Appl Biochem Biotechnol (2012) 166:176–186
DOI 10.1007/s12010-011-9414-9

Maximum Saccharification of Cellulose Complex by an Enzyme Cocktail Supplemented with Cellulase from Newly Isolated *Aspergillus fumigatus* ECU0811

Dan Wang · Jie Sun · Hui-Lei Yu · Chun-Xiu Li · Jie Bao · Jian-He Xu

Received: 28 July 2011 / Accepted: 18 October 2011 /
Published online: 16 November 2011

Abstract Either the natural biodegradation process or the industrial hydrolytic process requires synergistic interactions between various cellulases. However, it is sometimes impeded by low hydrolytic rate of existing cellulases and the lack of accessory enzymes. Herein, the ability of a commercial cellulase (Spezyme CP, from Genencor) to degrade steam explosion-pretreated corn stover was significantly improved. Firstly, a fungal cellulase producer, *Aspergillus fumigatus* ECU0811, was isolated from hundreds of soil samples. A 96-deep-well microscale-based platform was developed here to reduce the labor-intensive screening work and proved to be consistent with macroscale screening work. After optimization of fermentation, 3% corn cob could induce *A. fumigatus* ECU0811 to yield the highest cellulase production. Based on the high activities of β-glucosidase and xylanase by *A. fumigatus* ECU0811, 0.91 and 125 U/mg protein, respectively, an enzyme cocktail was composed with a fixed dosage of Spezyme CP (CPCel) at 14.2 filter paper units (FPU)/g glucan and varied dosages of *A. fumigatus* cellulase (AFCel). Consequently, the glucan-to-glucose conversion of corn stover was increased from 25.6% in the presence of CPCel at a dosage of 14.2 FPU/g glucan to 99.5% in the presence of the enzyme cocktail (14.2 FPU CPCel plus 1.21 FPU AFCel per gram of glucan). On the other side, it reduced the total protein amount of CPCel by as much as tenfold, which extremely improved the hydrolytic rate of Spezyme CP and reduced its dosage.

Keywords *Aspergillus fumigatus* · β-glucosidase · Microplate · Enzyme cocktail · Corn stover

Dan Wang and Jie Sun contributed equally to this work.

D. Wang · J. Sun · H.-L. Yu · C.-X. Li · J. Bao · J.-H. Xu (✉)
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
e-mail: jianhexu@ecust.edu.cn

Introduction

Bioconversion of the lignocellulosic biomass in biorefineries is attractive for its advantages of being environmentally friendly and its low cost [1, 2]. The major polysaccharides comprising different lignocellulosic residues are cellulose and hemicellulose which could be finally hydrolyzed into glucose, xylose, and other monosaccharides [3, 4]. However, the natural biodegradation is a rather slow process: the required synergistic interactions among cellulase and variety accessory enzymes are not well optimized; the complicated compositions of natural biomass always exhibit as a heterologous matrix, impeding in complete hydrolysis [5, 6]. So *in vitro* loading of optimized enzyme cocktails to the pretreated lignocelluloses is becoming a primary protocol to overcome those bottlenecks [7–9].

The commercial cellulase, Genencor Spezyme CP (from *Trichoderma reesei*), has been studied for decades due to its distinguished cellulase activity [10–12]. Nevertheless, the Spezyme CP (CPCel) does not completely cleavage cellobiose into glucose since *T. reesei* produces β-glucosidase at low levels compared to other fungi such as *Aspergillus niger* [13]. Furthermore, β-glucosidase of *T. reesei* would cause glucose inhibition [14], which leads to insufficient saccharification of cellulose. As a result, such a disadvantage will include an extra expensive preparation of glucosidase or coupling with a cellobiose-utilizing strain [15, 16]. On an industrial scale, *T. reesei* cellulase preparations, supplemented with β-glucosidase, are most often considered [13, 17]. Driven by this motivation, we focused on discovering novel strains from unexploited natural wealth, with high cellulase activities, that will realize maximum biodegradation when supplying to commercial Spezyme CP.

Considering hundreds of environmental strains screening, subsequent biomass hydrolysis, and enzyme cocktail optimization, a microscale-based strategy is necessary to reduce these labor-intensive work. Several high-throughput platforms on the aspects of cellulase screening [18], cellulase activity assay [19], and enzyme cocktail hydrolysis [20] have been developed. More importantly, the data from such microscale researches are somehow consistent to macroscale researches [21, 22]. Herein, these reported strategies were modified in this study to adapt to 96-deep-well microplate, handling hundreds of strains cultivation, activity assay, enzyme cocktail, and product analysis, as illustrated in Fig. 1. As a result, a fungal strain, *Aspergillus fumigatus* ECU0811, was successively derived from the

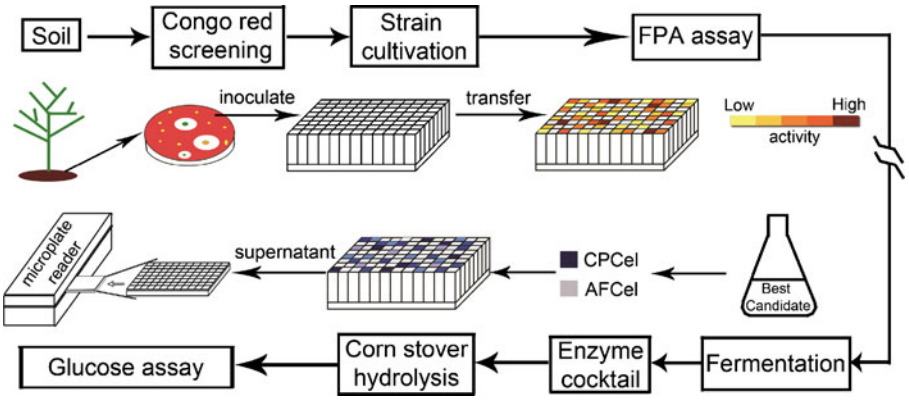

Fig. 1 The scheme of 96-deep-well microplate-based screening platform, including strain cultivation, activity assay, enzyme cocktail, and glucose analysis

environment, which contained higher activity of β-glucosidase and even xylanase activity as the same order of magnitude as Multifect xylanase (Genencor). Under optimized cultivation, the crude enzyme from scaled-up preparation could extremely improve the glucan-to-glucose conversion of corn stover to approximately 99.5%.

Materials and Methods

Enzymes, Substrates, and Reagents

Strain *T. reesei* (GIM3.141) was obtained from Guangdong Institute of Microbiology. Spezyme CP was donated by Genencor Danisco (Shanghai, China). The lignocellulosic materials, corn cob, wheat straw, and rice straw, were collected from farms in Jiangsu province. Steam explosion-pretreated corn stover was a generous gift provided by Prof. Bao (East China University of Science and Technology, Shanghai). All the dried substrates were milled and sieved to obtain powders with particle size less than 100 μm, with an average content of 32.6% cellulose, 26.4% hemicellulose, and 8.1% lignin. All the other substrates and chemical reagents were obtained from either Sigma (Shanghai, China) or National Medicines Group Shanghai Chemical Reagent Company (Shanghai, China).

Strain Screening

Hundreds of soil samples were collected and streaked to select the colonies with halo-forming activity, according to the Congo red staining method [23]. The colonies with higher D/d (D is the diameter of clear zone, and d is the diameter of colony) values entered second-stage screening. For the second-stage enrichment, the basic medium used for screening was a slightly modified Mandels medium [24]: 0.05% yeast extract, 0.3% peptone, 0.2% KH_2PO_4, 0.15% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \cdot 7H_2O$, and 0.03% $CaCl_2$. To 1 L of this medium, 1 mL of Mandels trace element solution (0.16% $MnSO_4$, 0.14% $ZnSO_4$, 0.5% $FeSO_4$, and 0.2% $CoCl_2$) was added. The second-stage screening was carried out in a 96-deep-well microplate (Corning, Shanghai, China). After sterilization, 1 mL of the above medium was transferred to each well with 2% dried corn stover powder, followed by aspirating three times to ensure a homogeneous solid suspension. A single colony was inoculated into each well using a toothpick. The microplate was shaken at 30 °C, 500 rpm for 2 days on a microplate shaker (HTC Biotech, Bovenden, Germany). Then, the supernatant was aliquoted for the filter paper activity (FPA) assay in a 96-deep-well microplate.

For the microplate-based FPA assay, it was performed according to the previous study [25] and adapted to a 96-deep-well microplate as follows. A 20-μL aliquot of supernatant was added into each well, with 40 μL of 50 mM sodium acetate buffer and a 7.0-mm-diameter filter paper disk prepared by an office paper punch. After incubation at 50 °C for 60 min, 120 μL of 3,5-dinitrosalicylic acid (DNS) was added into each well and incubated at 95 °C for 5 min to stop the reaction. And then the mixture was diluted with 160 μL of H_2O to measure the absorbance at 540 nm by a microplate spectrophotometer (BioTek, Beijing, China). One activity unit is defined as the quantity of enzymes required to liberate 1.0 μmol glucose from substrate per minute. For all the heating procedures, the microplate was sealed with a plastic mat and a closed lid (Corning, Shanghai, China).

Strain Identification

The genomic DNA was first extracted and used as the template to amplify internal transcribed spacer (ITS) rDNA sequence. The PCR primer pairs were designed as: Forward, 5′-TCCGTAGGTGAACCTGCGG-3′; Reverse, 5′-TCCTCCGCTTATT GATATGC-3′. The species of the best strain was identified by sequencing and sequential Basic Local Alignment Search Tool (BLAST) of the enzymatically amplified ITS rDNA [26].

Crude Enzyme Preparation

The optimized medium for fermentation contained 3.0% corn cob, 0.3% peptone, 0.05% yeast extract, 0.2% Tween-80, 0.2% K_2HPO_4, 0.15% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, and 0.1% Mandels trace element solution. The strain was fermented to produce cellulases in 250-mL shake flasks, incubated at 250 rpm, 30 °C for 5 days. Preparation of crude enzyme involved centrifugation (10,000×*g*, 20 min), ultrafiltration, and lyophilization. The lyophilized enzyme was stored at 4 °C for subsequent experiments. Protein concentration was determined using the conventional BCA assay method [27].

Enzyme Activity Assay

For the assays of Avicelase, CMCase, FPA, and xylanase activity, it generally followed the DNS assay protocol [28], however, with different substrates of 5.0 mg/mL avicel, 1.0% carboxymethyl cellulose sodium (CMC), 1.0×6.0 cm filter paper, and 1.0 mg/mL birchwood xylan, respectively. One activity unit is defined as the quantity of enzymes required to liberate 1.0 μmol glucose or xylose from substrate per minute. The hydrolytic activity of β-glucosidase was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside [29]. One unit of β-glucosidase was defined as the amount of enzyme that releases 1.0 μmol *p*-nitrophenol per minute.

Hydrolysis of Corn Stover

In brief, 5 mg/mL corn stover was dispensed into a 96-deep-well microplate. Subsequently, appropriate enzyme dosage was supplemented into each well with 50 mM citrate buffer (pH 4.8), up to a total volume of 1 mL. The reaction was carried out at 50 °C with continuous agitation at 800 rpm for 24 h. The hydrolysate was then centrifuged (3,000 rpm, 10 min), and the supernatant was used for the glucose assay.

Determination of Glucan-to-Glucose Conversion

The glucose assay kit (Shensuo Bio-tech, Shanghai, China) uses glucose oxidase–peroxide reaction for the determination of glucose concentrations, which was scaled down to a total volume of 750 μL. To a new microplate, 5 μL supernatant was added from a hydrolysis microplate, while 5 μL H_2O was used as a blank. The assay was performed at 37 °C for 20 min and cooled down to room temperature for measuring the absorbance at 505 nm. Percentage of glucan-to-glucose conversion (in percent) was calculated from the released amount of glucose (milligrams per milliliter) divided by the added amount of cellulose in substrate (milligrams per milliliter) multiplied by 1.1 (180/162) [30].

Results

Strain Screening and Identification

After the first-stage screening, in total, 145 colonies with relatively higher *D*/*d* value were selected to determine the total cellulase activity. Figure 2 shows the distribution of those strains with FPA activity less than 0.3 FPU/mL. One hundred fifteen of the counted strains exhibited lower FPA activity of 0–0.05 FPU/mL, while 24 showed slightly higher activity of 0.05–0.1 FPU/mL. The remaining six strains with obvious filamentous characteristics showed a higher FPA activity of 0.1–0.3 FPU/mL, and the highest one was marked as ECU0811. It is worthy to note that ECU0811 has a similar FPA activity of 0.25 FPU/mL, as compared to the well-known cellulase-producing fungi *T. reesei*.

The top six candidates displaying cellulase activities greater than 0.1 FPU/mL were further selected to investigate cellulase and β-glucosidase activities. Figure 3 indicates the cellulase and β-glucosidase activities of the best two candidates, ECU0811 and F13, using *T. reesei* as a reference. Since the different sources of substrate might affect the production of cellulases [31, 32], three raw lignocelluloses, i.e., corn cob, wheat straw, and rice straw, together with a model substrate α-cellulose, were used to induce the secretion of cellulase. The highest cellulase and β-glucosidase activities were both obtained in the presence of corn cob, while α-cellulose hardly improved the enzyme activities. One possible reason is that the raw substrate is usually a complicated matrix and might have free cellooligosaccharides of lower degrees to induce cellulases [33]. By contrast, α-cellulose has less amorphous structure to be degraded into cellooligosaccharides. So for all the following experiments, we used corn-derived materials as carbon source. Surprisingly, the strain ECU0811 not only had a compatible cellulase activity to that of *T. reesei* but also had a β-glucosidase activity of 2.58 U/mL, which is 23.4- and 42.9-fold higher than those of strains *T. reesei* and F13 (Fig. 3), respectively. As a consequence, the strain ECU0811 was successfully derived from soil and exhibited higher β-glucosidase activity for supplement to commercial cellulase preparations. Identification of the strain ECU0811 was achieved by BLAST search of ITS rDNA, which shared 100% homologous identity to the fungi *A. fumigatus*.

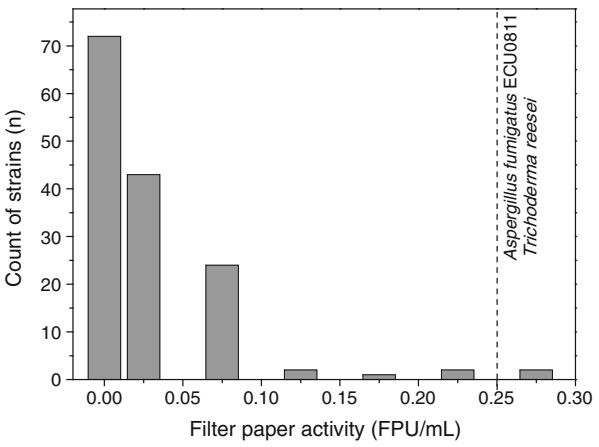

Fig. 2 Distribution of strains with respect to their filter paper activities (FPA). *Y*-axis represents the colony number within specific range of the FPA activity, *X*-axis represents the range of FPA activity

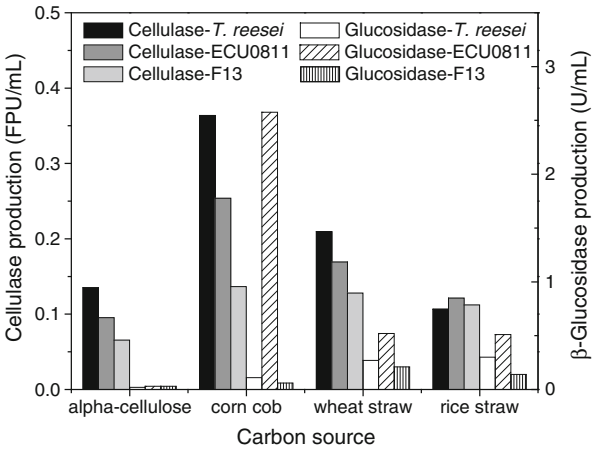

Fig. 3 Comparison of cellulase and β-glucosidase production by strains *T. reesei*, ECU0811 and F13 grown on four different carbon sources of α-cellulose, corn cob, wheat straw, and rice straw

Medium Optimization for *A. fumigatus* Cellulase Production

Large-scale cultivation of the fungal strain *A. fumigatus* ECU0811 was optimized by monitoring its cellulase production under various carbon sources, concentrations of carbon source, initial pH, and additives. Various carbon sources (i.e., glucose, xylose, sucrose, maltose, lactose, galactose, α-cellulose, corn cob, starch, rice straw, CMC, wheat straw, avicel, dextrin, and β-cyclodextrin) for cellulase production were examined. The highest cellulase production was observed using corn cob as the substrate (data not shown). The effect of initial pH was observed ranging from 2.0 to 8.0, as shown in Fig. 4a. As a result, the optimal initial pH was 3.0. The effect of concentration of corn cob was shown in Fig. 4b. The substrate corn cob powder with a concentration of 3% could maximally induce cellulase production. The effect of additives supplemented to medium, i.e., Triton X-100, glycerol, Tween-80, olive oil, SDS, and EDTA, was also examined (data not shown). The extra 0.2% (*w*/*v*) addition of Tween-80 seemed to increase the permeability of cell membrane [10, 34], resulting in 50% increase in cellulase production compared to the control. The whole fermentation lasted for 5 days.

Compositions of *A. fumigatus* Cellulase

The crude enzyme was prepared from the optimized medium, as determined above. The enzyme compositions in Spezyme CP and *A. fumigatus* ECU0811 were compared in Table 1. The commercial enzyme Spezyme CP showed higher cellulase activities, such as FPA (0.49 U/mg), Avicelase (0.05 U/mg), and CMCase (1.34 U/mg). By contrast, *A. fumigatus* contained a higher β-glucosidase activity of 0.91 U/mg and a xylanase activity of 125 U/mg, which were 91- and 49-fold higher than those of Spezyme CP, respectively. This corresponded to the diverse enzymes produced by *A. fumigatus* strain as reported previously [35, 36].

Feasibility of 96-Deep-Well Microplate-Based Platform

Another noticeable study in this paper is that we constructed a microplate-based platform containing hundreds of strains cultivation, activity assay, enzyme cocktail, and product

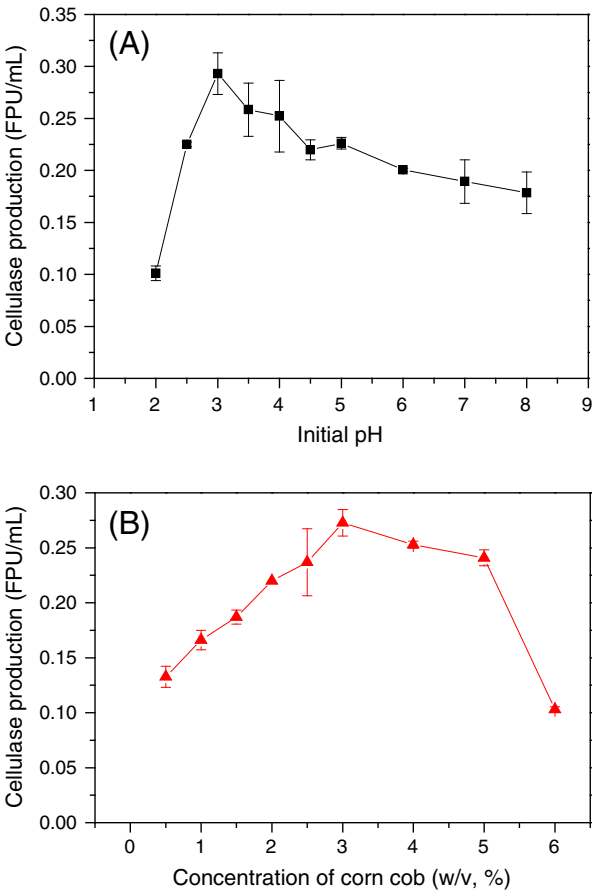

Fig. 4 Optimization of initial pH (**a**) and corn cob concentration (**b**) in the cellulase production by *A. fumigatus* ECU0811. The experiments were carried out in 250-mL shake flasks. *Error bars* indicate standard derivation

analysis (Fig. 1). The conventional strain screening was time-consuming and labor-intensive because of numerous samples. To make it more convenient, we carried out the liquid spawn cultivation and FPA activity measurement by a microplate-based method. The reproducibility and accuracy of the 60-μL FPA format were verified by a previous report [37]. In this work, the FPA activity was generally higher than that from tube assay, however, with the same trend (data not shown). In other words, the slight variations do not significantly affect the selection of high cellulase-producing strains. Therefore, the microplate-based assay method can be used for rapid strain screening.

Table 1 Assay of various enzymes activity (units per milligram protein) in Spezyme CP (CPCel) and cell-free extract of *A. fumigatus* ECU0811 (AFCel)

Enzyme	FPA	β-Glucosidase	Avicelase	CMCase	Xylanase
Spezyme CP (CPCel)	0.49	0.01	0.05	1.34	2.53
A. fumigatus ECU0811 (AFCel)	0.16	0.91	0.02	0.39	125

FPA filter paper activity

Next, once the lignocelluloses were metered into a microplate, the saccharification studies can be performed, which enabled the large amount of parametric optimization in parallel. A *t* test performed on the data demonstrated that there was no significant difference at the 95% confidence level between the shake flask (20 mL) and microplate (1 mL) experiments. The calculated $P=0.74>0.05$ ($df=23$) and the difference between the two systems can be accepted. Therefore, the proposed microplate-based system can be adapted to the enzymatic hydrolysis of corn stover. We believed this proposed microplate-based platform could be also extended to a wider set of enzymes, enzyme compositions, and various substrates as a preliminary test for more economic application.

Maximum Enzymatic Hydrolysis of Corn Stover

A preliminary enzymatic hydrolysis of corn stover for 24 h using different dosages of Spezyme CP (CPCel) was performed in microplates, as shown in Fig. 5a. The maximum glucan-to-glucose conversion with 147 FPU/g glucan could quickly reach 81.5% at 6 h (data not shown). When we reduced the enzyme loading by tenfold (14.2 FPU/g glucan), the maximum glucan-to-glucose yield was 25.6% after 24 h. Any enzyme loading less than

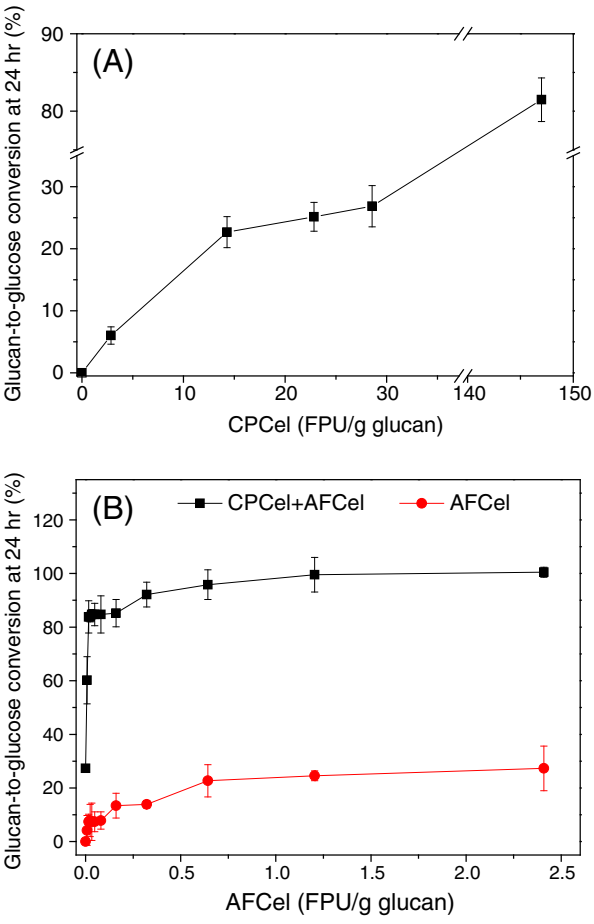

Fig. 5 Hydrolytic reaction profiles of pretreated dried corn stover. **a** Different dosages (filter paper units per gram of glucan) of Spezyme CP (CPCel) were loaded. **b** The dosage of CPCel was fixed at 14.2 FPU/g glucan, while *A. fumigatus* cellulase (AFCel) was supplemented within a range of 0–2.5 FPU/g glucan. *X*-axis represents the enzyme dosage (filter paper units per gram of glucan). *Y*-axis represents the glucan-to-glucose conversion (percentage). All the reactions were stopped at 24 h. *Error bars* indicate standard derivation

14.2 FPU/g glucan could hardly achieve a conversion over 10% as 2.94 FPU/g glucan only gave 6.02% conversion after 24 h. On the other hand, overloading of CPCel (573 FPU/g glucan) even inhibited the hydrolysis of corn stover, resulting in a lower glucose yield (ca. 78.2%). This phenomenon can be also found in previous reports [38], indicating that the Spezyme CP does not contain adequate β-glucosidase to hydrolyze cellobiose, and the eventually accumulated cellobiose will inhibit enzyme activity and hydrolytic rate.

Subsequently, we fixed the dosage of CPCel at 14.2 FPU/g glucan for the following enzyme cocktail study. Supplementation of *A. fumigatus* cellulase (AFCel) to CPCel resulted in a significant improvement of corn stover hydrolysis, as shown in Fig. 5b. AFCel alone did contain cellulase activity to eventually deconstruct glucan (circle symbol), even at a higher rate than CPCel. Surprisingly, extra addition of 0.32 FPU/g glucan AFCel into CPCel could dramatically increase the glucan-to-glucose conversion up to 92.1% (square symbol). Further increased loading of AFCel dosages could allow approximately 100% glucan conversion (ca. 1.21 FPU/g glucan, 99.5%), 73.9% higher from 25.6%. These data corresponded to the high activities of accessory enzymes from *A. fumigatus* ECU0811 (Table 1). Noteworthily, ca. 0.02 FPU/g glucan AFCel loading into 14.2 FPU/g glucan CPCel could lead to a 83.8% glucan-to-glucose conversion after 24 h, which was higher than loading 147 FPU/g glucan CPCel alone and also reduced total protein dosage by tenfold. To conclude, it is obviously observed that the strain *A. fumigatus* not only could provide glucosidase to release more glucose from cellooligosaccharides but also involve xylanase to deconstruct xylan matrix, resulting in a much higher glucan-to-glucose conversion.

Discussion and Concluding Remark

Traditional strain screening method usually takes weeks for one cycle. A rapid screening platform was developed by using 96-deep-well microplate, which reduces the time to 4–5 days. By the high-throughput method, hundreds of samples could be simultaneously screened, and it helps to cover a wider range of natural samples. Also, this platform could be adjusted by measuring other reducing sugars or substrates, and further applied in screening of other enzymes.

The complete hydrolysis of biomass usually requires a specific cocktail of cellulolytic enzymes acting synergistically. In this work, we successfully isolated a filamentous fungus *A. fumigatus* ECU0811 which exhibited various cellulases activities, especially higher β-glucosidase activity than *T. reesei*. As a cellulase complex, the crude enzyme preparation (AFCel) afforded 27.3% conversion of corn stover hydrolysis at an enzyme dosage of 2.41 FPU/g glucan, while the commercial enzyme (CPCel) required 14.2 FPU/g glucan to obtain a similar conversion of 25.6%, almost sixfold protein amount. Furthermore, a supplement of AFCel at merely 1.21 FPU/g glucan could significantly elevate the glucan-to-glucose conversion from 25.6% up to 99.5% in the presence of CPCel at 14.2 FPU/g glucan, which also maximally reduced the amount of commercial enzyme (CPCel) by as much as tenfold (Fig. 5b) to achieve a complete hydrolysis of corn stover. The advantage of AFCel supplement was further verified by an enzyme cocktail with other two enzymes, Multifect xylanase (Genencor Danisco, Shanghai, China) and almond β-glucosidase (Sigma, Shanghai, China), that AFCel could already offer adequate xylanase and glucosidase to achieve the maximum glucan-to-glucose conversion (data not shown). Such a distinguished property enabled AFCel to be an excellent cellulase candidate, which will reduce the cost of expensive cellulase preparation and provide a platform for further application.

Interestingly, the glucan-to-glucose conversion can reach up to 100% by the optimized enzyme cocktail, whereas it just attained about 80% when using the CPCel alone (Fig. 5). Moreover, a negative effect was observed when the accessory enzymes were overloaded. The mechanism underlying this phenomenon is not yet clear so far. However, the results indicate that it is better to avoid excessive addition of accessory enzymes [8].

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (Nos. 20902023 & 31071604), Ministry of Science and Technology, P.R. China (Nos. 2009CB724706), and China National Special Fund for State Key Laboratory of Bioreactor Engineering (No. 2060204).

References

1. Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., et al. (2007). *Science, 315*, 804–807.
2. Percival Zhang, Y. H., Himmel, M. E., & Mielenz, J. R. (2006). *Biotechnology Advances, 24*, 452–481.
3. Perez, J., Munoz-Dorado, J., de la Rubia, T., & Martinez, J. (2002). *International Microbiology, 5*, 53–63.
4. Service, R. F. (2007). *Science, 315*, 1488–1491.
5. Lynd, L. R., Weimer, P. J., van Zyl, W. H., & Pretorius, I. S. (2002). *Microbiology and Molecular Biology Reviews, 66*, 506–577.
6. Sanchez, O. J., & Cardona, C. A. (2008). *Bioresource Technology, 99*, 5270–5295.
7. Baker, J. O., Ehrman, C. I., Adney, W. S., Thomas, S. R., & Himmel, M. E. (1998). *Applied Biochemistry and Biotechnology, 70–72*, 395–403.
8. Berlin, A., Maximenko, V., Gilkes, N., & Saddler, J. (2007). *Biotechnology and Bioengineering, 97*, 287–296.
9. Meyer, A. S., Rosgaard, L., & Sorensen, H. R. (2009). *Journal of Cereal Science, 50*, 337–344.
10. Tu, M. B., Chandra, R. P., & Saddler, J. N. (2007). *Biotechnology Progress, 23*, 1130–1137.
11. Wu, Z., & Lee, Y. Y. (1998). *Applied Biochemistry and Biotechnology, 70–72*, 479–492.
12. Yang, B., Willies, D. M., & Wyman, C. E. (2006). *Biotechnology and Bioengineering, 94*, 1122–1128.
13. Reczey, K., Brumbauer, A., Bollok, M., Szengyel, Z., & Zacchi, G. (1998). *Applied Biochemistry and Biotechnology, 70–72*, 225–235.
14. Chen, H. Z., Hayn, M., & Esterbauer, H. (1992). *Biochimica et Biophysica Acta, 1121*, 54–60.
15. Golias, H., Dumsday, G. J., Stanley, G. A., & Pamment, N. B. (2000). *Biotechnology Letters, 22*, 617–621.
16. Nieves, R. A., Ehrman, C. I., Adney, W. S., Elander, R. T., & Himmel, M. E. (1998). *World Journal of Microbiology and Biotechnology, 14*, 301–304.
17. Sternberg, D., Vijayakumar, P., & Reese, E. T. (1977). *Canadian Journal of Microbiology, 23*, 139–147.
18. Chandrasekaran, A., Bharadwaj, R., Park, J. I., Sapra, R., Adams, P. D., & Singh, A. K. (2010). *Journal of Proteome Research, 9*, 5677–5683.
19. Chundawat, S. P., Balan, V., & Dale, B. E. (2008). *Biotechnology and Bioengineering, 99*, 1281–1294.
20. King, B. C., Donnelly, M. K., Bergstrom, G. C., Walker, L. P., & Gibson, D. M. (2009). *Biotechnology and Bioengineering, 102*, 1033–1044.
21. Bharadwaj, R., Wong, A., Knierim, B., Singh, S., Holmes, B. M., Auer, M., et al. (2011). *Bioresource Technology, 102*, 1329–1337.
22. Cianchetta, S., Galletti, S., Burzi, P. L., & Cerato, C. (2010). *Biotechnology and Bioengineering, 107*, 461–468.
23. Kim, Y. S., Jung, H. C., & Pan, J. G. (2000). *Applied and Environmental Microbiology, 66*, 788–793.
24. Mandels, M., & Weber, J. (1969). *Journal of the American Chemical Society, 95*, 391–414.
25. Xiao, Z., Storms, R., & Tsang, A. (2004). *Biotechnology and Bioengineering, 88*, 832–837.
26. Anderson, I. C., Campbell, C. D., & Prosser, J. I. (2003). *Environmental Microbiology, 5*, 36–47.
27. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). *Analytical Biochemistry, 150*, 76–85.
28. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428.
29. Yu, H. L., Xu, J. H., Lu, W. Y., & Lin, G. Q. (2007). *Enzyme and Microbial Technology, 40*, 354–361.
30. Shi, Q. Q., Sun, J., Yu, H. L., Li, C. X., Bao, J., & Xu, J. H. (2011). *Applied Biochemistry and Biotechnology, 164*, 819–830.

31. Juhász, T., Szengyel, Z., Réczey, K., Siika-Aho, M., & Viikari, L. (2005). *Process Biochemistry, 40*, 3519–3525.
32. Olsson, L., Christensen, T. M. I. E., Hansen, K. P., & Palmqvist, E. A. (2003). *Enzyme and Microbial Technology, 33*, 612–619.
33. Wang, C. H., Hseu, T. H., & Huang, C. M. (1988). *Journal of Biotechnology, 9*, 47–59.
34. Reese, E. T., & Maguire, A. (1969). *Applied and Environmental Microbiology, 17*, 242–245.
35. Sherief, A. A., El-Tanash, A. B., & Atia, N. (2010). *Research Journal of Microbiology, 5*, 199–211.
36. Wase, D. A. J., Raymahasay, S., & Wang, C. W. (1985). *Enzyme and Microbial Technology, 7*, 225–229.
37. Gusakov, A. V., Salanovich, T. N., Antonov, A. I., Ustinov, B. B., Okunev, O. N., Burlingame, R., et al. (2007). *Biotechnology and Bioengineering, 97*, 1028–1038.
38. Zhang, M., Su, R., Qi, W., & He, Z. (2010). *Applied Biochemistry and Biotechnology, 160*, 1407–1414.