J Ind Microbiol Biotechnol (2013) 40:805–810
DOI 10.1007/s10295-013-1290-6

BIOENERGY/BIOFUELS/BIOCHEMICALS

A comprehensive analysis of the effects of the main component enzymes of cellulase derived from *Trichoderma reesei* on biomass saccharification

Tetsushi Kawai · Hikaru Nakazawa · Noriko Ida · Hirofumi Okada · Wataru Ogasawara · Yasushi Morikawa · Yoshinori Kobayashi

Received: 22 January 2013 / Accepted: 10 May 2013 / Published online: 4 June 2013
© Society for Industrial Microbiology and Biotechnology 2013

Abstract The aim of this study was a comprehensive analysis of the effects of the component enzymes of cellulase derived from *Trichoderma reesei* strain PC-3-7 on biomass saccharification. We used cellulases with deleted CBH I, CBH II, or EG I, which contain all other component enzymes, for saccharification of differently pretreated biomasses of rice straw, Erianthus, eucalyptus, and Japanese cedar. We found that CBH I was the most effective in saccharification of all pretreated cellulosic biomasses, although the effect was weaker in saccharification of sulfuric acid- and hydrothermally pretreated rice straw than of others; CBH II was more effective for rice straw than for eucalyptus, and was the most effective at the early stages of biomass degradation; EG I had little effect on pretreated biomasses, in particular, it had no effect on steam-exploded Japanese cedar. Thus, the effects of the main component enzymes depend on the biomass source and pretreatment. These findings will likely help to improve cellulase for industrial use.

Keywords Cellulase · *Trichoderma reesei* · Component enzymes · Bioconversion · Enzymatic saccharification

T. Kawai · N. Ida · Y. Morikawa · Y. Kobayashi (✉)
Japan Bioindustry Association, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
e-mail: yn-kobayashi@aist.go.jp

H. Nakazawa · H. Okada · W. Ogasawara
Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka 940-2188, Japan

Introduction

The sugar derived from cellulosic biomass carbohydrates can be converted into bio-ethanol and bio-chemicals [1]. Cellulosic biomass is a particularly attractive new energy resource for human beings because (a) it is available in large quantities; (b) unlike some other potential sugar resources (like starch, for example) it is not suitable as food or forage. Therefore, its future industrial use can be expected. However, the production of sugar from cellulosic biomass requires large amounts of saccharification enzymes, and their high cost restricts the industrial utilization of cellulosic biomass. To reduce the cost, it is desirable to develop a highly efficient cellulase, small amounts of which would be sufficient to degrade cellulosic biomass. The filamentous fungus *Trichoderma reesei* secretes large quantities of cellulase. Almost all secreted *T. reesei* proteins are glycoside hydrolases capable of degrading cellulosic biomass [12]. In recent years, *T. reesei* cellulase for industrial use became available on the market. We previously developed a cellulase with higher saccharification activity than that of commercially available cellulases [14, 21]. However, the industrial use of cellulosic biomass requires a further cost reduction by producing more powerful cellulases.

Cellulase derived from *T. reesei* contains various kinds of component enzymes [20]. Cellobiohydrolase (CBH), endoglucanase (EG), and β-glucosidase (BGL) are considered the most important components [5, 8, 30]. CBH produces cellobiose by processive hydrolysis of the reducing end (CBH I) or non-reducing end (CBH II) of the cellulose chain [3]; EG cleaves cellulose chains in non-crystalline regions by endo-type hydrolysis [17]; BGL cleaves the oligosaccharides produced by CBH and EG, and produces glucose [7]. EG IV and VII, which belong to

the glycoside hydrolase family (GHF) 61, have been reported to oxidatively degrade cellulose and to show a synergistic effect with other cellulase components [23]. In addition, cellulase contains an expansin-like protein, swollenin, which also shows synergistic effects with other cellulase components, despite exhibiting little cellulase activity [26].

The extent of the contribution of the various component enzymes to cellulosic biomass saccharification is not well understood. Previous studies used a cocktail of purified component enzymes to clarify their relative contributions [2, 4]; however, such cocktails cannot fully recreate the activity of the conditioned medium in many cases, since they do not include all of the component enzymes derived from *T. reesei*, and unidentified additional enzymes may affect the saccharification of cellulosic biomass.

The effects of individual component enzymes on saccharification can be obscured by the complexity of the cellulosic biomass [31]. Cellulose itself consists of crystalline and non-crystalline regions, and its crystalline structure is different in each plant species [10]. Not only cellulose but also other cellulosic polymers (hemicellulose and lignin) also differ in different plants. Furthermore, the biomass composition changes considerably depending on the type of pretreatment. Pretreatment with NaOH, for example, dissolves lignin, and diluted sulfuric acid and hydrothermal pretreatment degrade hemicellulose depending on the temperature [27]. Therefore, the effects of component enzymes can differ depending on the plant species and pretreatment method.

In this study, we used biomass derived from four plant species (rice straw, Erianthus, eucalyptus, and Japanese cedar) with four pretreatments (NaOH, diluted sulfuric acid, hydrothermal treatment, and steam explosion) to comprehensively analyze the effects of the major component enzymes by using cellulases derived from *T. reesei cbh1*, *cbh2*, and *egl1* disruption mutants, which secrete cellulases containing all of the component enzymes except for the disrupted ones [24]. This work will pave the way for further improvements in cellulase efficiency.

Materials and methods

Strain and enzyme preparation

The parent strain (WT) and transformants of *T. reesei* PC-3-7 [24] were maintained on Potato dextrose agar plates or slants; 10^7 conidia were inoculated into 50 ml of a basal medium [15] containing 1 % Avicel as a carbon source and incubated with shaking at 220 rpm for 1 week. The supernatants obtained from this standard culture were used as enzymes.

Enzyme assays and protein analysis

The reducing sugar was measured by using the 3, 5-dinitrosalicylic acid (DNS) method [19]. Protein concentration was determined by use of a Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cellobiase activity was determined as described previously [21].

Biomass pretreatment and composition analysis

Prior to saccharification, cellulosic biomass was subjected to hydrothermal, acid, alkaline, or steam-explosion treatments as previously reported [14]. The composition of the pretreated biomass was determined by high-performance liquid chromatography by using two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory [28].

Enzymatic saccharification

Saccharification by cellulases was performed in 20-ml plastic bottles in 100 mM sodium acetate buffer (pH 5.0). Substrate concentrations were 5.0 % dry mass (w/w). Reactions were performed at 50 °C with shaking (150 rpm) for 0, 6, 24, 48, and 72 h, with enzyme loading at 0.6–3.6 U-cellobiase per gram of dry biomass. The supernatants were boiled for 5 min to inactivate the enzymes, and the produced sugar was measured by the DNS method. The biomass saccharification ratio (%) was calculated as the ratio of the sugar content to cellulose and hemicellulose in the dry mass.

Results and discussion

Cellobiase activity of cellulases lacking individual components

We previously reported that cellulases with deleted individual components appeared to contain about the same ratio of other component enzymes as those derived from WT *T. reesei* [24]. Because CBH I is a major cellulase component [25], the use of total protein amounts for enzyme dosage of *cbh1* deletion cellulase (ΔCBH I) is not adequate, because it would lead to an overestimation of other component enzymes in the absence of CBH I. Therefore, in this study, the enzyme dosage for biomass saccharification was based on the cellobiase activity of cellulases, because the deletion of individual component enzymes has little effect on this activity [32]. Table 1 shows specific cellobiase activities in WT cellulase and in cellulases with deleted individual components. The protein

Table 1 Cellobiase activity of wild-type cellulase and cellulases with deleted component enzymes

Enzyme	U/mg (ratio to WT)
WT	0.12 (1.0)
ΔCBH I	0.30 (2.5)
ΔCBH II	0.21 (1.8)
ΔEG I	0.13 (1.1)

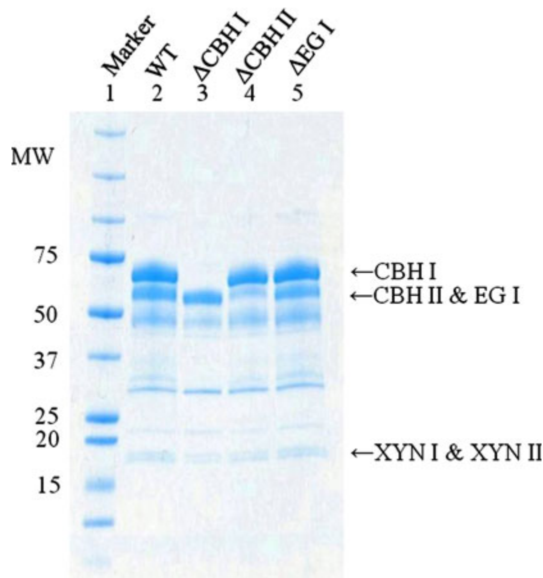

Fig. 1 Analysis of cellulases with deleted individual components by SDS-PAGE. The amounts of protein applied corresponds to equal cellobiase activity in each sample. *1*, molecular weight markers (kDa); *2*, WT; *3*, ΔCBH I; *4*, ΔCBH II; *5*, ΔEG I

ratio of component enzymes in WT cellulase normalized to cellobiase activity was CBH I : CBH II : EG I = 60 : 43 : 9. The proportion of CBH II was greater than reported previously [25]. One possible reason for this difference may be that cellulase induction by Avicel used in this study differs from the conditions used in the above publication; the mechanisms of cellulase induction by various cellulose derivatives are still unknown [18, 22]. Separation of these cellulases by electrophoresis (with loading normalized according to cellobiase activities) confirmed a similar proportion of the component enzymes in WT and in deletion mutants, although the bands corresponding to xylanase I (XYN I) and xylanase II (XYN II) appeared to be slightly weaker in ΔCBH I than in WT (Fig. 1). Thus, normalization to cellobiase activity is adequate for evaluation of the effect of the component enzymes on biomass saccharification. Using this procedure, we estimated that 1.0 U of cellobiase activity corresponds to 8.3 mg total protein in WT, 3.3 mg in ΔCBH I, 4.8 mg in ΔCBH II, and 7.7 mg in ΔEG I (*egl1* deletion). The protein dosages were defined according to the saccharification rates of particular substrates (not the protein amounts), because the same protein amount would be excessive for rice straw and insufficient for eucalyptus. We chose the protein dosage so that the saccharification yield was about 80 % at 24 h when the reaction was carried out with WT cellulase. The chosen extent of saccharification was high, because we wished to get the information about cellulase ability to degrade a large proportion of biomass.

Saccharification of NaOH-pretreated rice straw, eucalyptus, and Erianthus

Saccharification of NaOH-pretreated rice straw, eucalyptus (Fig. 2) and Erianthus (data not shown) was carried out to reveal the relative effects of the component enzymes. The biomass composition after pretreatment observed in our experiments was similar to that reported previously [14]. The relative saccharification rate of ΔCBH II cellulase was 0.95 of WT at 72 h for rice straw (Fig. 2a). This result was unexpected because CBH II has been reported to be an important factor for cellulose degradation [6]. CBH II played a role at early stages (6 h and 24 h), but other component enzymes probably substituted for CBH II later (72 h). CBH II showed a more pronounced effect on eucalyptus (Fig. 2b), which indicates that its role varies with the type of biomass. The greater effect of CBH I was likely due to both its function such as processivity and abundance. The effect of EG I was the smallest for both biomass types, although EG I has been reported to be important for biomass degradation [11]. Whether EG I does not contribute significantly, or other component enzymes can compensate for its absence, remains to be established. Saccharification of NaOH-pretreated Erianthus showed the same patterns as NaOH-pretreated rice straw (data not shown).

Saccharification of sulfuric acid- and hydrothermally pretreated rice straw and eucalyptus

We used the same approach for sulfuric acid- and hydrothermally pretreated biomasses (Figs. 3, 4). Although the importance of CBH I for cellulose degradation has been reported [2, 16], the effect of ΔCBH I varied remarkably between different types of biomass and pretreatments. It was considerably weaker for rice straw after sulfuric acid and hydrothermal pretreatments (especially at later time points) than after NaOH pretreatment (compare Figs. 3a, 4a with Fig. 2a). This suggests that CBH I can be substituted to some extent by another component at late stages. The effect of CBH II was weak for rice straw and moderate for eucalyptus irrespective of the pretreatment method used, which indicates that CBH II can be also substituted

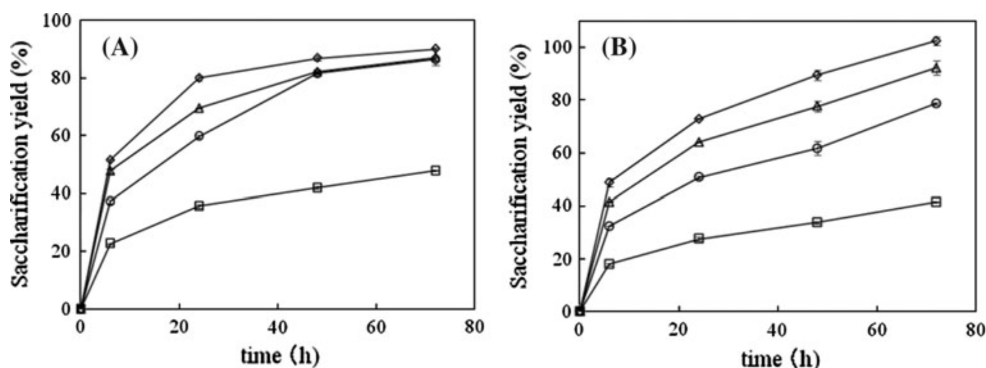

Fig. 2 Saccharification of NaOH-pretreated rice straw (**a**) and eucalyptus (**b**) by *T. reesei* cellulases. Proteins were loaded at 0.6 U-cellobiase/g-biomass; WT: 5 mg/g-biomass, ΔCBH I: 2 mg/g-biomass, ΔCBH II: 2.8 mg/g-biomass and ΔEG I: 4.5 mg/g-biomass (**a**) or 2.4 U-cellobiase/g-biomass; WT: 20 mg/g-biomass, ΔCBH I: 8 mg/g-biomass, ΔCBH II: 11 mg/g-biomass and ΔEG I: 18 mg/g-biomass (**b**). *Diamonds*: WT; *squares*: ΔCBH I; *circles*: ΔCBH II; *triangles*: ΔEG I. *Error bars* represent standard deviations

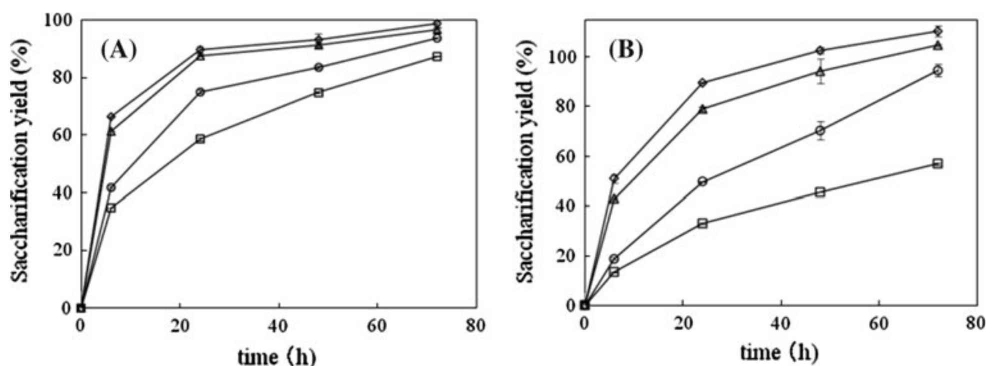

Fig. 3 Saccharification of sulfuric acid-pretreated rice straw (**a**) and eucalyptus (**b**). Proteins were loaded at 1.8 U-cellobiase/g-biomass; WT: 15 mg/g-biomass, ΔCBH I: 6 mg/g-biomass, ΔCBH II: 8.6 mg/g-biomass and ΔEG I: 14 mg/g-biomass (**a**) or 3.2 U-cellobiase/g-biomass; WT: 27 mg/g-biomass, ΔCBH I: 11 mg/g-biomass, ΔCBH II: 15 mg/g-biomass and ΔEG I: 25 mg/g-biomass (**b**). *Symbols* are the same as in Fig. 2. *Error bars* represent standard deviations

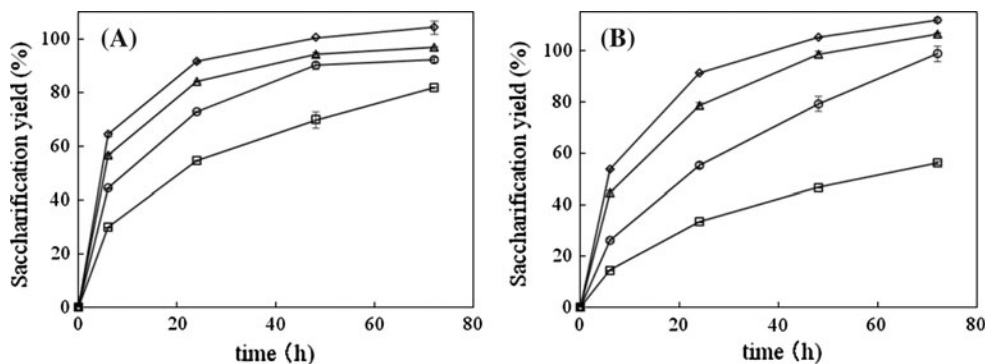

Fig. 4 Saccharification of hydrothermally pretreated rice straw (**a**) and eucalyptus (**b**). Proteins were loaded at 1.8 U-cellobiase/g-biomass; WT: 15 mg/g-biomass, ΔCBH I: 6 mg/g-biomass, ΔCBH II: 8.6 mg/g-biomass and ΔEG I: 14 mg/g-biomass (**a**) and 2.4 U-cellobiase/g-biomass; WT: 20 mg/g-biomass, ΔCBH I: 8 mg/g-biomass, ΔCBH II: 11 mg/g-biomass and ΔEG I: 18 mg/g-biomass (**b**). *Symbols* are the same as in Fig. 2. *Error bars* represent standard deviations

by other components, particularly for eucalyptus (Figs. 3b, 4b). The effect of EG I was small, and varied with different types of biomass or pretreatments (Figs. 3, 4). EG I had no effect even at early stages of degradation, and thus seems to be dispensable for sulfuric acid-pretreated rice straw (Fig. 3a).

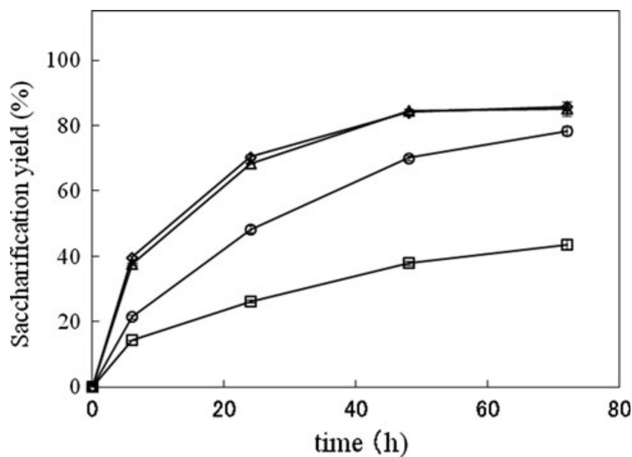

Fig. 5 Saccharification of steam-exploded Japanese cedar. Proteins were loaded at 3.6 U-cellobiase/g-biomass; WT: 30 mg/g-biomass, ΔCBH I: 12 mg/g-biomass, ΔCBH II: 17 mg/g-biomass and ΔEG I: 28 mg/g-biomass. *Symbols* are the same as in Fig. 2. *Error bars* represent standard deviations

Saccharification of steam-exploded Japanese cedar

Steam-exploded Japanese cedar was saccharified (Fig. 5) to compare the effects of the component enzymes on soft wood (Japanese cedar) and hard wood (eucalyptus). The saccharification pattern of Japanese cedar resembled that of eucalyptus for ΔCBH I and ΔCBH II, but not for ΔEG I. The effect of EG I was detected to some extent for all types of pretreated eucalyptus, but not for Japanese cedar. This may be due to the crystalline structure of cellulose, to structural changes in the biomass after pretreatment, or to substitution with other component enzymes [9]. This phenomenon was also seen for Avicel saccharification (data not shown).

Conclusions and future perspectives

In this study, we found that the effects of the main component enzymes depend on biomass origin and pretreatment type, which is in agreement with earlier reports that activity towards such substrates as filter paper, Avicel, or CMC is not proportional to the biomass saccharification ability [13, 14]. Therefore, our findings provide useful information compared to the results based on measuring only enzymatic activity [29]. Our study may help to develop a highly efficient cellulase by optimization of the component enzymes in several ways, exemplified below. (1) Although EG I had no or little effect in our experiments, the *egl1* expression level was high (the third highest after *cbh1* and *cbh2*) [24]. Therefore, the *egl1* promoter might be useful for the expression of other valuable component enzymes like β-glucosidase or hemicellulases. (2) Although we did not verify the effect of the component enzyme dosage, the increase in CBH II might enhance sugar production in the short term because the effect of CBH II is more pronounced at early degradation stages. (3) Even CBH I (the component enzyme most important for cellulose degradation) could be substituted to a certain extent with other enzymes for saccharification of sulfuric acid-pretreated herbaceous biomass. In these cases, the *cbh1* promoter would be attractive, because its activity is the highest among *T. reesei* cellulase promoters [24]. It is important to have a choice of promoters for the expression of other valuable component enzymes adapted to specific target biomasses.

This knowledge will greatly facilitate the development of highly efficient saccharification enzymes for particular biomass types. We expect that our findings will help to develop more efficient saccharification enzymes. The analyses of other component enzymes, for example EG II, EG IV, and xylanases, are underway.

Acknowledgments This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO) Project.

References

1. Balat M, Balat H (2009) Recent trends in global production and utilization of bio-ethanol fuel. Appl Energ 86:2273–2282
2. Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Bongers M, Walton JD (2010) Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresour Biotechnol 101:9097–9105
3. Barr BK, Hsieh YL, Ganem B, Wilson DB (1996) Identification of two functionally different classes of exocellulases. Biochemistry-US 35:586–592
4. Billard H, Faraj A, Lopes Ferreira N, Menir S, Heiss-Blanquet S (2012) Optimization of a synthetic mixture composed of major *Trichoderma reesei* enzymes for the hydrolysis of steam-exploded wheat straw. Biotechnol Biofuels 5:9
5. Boer H, Teeri TT, Koivula A (2000) Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol Bioeng 69:489–494
6. Boisset C, Fraschini C, Schülein M, Henrissat B, Chanzy H (2000) Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. Appl Environ Microb 66:1444–1452
7. Chen H, Hayn M, Esterbauer H (1992) Purification and characterization of two extracellular beta-glucosidases from *Trichoderma reesei*. Biochim Biophys Acta 1121:54–60
8. Chirico WJ, Brown RD Jr (1987) β-glucosidase from *Trichoderma reesei*. Substate-binding region and mode of action on [1-^{3}H] cello-oligosaccharides. Eur J Biochem 165:343–351
9. Donaldson LA, Wong KKY, Mackie KL (1988) Ultrastructure of steam-exploded wood. Wood Sci Technol 22:103–114
10. Elazzouzi-Hafraoui S, Nishiyama Y, Putaux JL, Heux L, Dubreuil F, Rochas C (2008) The shape and size distribution of crystalline nanoparticles prepared by acid hydrolysis of native cellulose. Biomacromolecules 9:57–65

11. Eriksson T, Karlsson J, Tjerneld F (2002) A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. Appl Biochem Biotechnol 101:41–60
12. Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, Mathis H, Sigoillot J, Monot F, Asther M (2008) Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 1:18
13. Kabel MA, Maarel MJEC, Klip G, Voragen AGJ, Schols HA (2006) Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. Biotechnol Bioeng 93:56–63
14. Kawai T, Nakazawa H, Ida N, Okada H, Tani S, Sumitani J, Kawaguchi T, Ogasawara W, Morikawa Y, Kobayashi Y (2012) Analysis of the saccharification capability of high-functional cellulase JN11 for various pretreated biomasses through a comparison with commercially available counterparts. J Ind Microbiol Biotechnol 39:1741–1749
15. Kawamori M, Morikawa Y, Takasawa S (1986) Induction and production of cellulases by L-sorbose in *Trichoderma reesei*. Appl Microbiol Biotechnol 24:449–453
16. Kipper K, Väljamäe P, Johansson G (2005) Processive action of cellobiohydrolase Cel7A from *Trichoderma reesei* is revealed as ‘burst’ kinetics on fluorescent polymeric model substrates. Biochem J 385:527–535
17. Kleman-Leyer KM, Siika-Aho M, Teeri TT, Kirk TK (1996) The Cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. Appl Environ Microb 62:2883–2887
18. Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. Appl Microbiol Biotechnol 60:515–522
19. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
20. Nagendran S, Hallen-Adams HE, Paper JM, Aslam N, Walton JD (2009) Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of *Trichoderma reesei*. Fungal Genet Biol 46:427–435
21. Nakazawa H, Kawai T, Ida N, Shida Y, Kobayashi Y, Okada H, Tani S, Sumitani J, Kawaguchi T, Morikawa Y, Ogasawara W (2011) Construction of a recombinant *Trichoderma reesei* strain expressing *Aspergillus aculeatus* β-glucosidase 1 for efficient biomass conversion. Biotechnol Bioeng 109:92–99
22. Nogawa M, Goto M, Okada H, Morikawa Y (2001) L-Sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei*. Curr Genet 38:329–334
23. Quinlan RJ, Sweeney MD, Leggio LL, Otten H, Poulsen JCN, Johansen KS, Krogh KBRM, Jørgensen CI, Tovborg M, Anthonsen A, Tryfona T, Walter CP, Dupree P, Xu F, Davies GJ, Walton PH (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. PNAS 108:15079–15084
24. Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa Y (2009) Evaluation and characterization of *Trichoderma reesei* cellulase and xylanase promoters. Appl Microbiol Biotechnol 82:899–908
25. Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS (2007) Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. Biotechnol Progr 23:1270–1276
26. Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssönen E, Bhatia A, Ward M, Penttilä M (2002) Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. Eur J Biochem 269:4202–4211
27. Silverstein RA, Chen Y, Sharma-Shivappa RR, Boyette MD, Osborne J (2007) A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour Technol 98:3000–3011
28. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. NREL laboratory analytical procedures. http://www.eere.energy.gov/biomass/analytical_procedures.html#LAP-002
29. Suominen PL, Mäntylä AL, Karhunen T, Hakola S, Nevalainen H (1993) High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. Mol Gen Genet 241:523–530
30. Vlasenko E, Schülein M, Cherry J, Xu F (2010) Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour Technol 101:2405–2411
31. Zhang Y (2008) Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J Ind Microbiol Biotechnol 35:367–375
32. Zhao Y, Wu B, Yan B, Gao P (2004) Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Sci China Ser C 47:18–24