15%, 20%, 25%, and 30% (*w*/*w*) solid concentrations were investigated in shake flask using traditional baker's yeast (*Saccharomyces cerevisiae*). Compared to the water-washed material, unwashed steam-exploded corn stover was also tested as control to evaluate the effect of by-products derived from the process of steam explosion on the enzymatic hydrolysis and fermentation.

Materials and Methods

Raw Material and Pretreatment

All the investigations were performed using corn stover from the northeast of China as raw material. After collection, the corn stover was chopped, air-dried, and then stored at room temperature. Steam-explosion pretreatment was performed in StakeTech batch system (SunOpta Bioprocess, Canada) at the condition of 205 °C, 2.0 MPa for 6 min. In the process, only vapor was used as catalyst and to achieve high pressure. The exploded material (49.8% DM) was recovered in a cyclone and stored in a plastic bag. The recovery of glucose/xylose following the pretreatment was 98.6%/77.2%.

Water-wash process of the steam-exploded corn stover was carried out at a custom-built tank with stirring impeller. The water was added until the slurry reached 10% (*w*/*w*) solid concentration (equaled 9.3% WIS). The steam-exploded stover was washed for 10 min at 100 rpm to remove toxic degradation from steam-explosion process. After being washed, the corn stover was pressed to expel water, air-dried, and then stored at room temperature (78.4% DM). The liquid fraction from water-wash process contained 0.3 g/l glucose, 7.2 g/l xylose, 0.81 g/l acetic acid, and 36 mg/l furfural.

Enzymatic Hydrolysis

The pretreated material was thoroughly put to enzymatic hydrolysis using commercial acidic cellulase (121.8 FPU/g mixture, 46.1 Cellobiase IU/g mixture, 476 CMC IU/ g mixture, Global Green Tech, China). Hydrolysis was performed at 50 °C and 220 rpm for 96 h in 250-ml shake flasks (working weight 175 g) with different solid concentrations of 10%, 15%, 20%, 25%, and 30% (*w*/*w*), respectively. In this work, both water-washed and unwashed steam-exploded corn stovers were tested. For the washed material, solid concentration equaled water insoluble solids (WIS); for unwashed stover, WIS equaled 0.93× solid concentration (100 g dried unwashed material remained 93.0 g after being washed and dried). In the process, acidic cellulase (20 FPU/g dry stover) and 0.1 M citric buffer (pH 4.8) was employed. Two different strategies were used with various solid levels. For relatively lower solid concentrations (10% and 15%, *w*/*w*), material was thoroughly loaded at the beginning of hydrolysis reaction. For higher solid levels, the hydrolysis was initially loaded at 15% (*w*/*w*), then after 2-h hydrolysis, 8.8 g/16.5 g dry material was added to reach 20%/25% (*w*/*w*) respectively. For the 30% (*w*/*w*) solid level, 13.1 g dry material was added after 2-h hydrolysis, and another 13.1 g dry material was added after the second 2-h hydrolysis.

Fermentation

The fermentation process was also performed in 250-ml shake flasks with work weight of 175 g using the whole slurry from the enzymatic hydrolysis stage. In each shake flask, 0.1% (*w*/*w*) active dry baker's yeast (*S. cerevisiae*; Angel Yeast, China) and 0.1% (*w*/*w*) urea were inoculated. The whole system was kept at 30 °C and 100 rpm for 48 h in the process of fermentation.

Analytical Methods

The cellulose content of corn stover before steam explosion, water-washed and unwashed steam-exploded corn stover, was determined according to the National Renewable Energy Laboratory's analytical methods for biomass [25]. The sugar composition of the water extract was determined by high-performance liquid chromatography (HPLC) in an Agilent 1200 liquid chromatograph with refractive index detector. An AMINEX HPX-87P carbohydrate column (Bio-Rad, Hercules, CA, USA) operating at 85 °C with ultra pure water as a mobile phase (0.6 ml/min) was used. The cellulose and hemicellulose content of the extracted solid residue was determined based on monomer content measured after a two-step acid hydrolysis procedure to fractionate the fiber. A first step with 72% (*w*/*w*) H_2SO_4 at 30 °C for 60 min was used. In a second step, the reaction mixture was diluted to 4% (*w*/*w*) H_2SO_4 and autoclaved at 121 °C for 1 h. This hydrolysis liquid was then analyzed for sugar content by HPLC as described above. Glucose concentration from enzymatic hydrolysis and fermentation samples was measured by an enzymatic determination glucose assay kit (Sigma GAHK-20). The slurry viscosity was determined by NDJ-1 rotary viscosimeter (Shanghai geology instrument institute, Shanghai, China). The ethanol concentration and the by-product contents (acetic acid and furfural) were determined by GC-920 gas chromatography (Haixin Instrument, Shanghai, China). All of the analytical determinations were performed in duplicate. Relative standard deviations were in all cases below 5%.

Results and Discussion

Raw Material and Pretreatments

Corn stover from the northeast of China was used as raw material. After the steam-explosion pretreatment and water-wash process, the carbohydrate content of the substrates was different. The carbohydrate and lignin content were presented in Table 1.

Enzymatic Hydrolysis on Steam-Exploded Stover

During the process of enzymatic hydrolysis, the material changed from a solid into a liquid. The structure of the substrate was degraded (Fig. 1). The SEM image indicated steam-

Table 1 Composition of corn stover.

	Composition (%)[a]					
	Glucan	Mannan	Xylan	Arabinan	Galactan	Lignin
Raw material	36.9	0.8	24.7	3.2	1.7	19.9
Pretreated material (unwashed)[b]	38.6	0.9	24.3	3.9	2.4	21.7
Pretreated material (water-washed)	42.8	0.8	17.1	2.5	1.6	27.4

[a] Content based on dry matter

[b] Content involved monomeric sugars obtained from pretreatment

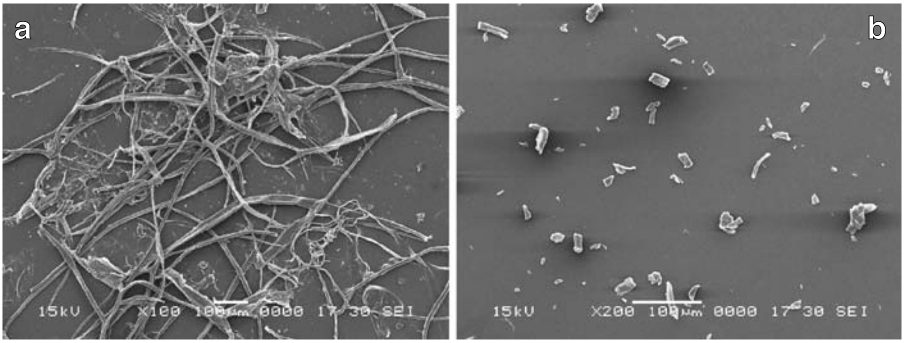

Fig. 1 SEM image of the corn stover substrate before (**a**) and after (**b**) enzymatic hydrolysis

exploded stover wound together and formed a complex structure before enzymatic hydrolysis. However, after saccharification process, the fabric totally disappeared; only some small pieces of stover were observed.

When water-washed steam-exploded stover was used as substrate, increasing solid concentration led to a highly concentrated glucose solution (Fig. 2a). The highest glucose concentration was obtained after 96 h of enzymatic hydrolysis using 30% substrate loading, and the final glucose level reached 103.3 g/l. It was shown that above 93% total glucose released in first 72 h and the total enzymatic hydrolysis was not a long time compared to other group's report at high solid level [26] (12% WIS, 96 h SSF). A similar tendency was found when hydrolyzing unwashed steam-exploded corn stover at varying solid levels (Fig. 2b). The final glucose concentration reached 85.1 g/l at 30% solid loading (27.9% WIS).

From the data of glucose concentration at different enzymatic hydrolysis time, the cellulose conversions were evaluated according to the glucan content and WIS in pretreated material (Table 1). The results are shown in Table 2. In the present work, it clearly appeared that glucose concentration followed the typical batch hydrolysis pattern and at lower solid levels, there is a rapid glucose release at the beginning of the process, then the glucose generation rate slowed down. The result was similar with the reports by other researchers

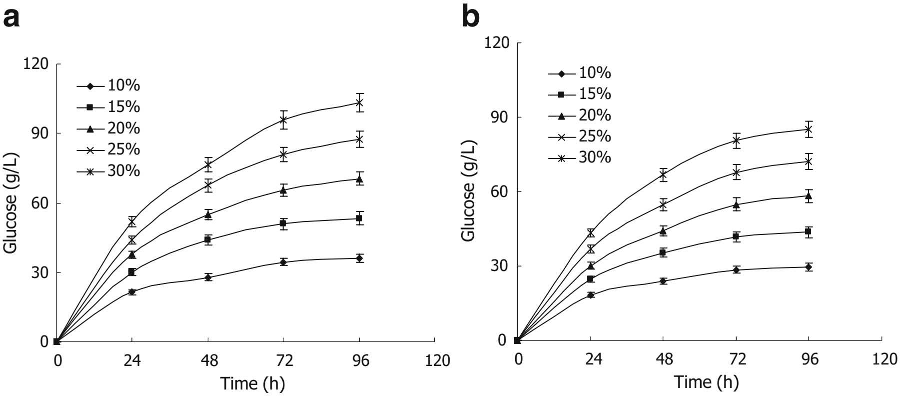

Fig. 2 Hydrolysis profiles of pretreated corn stover at different solid loadings (percent, *w*/*w*) under the condition of 20 FPU/g substrate and 50 °C. **a** water-washed stover; **b** unwashed stover

Table 2 Cellulose conversion at varying solid loadings.

Hydrolysis time (h)	Cellulose conversion (%)									
	Water-washed solid concentration (%)					Unwashed solid concentration (WIS), (%)				
	10	15	20	25	30	10 (9.3)	15 (14.0)	20 (18.6)	25 (23.3)	30 (27.9)
24	47.6	43.4	40.1	36.2	31.1	45.7	41.1	37.4	36.5	35.6
48	61.7	59.1	55.9	54.5	52.2	60.0	58.8	55.2	54.5	55.4
72	73.3	71.9	69.7	68.8	67.9	70.9	69.5	68.5	67.8	67.0
96	75.6	75.0	74.2	73.7	72.5	74.1	72.8	73.0	72.2	70.9

[27, 28]. For instance, 63.0% of the total glucose released in the whole 96-h hydrolysis was liberated within the first 24 h when using a water-washed substrate concentration of 10%. But for higher solid levels, the behavior was changed; the relative hydrolysis yield at the beginning of the process markedly reduced. For example, only 42.9% of total glucose hydrolyzed in the first 24 h at 30% solid loading. Interestingly, regardless of the initial hydrolysis rates, we found that the final glucose yield was very similar at varying solid loadings; the data ranged from 72.5% to 75.6% with washed stover. It indicated that the problem of product inhibition was not serious during our experiments, and high solid hydrolysis might reach the same level as the case of lower solid level after a relatively long hydrolysis process. And the cellulose conversion of unwashed material (based on WIS and the glucose released in pretreatment was subtracted according to the content of the liquid fraction from water-wash process) was similar as the washed pretreated stover.

Slurry Viscosity and Degradation Level During Enzymatic Hydrolysis

As shown in Fig. 3, the viscosity raised with the increasing solid loading. For the unwashed material, the initial viscosity reached 10,000 mPa·s with the 30% (*w*/*w*) solid concentration admixture. But, it dropped in the hydrolysis stage; finally, the data decreased to 2,000 mPa·s. Remarkably, the dispersion of viscosity between the samples with varying solid loadings was much closer after a 96-h hydrolysis reaction. For water-washed material, though the initial viscosity was evidently lower than the unwashed substrate at the same solid level, the final viscosity was nearly the same. These results indicated that at the beginning of enzymatic hydrolysis, the viscosity was very high, which might cause mass transfer problem in large-scale enzymatic hydrolysis process.

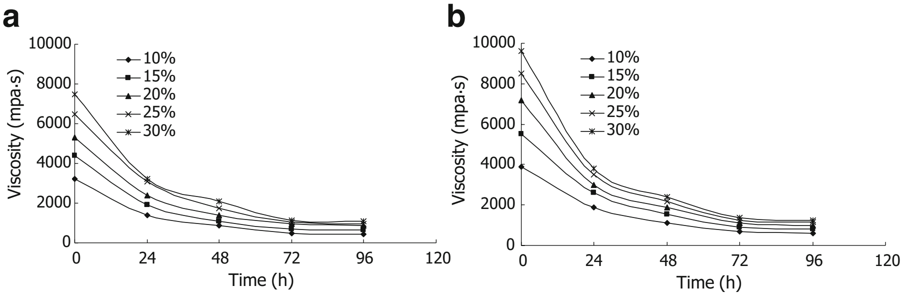

Fig. 3 Slurry viscosity during enzymatic hydrolysis. **a** water-washed stover; **b** unwashed stover

The slurry from pretreatment usually contains various toxic degradations. These by-products have a negative effect on subsequent fermentation process. During the operation at low solid level, the fact might be easily neglected. However, under high solid loading condition, it became a more noticeable problem. In present work, for both washed and unwashed steam-exploded corn stover, toxic by-products concentration raised with the increasing solid concentration (Fig. 4). Notably, both acetic acid and furfural level reduced by 2:3 ratio after water-wash process. For instance, the acetic acid concentration reached 4.7 g/l at 96 h when 30% unwashed pretreated stover was used, and the data became 1.5 g/l for water-washed substrate. It clearly demonstrated that pre-wash treatment might efficiently decrease the concentrations of most toxic by-products produced in steam-explosion stage.

Fermentation

After enzymatic hydrolysis, fermentation with different solid concentrations was evaluated with regard to ethanol yield. Figure 5 showed the profiles of glucose concentration and ethanol in fermentation processes. As shown in the figure, when washed stover was used as substrate, the ethanol concentration increased rapidly at the beginning of the process, and then ethanol generation slowed down. The overall ethanol yield reached 92% to 94% based on theoretical production from glucose (Fig. 5a,c). A different behavior was found when fermenting unwashed stover hydrolyzed slurry (Fig. 5b,d). The ethanol's final yield reached 88% and 86% at 10% and 15% solid level, and the yield dropped to 68% at 20% solid loading after 48-h fermentation process. When the solid concentration reached 25% and above, no ethanol was detected from the samples. It clearly showed that high toxic by-product concentration had profound negative effect on the fermentation stage. According to the former result showed in Fig. 4, when acetic acid and furfural reached 3.3 g/l and 145 mg/l respectively, the inhibitory effect on ethanol production was clearly observed. So,

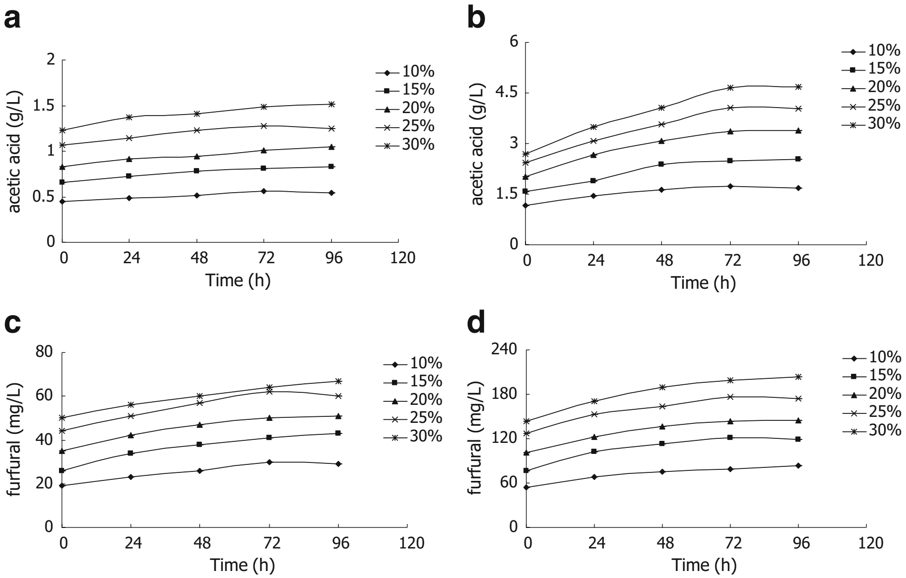

Fig. 4 Profiles of toxic degradation concentration during enzymatic hydrolysis. **a** acetic acid, water-washed; **b** acetic acid, unwashed; **c** furfural, water-washed; **d** furfural, unwashed

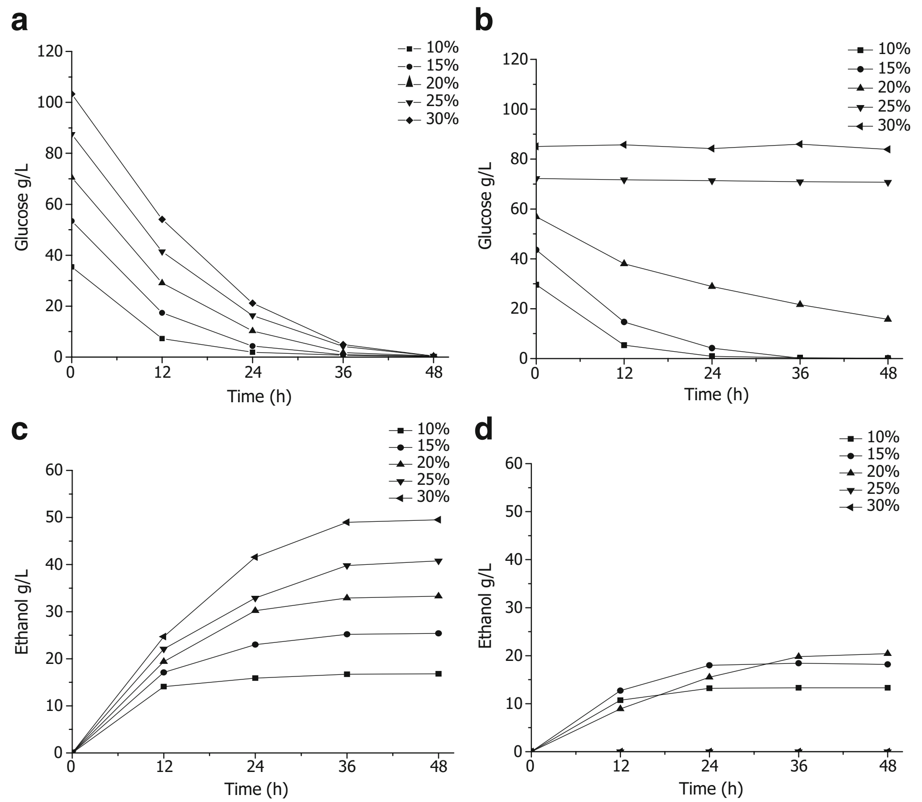

Fig. 5 Fermentation profile of pretreated corn stover with washing (**a** and **c**) and without washing treatment (**b** and **d**) at different solid loadings (%, *w*/*w*)

compared to unwashed stover, water-washed material was more suitable for fermentation stage.

Conclusion

High solid operation was a significant way to elevate the final ethanol concentration. For water-washed stover, when we used 30% solid instead of familiar 10% to 15% solid concentration, the final ethanol yield nearly did not decrease, and a proportional increase of final ethanol concentration was obtained. For instance, the final ethanol concentration increased by 195% when solid concentration was raised from 10% (*w*/*w*) to 30% (*w*/*w*). In present work, high solid concentration up to 30% (*w*/*w*) seemed feasible when water-washed stover was used as substrate in the process of bioethanol production. While solid concentration reached 30% (*w*/*w*), corresponding glucose concentration reached 103.3 g/l, then a final ethanol concentration up to 49.5 g/l was obtained. The glucose yield in the enzymatic hydrolysis process and ethanol yield in the fermentation reached 72.5% and 94.0%, respectively. And the overall yield reached 0.151 g ethanol/g raw stover based on the composition of the material. To the best of our knowledge, this result is higher than any other previous report under such a high solid concentration.

In our research, in the shake-flask system, not only mass and heat transfer problem was solved by fed-batch mode but also enough amount of cellulases was provided for enzymatic hydrolysis reaction. So, contrary to the results of Jørgensen et al. [19], the glucose yield did not linearly decrease with the increasing solid concentration. This implicated that the amount and efficiency of enzyme was also an important issue to be considered except the mixing problem.

In this work, for the water-washed pretreated material, about 2:3 acetic acid and furfural was washed-out during pre-wash step. Thus, the fermentation of hydrolysate at 30% solid level resulted in an ethanol yield up to 94.0%. On the other hand, with unwashed stover, the ethanol yield in the fermentation process profoundly decreased to 68% at 20% solid loading when the acetic acid and furfural concentration were only 3.3 g/l and 145 mg/l, respectively. These indicated that normal industrial *S. cerevisiae* strain for ethanol production based on starch was too sensitive to the degradation from pretreatment of lignocellulosic biomass, so breeding strains resisting to those toxic degradation by traditional or modern molecular method becomes inevitable and urgent.

Though we had succeeded in achieving high cellulose conversion and ethanol yield under high solid level in shake-flask scale, it still has a long way to go in large-scale industrial bioethanol production. Further researches on developing high efficiency and power-saving reactor and strain with inhibitor-tolerance are needed for the factory-scale bioethanol process.

Acknowledgements This work was financially supported by the National Basic Research Program (973 Program 2007CB714303) and Shanghai Leading Academic Discipline Project B505. We thank COFCO Bio-energy (Zhaodong) Limited for supplying the steam-exploded corn stover material and Green Global Inc. for kindly providing the enzyme.

References

1. Galbe, M., & Zacchi, G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production. *Advances in Biochemical Engineering/Biotechnology*, *108*, 41–65.
2. Varga, E., Klinke, H., Réczey, K., & Thomsen, A. B. (2004). High solids simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. *Biotechnology and Bioengineering*, *88*, 567–574. doi:10.1002/bit.20222.
3. Alexander, E., Farrell, D., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M., et al. (2006). Ethanol can contribute to energy and environmental goals. *Science*, *311*, 506–508. doi:10.1126/science.1121416.
4. Öhgren, K., Rudolf, A., Galbe, M., & Zacchi, G. (2006). Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content. *Biomass and Bioenergy*, *30*, 863–869. doi:10.1016/j.biombioe.2006.02.002.
5. Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., & Lee, Y. Y. (2005). Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, *96*, 1959–1966. doi:10.1016/j.biortech.2005.01.010.
6. Wingren, A., Galbe, M., & Zacchi, G. (2003). Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. *Biotechnology Progress*, *19*, 1109–1117. doi:10.1021/bp0340180.
7. Torget, R., Walter, P. J., Himmel, M., & Grohmann, K. (1991). Dilute-acid pretreatment of corn residues and short rotation woody crops. *Applied Biochemistry and Biotechnology*, *28/29*, 75–86.
8. Schell, D. J., Walter, P. J., & Johnson, D. K. (1992). Dilute sulfuric acid pretreatment of corn stover at high solids concentrations. *Applied Biochemistry and Biotechnology*, *34/35*, 659–665.
9. Kaar, W. E., & Holtzapple, M. T. (2000). Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover. *Biomass and Bioenergy*, *18*, 189–199. doi:10.1016/S0961-9534(99)00091-4.
10. Esteghlalian, A., Hashimoto, A. G., Fenske, J. J., & Penner, M. H. (1997). Modelling and optimization of the diluted-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresource Technology*, *59*, 129–136. doi:10.1016/S0960-8524(97)81606-9.

11. Kalman, G., Varga, E., & Reczey, K. (2002). Diluted sulphuric acid pretreatment of corn stover at long residence times. *Chem Biochem Eng Q*, *16*, 151–157.
12. Bayrock, D. P., & Ingledew, W. M. (2001). Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. *Journal of Industrial Microbiology & Biotechnology*, *27*, 87–93. doi:10.1038/sj.jim.7000167.
13. Schell, D. (2005). 2005 OBP biennial peer review, energy efficiency and renewable energy. http://programreview.biomass.govtools.us/%5Cdocuments%5C11e80e62-6e8a-4858-a0e5-1fa75a591116.ppt
14. Zacchi, G., & Axelsson, A. (1989). Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions. *Biotechnology and Bioengineering*, *34*, 223–233. doi:10.1002/bit.260340211.
15. Fan, Z. L., South, C., Lyford, K., Munsie, J., van Walsum, P., & Lynd, L. R. (2003). Conversion of paper sludge to ethanol in a semicontinuous solids-fedreactor. *Bioprocess and Biosystems Engineering*, *26*, 93–101. doi:10.1007/s00449-003-0337-x.
16. Rudolf, A., Alkasrawi, M., Zacchi, G., & Liden, G. (2005). A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. *Enzyme and Microbial Technology*, *37*, 195–204. doi:10.1016/j.enzmictec.2005.02.013.
17. Varga, E., Klinke, H. B., Reczey, K., & Thomsen, A. B. (2004). High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. *Biotechnology and Bioengineering*, *88*, 567–574. doi:10.1002/bit.20222.
18. Wayman, M., Parekh, S., Chornet, E., & Overend, R. P. (1986). SO_2-catalysed prehydrolysis of coniferous wood for ethanol production. *Biotechnology Letters*, *8*, 749–752. doi:10.1007/BF01032576.
19. Jørgensen, H., Vibe, J., Larsen, J., & Felby, C. (2007). Liquefaction of lignocellulose at high-solids concentrations. *Biotechnology and Bioengineering*, *96*, 862–870. doi:10.1002/bit.21115.
20. Tolan, J. S. (2002). Iogen's process for producing ethanol from cellulosic biomass. *Clean Technol Environ Policy*, *3*, 339–345. doi:10.1007/s10098-001-0131-x.
21. Lu, Y., Yang, B., Gregg, D., Saddler, J. N., & Mansfield, S. D. (2002). Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. *Applied Biochemistry and Biotechnology*, *98/100*, 641–654. doi:10.1385/ABAB:98-100:1-9:641.
22. Dien, B. S., Li, L., Iten, L. B., Jordan, D. B., Nichols, N. N., O'Bryan, P. J., et al. (2006). Enzymatic saccharification of hot-water pretreated corn fiber for production of monosaccharides. *Enzyme and Microbial Technology*, *39*, 1137–1144. doi:10.1016/j.enzmictec.2006.02.022.
23. Klinke, H. B., Thomsen, A. B., & Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass. *Applied Microbiology and Biotechnology*, *66*, 10–26. doi:10.1007/s00253-004-1642-2.
24. Palmqvist, E., Grage, H., Meinander, N. Q., & Hahn-Hagerdal, B. (1999). Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. *Biotechnology and Bioengineering*, *63*, 46–55. doi:10.1002/(SICI)1097-0290(19990405)63:1<46::AID-BIT5>3.0.CO;2-J.
25. National Renewable Energy Laboratory (NREL). Chemical analysis and testing laboratory analytical procedures, LAP-002 (1996), LAP-003 (1995), LAP-004 (1996), LAP-005 (1994), LAP-010 (1994) and LAP-017 (1998), NREL, Golden, CO, USA. http://www.eere.energy.gov/biomass/analytical_procedures.html.
26. Öhgren, K., Bengtsson, O., Gorwa, M. F., Galbe, M., Hahn, B., & Zacchi, G. (2006). Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. *Journal of Biotechnology*, *126*, 488–498. doi:10.1016/j.jbiotec.2006.05.001.
27. Linde, M., Galbe, M., & Zacchi, G. (2007). Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme and Microbial Technology*, *40*, 1100–1107. doi:10.1016/j.enzmictec.2006.08.014.
28. Kim, S. H., & Holtzapple, M. T. (2005). Lime pretreatment and enzymatic hydrolysis of corn stover. *Bioresource Technology*, *96*, 1994–2006. doi:10.1016/j.biortech.2005.01.014.