Journal of Microbiology (2020) Vol. 58, No. 8, pp. 687–695
DOI 10.1007/s12275-020-9630-5
eISSN 1976-3794
pISSN 1225-8873

The effects of deletion of cellobiohydrolase genes on carbon source-dependent growth and enzymatic lignocellulose hydrolysis in *Trichoderma reesei*[§]

Meibin Ren[1†], Yifan Wang[1†], Guoxin Liu[2], Bin Zuo[3], Yuancheng Zhang[3], Yunhe Wang[3], Weifeng Liu[1], Xiangmei Liu[1*], and Yaohua Zhong[1*]

[1]*State Key Laboratory of Microbial Technology, Institute of Microbial Technology, Shandong University, Qingdao 266237, P. R. China*
[2]*Zibo Center Hospital, Zi Bo 255036, P. R. China*
[3]*Leling Shengli New Energy Co., Ltd., Leling 253600, P. R. China*

(Received Dec 30, 2019 / Revised Apr 22, 2020 / Accepted May 4, 2020)

The saprophytic fungus *Trichoderma reesei* has long been used as a model to study microbial degradation of lignocellulosic biomass. The major cellulolytic enzymes of *T. reesei* are the cellobiohydrolases CBH1 and CBH2, which constitute more than 70% of total proteins secreted by the fungus. However, their physiological functions and effects on enzymatic hydrolysis of cellulose substrates are not sufficiently elucidated. Here, the cellobiohydrolase-encoding genes *cbh1* and *cbh2* were deleted, individually or combinatively, by using an auxotrophic marker-recycling technique in *T. reesei*. When cultured on media with different soluble carbon sources, all three deletion strains (Δ*cbh1*, Δ*cbh2*, and Δ*cbh1*Δ*cbh2*) exhibited no dramatic variation in morphological phenotypes, but their growth rates increased apparently when cultured on soluble cellulase-inducing carbon sources. In addition, Δ*cbh1* showed dramatically reduced growth and Δ*cbh1*Δ*cbh2* could hardly grew on microcrystalline cellulose (MCC), whereas all strains grew equally on sodium carboxymethyl cellulose (CMC-Na), suggesting that the influence of the CBHs on growth was carbon source-dependent. Moreover, five representative cellulose substrates were used to analyse the influence of the absence of CBHs on saccharification efficiency. CBH1 deficiency significantly affected the enzymatic hydrolysis rates of various cellulose substrates, where acid pre-treated corn stover (PCS) was influenced the least. CBH2 deficiency reduced the hydrolysis of MCC, PCS, and acid pre-treated and delignified corncob but improved the hydrolysis ability of filter paper. These results demonstrate the specific contributions of CBHs to the hydrolysis of different types of biomass, which could facilitate the development of tailor-made strains with highly efficient hydrolysis enzymes for certain biomass types in the biofuel industry.

Keywords: *Trichoderma reesei*, *cbh1*, *cbh2*, biomass conversion, biofuels

Introduction

Lignocellulosic biomass is the most abundant sustainable and bio-renewable resource in nature. The major component is highly crystalline cellulose and embedded in a hemicellulose and lignin matrix, which makes it very resistant to hydrolysis by cellulases (Carroll and Somerville, 2009). Therefore, physicochemical pre-treatment is usually required to reduce cellulose crystallinity or to remove the components that resist degradation (Sathitsuksanoh *et al.*, 2011). For example, dilute-acid hydrolysis has been successfully developed for the pre-treatment of lignocellulosic materials, which removes most of the hemicellulose and increases the porosity of the materials (Karimi *et al.*, 2006). However, while these pre-treated substrates are relatively can be effectively hydrolyzed, saccharification of the materials is still not efficient enough for commercially viable bioconversion (Klein-Marcuschamer *et al.*, 2012). A major issue within this process is that natural enzyme mixtures do not efficiently break down a range of different pre-treated materials (Qian *et al.*, 2017; Soleimani and Ranaei-Siadat, 2017). Thus, it is important to develop highly efficient cellulase systems uniquely tailored to different pre-treated substrates to improve the hydrolytic efficiency and reduce the cost of bioconversion.

The saprophytic fungus *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is well known in the bioethanol industry for its ability to produce large amounts of cellulolytic enzymes to degrade lignocellulosic biomass (Kubicek *et al.*, 2009). The main cellulases secreted by *T. reesei* are exoglucanase or cellobiohydrolase (CBH, EC 3.2.1.91), endoglucanase (EG, EC 3.2.1.4), and β-glucosidase (BGL, EC3.2.1.21), which work synergistically in the enzymatic hydrolysis of cellulosic materials (Zhang *et al.*, 2006). CBHs including CBH1/Cel7A and CBH2/Cel6A account for more than 70% of total extracellular proteins (Seiboth *et al.*, 1992). Both CBHs have been shown to act progressively, where CBH1 and CBH2 cleave the cellobiose dimers from the cellulose chain at the reducing and non-reducing end, respectively (Liu *et al.*, 2011). Moreover, CBH1 and CBH2 show an essential synergy, which appears to catalyze most of the bond-cleavages in the hydrolysis process (Klein-Marcuschamer *et al.*, 2012; Qian *et al.*, 2017).

[†]These authors contributed equally to this work.
*For correspondence. (Y. Zhong) E-mail: zhongyaohua@sdu.edu.cn / (X. Liu) E-mail: liuxiangmei@sdu.edu.cn
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Accordingly, the depolymerization of crystalline cellulose to cellobiose by the cellobiohydrolases is considered the rate-limiting step (Zhang *et al.*, 2006). However, the extent of the contribution of the individual cellobiohydrolases to hydrolysis of cellulosic materials is not well elucidated.

Synthetic enzyme mixtures have been designed to explore the contribution of individual enzymes to the efficiency of hydrolysis. Construction of the enzymes *de novo* would allow the individual components and their relative proportions to be controlled (Qian *et al.*, 2017). However, it remains a challenge to demonstrate the relative contributions using such a cocktail of purified enzymes since more than 80 proteins have been identified in the enzymes secreted by *T. reesei* and additional unidentified proteins may also contribute to the hydrolysis process (Druzhinina and Kubicek, 2017). Deletion of corresponding genes can accurately characterise a protein's biological function. Recently, Kawai *et al.* (2013) analysed for the first time the effects of the *T. reesei* cellulases with deleted component enzymes, which contained all other individuals, on biomass conversion. They found that saccharification of sulfuric acid and hydrothermally pre-treated rice straw was less affected by CBH1 deletion than NaOH pre-treated ones while CBH2 was the most effective at the early stages of substrate degradation including *Erianthus*, *Eucalyptus*, and Japanese cedar (Kawai *et al.*, 2013). Compared to the biomass materials mentioned above, corn residues including corn stover and corncob are considered to be one of the most promising biofuel feedstocks because of their large quantity, relative homogeneity, and attractive price (Torney *et al.*, 2007). Thus, a better understanding of the influence of cellobiohydrolases on saccharification of differently pre-treated corn materials is necessary for optimal enzymatic hydrolysis in biofuel production.

Protein synthesis is an energy-intensive process, and is tightly associated with cell growth (Maitra and Dill, 2015). CBH1 and CBH2 are the proteins secreted in a large amount by *T. reesei* under proper induction conditions, whose synthesis and transport would drive the cells to expend energy in a great deal and may provoke cellular stress during growth. Pakula *et al.* (2005) detailedly examined the capacity of *T. reesei* to synthesize and secrete proteins and discovered that production of extracellular proteins was favoured at low specific growth rates. In addition, induction of cellulase genes in *T. reesei* is primarily carbon source-dependent (Kubicek *et al.*, 2009). Previous studies have explored the expression profile of CBHs associated with specific carbon sources (Zhang and Lynd, 2006; Kubicek *et al.*, 2009). Cellulose and lactose promote the formation of both cellobiohydrolases and is accompanied with relatively slower growth while easily metabolizable carbon sources like glucose and fructose enable fast fungal growth without formation of CBH1 (Ahamed and Vermette, 2008). It has also been shown that *T. reesei* is unable to digest crystalline cellulose in absence of both cellobiohydrolases (Mach and Zeilinger, 2003). The behaviour of cellulase knockout *T. reesei* on different carbon sources can help gaininsight into the relationship between cellulase secretion and growth. Although *T. reesei* strains lacking cellulase genes have been used in industry, public information on the strains is limited (Singh *et al.*, 2015).

In the present study, *T. reesei* with single and double knockout of *cbh* genes were constructed to analyse the influence of cellobiohydrolases on growth and capacity to utilise cellulose substrates with different carbon sources. Moreover, five representative biomasses (microcrystalline cellulose, filter paper, acid-pretreated corncob, delignified corncob, and acid-pretreated corn stover) were used to analyse the influence of the cellobiohydrolases on saccharification efficiency. It could be expected that this type of knowledge would contribute to the development of customized enzyme systems by strain improvement for economically viable biomass conversion processes.

Materials and Methods

Strains, cultural conditions, and enzyme preparation

T. reesei KU70, which lacks the *ku70* gene required for the nonhomologous end joining (NHEJ) pathway, was used as the parental strain for highly efficient gene targeting (Guangtao *et al.*, 2009). The fungal strains were maintained on potato dextrose agar plates (PDA) or slants supplemented with 10 mM uridine when necessary (for the uridine auxotrophic strain KU70). Conidia (10^8/ml) were collected and inoculated into 50 ml of minimal medium (MM, Penttilä *et al.*, 1987) and incubated on a rotary shaker at 200 rpm and 30°C for 36 h to prepare the seed culture. Then the mycelia were collected by filtration and washed twice with sterilised water. After that the equal mycelia (1 g) was transferred into each 200 ml inducing medium containing 4% lactose as the inducer (Lactose 40.0 g/L, $Na_3Citrate{\cdot}5H_2O$ 3.0 g/L, KH_2PO_4 5.0 g/L, $(NH_4)_2SO_4$ 2.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $CaCl_2$ 0.5 g/L, peptone 1.0 g/L, $FeSO_4{\cdot}7H_2O$ 0.0075 g/L, $MnSO_4{\cdot}H_2O$ 0.0025 g/L, $ZnSO_4{\cdot}7H_2O$ 0.0036 g/L, and $CoCl_2{\cdot}6H_2O$ 0.0037 g/L) and grown for 9 days. The culture supernatant was separated by centrifugation and used as the crude enzyme for the analysis of enzymatic properties and saccharification.

To make use of the recyclable marker *Aspergillus niger pyrG* (encoding orotidine 5′-phosphate carboxylase, which confers uracil/uridine prototrophy), 10^7 conidia were plated on minimal medium plates containing 1.5 g/L 5-fluoroorotic acid (5-FOA, Fermentas) and 10 mM uridine. 5-FOA resistant colonies were obtained after 3–4 days and transferred to PDA containing uridine for sporulation. Purified conidia were then tested for uridine auxotrophy on minimal medium plates.

Construction of cellobiohydrolases deletion strains

For the single gene deletion, the disruption cassette containing the genetic marker *pyrG* and homologous flanking regions of the target gene was constructed. To obtain the *cbh1* deletion strain, the *cbh1* disruption construct (Δ*cbh1*::*pyrG*) was designed as follows (Fig. 1A): the 5′- and 3′-flanking regions of the *cbh1* gene from the *T. reesei* genome were amplified using the primer pairs cbh1-5F/cbh1-5R and cbh1-3F/cbh1-3R, respectively (Supplementary data Table S1). The *pyrG* expression cassette was amplified with plasmid pAB4-1 (Mattern *et al.*, 1987) as a template and pyrG-S/pyrG-A as the primer pair. The direct repeat sequence (DR1), a DNA fragment of the *pyrG* gene and used for the removal of the *pyrG* marker gene by homologous recombination (HR), was

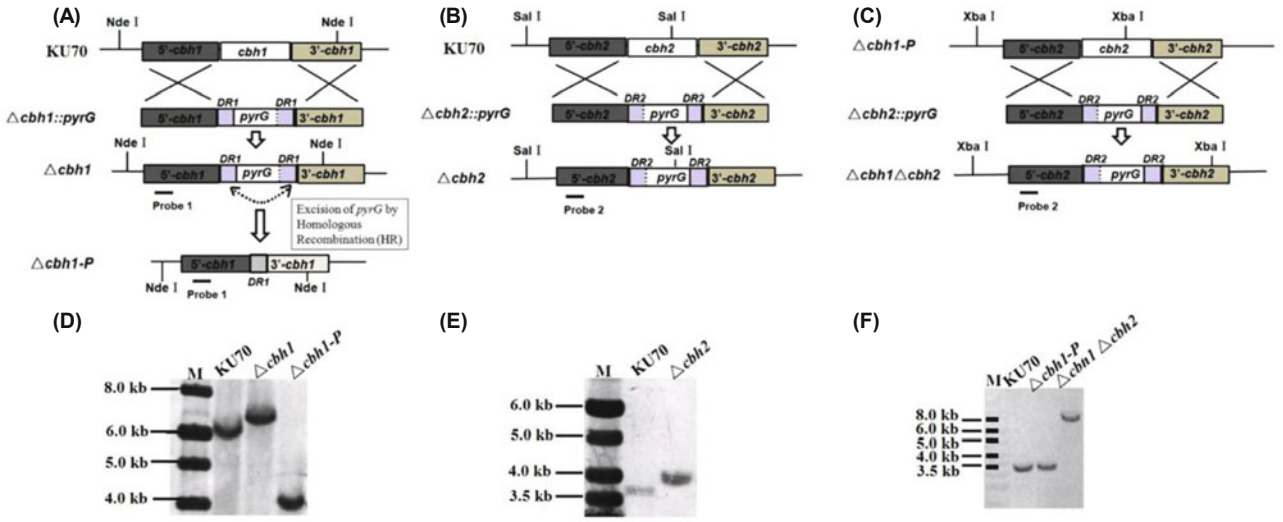

Fig. 1. Construction and identification of cellobiohydrolase knockouts Δ*cbh1*, Δ*cbh2*, and Δ*cbh1*Δ*cbh2*. The deletion of *cbh1* and/or *cbh2* in *T. reesei* were executed as shown in the graphical representation. Relative positions of the restriction sites are given. Southern blot analysis was used for the identification of Δ*cbh1* (A), Δ*cbh2* (B), and Δ*cbh1*Δ*cbh2* (C), respectively. (A) *Nde*I digested chromosomal DNA was probed with an upstream fragment of *cbh1*. Deletion of *cbh1* led to an increase of the original 6.1 kb *cbh1* fragment to 6.7 kb. (B) Genomic DNA of strain Δ*cbh2* was digested with *Sal*I. Filter was probed with a 1 kb-*Sal*I fragment comprising part of the upstream region of the *cbh1* gene. (C) Southern blot to verify *cbh2* was replaced in *T. reesei* Δ*cbh1* with hybridization probe 2. Chromosome was digested with *Xba*I. Fragment of 3.3 kb was observed in Δ*cbh1*Δ*cbh2,* while the size of hybridized fragment in the parent strain was 8.5 kb.

amplified using the primer pair pyrGh-S/pyrGh-A. The 5′ region of *cbh1* and the DR1 fragment were mixed and ligated by fusion PCR with the nested primers cbh1-S-N-S and cbh1-S-N-A. Then the fusion fragment, the *pyrG* expression cassette, and the 3′ region of *cbh1* were mixed and used as the template to amplify the deletion cassette Δ*cbh1*::*pyrG* by using the nested primer pair cbh1-L-N-S and cbh1-L-N-A. PCR experiments were carried out according to the manufacturer's recommendations and the PCR fragments were purified with Gel Extraction Kit (Omega). The Δ*cbh1*::*pyrG* cassette was then used to transform *T. reesei* KU70 protoplasts to obtain the *cbh1* deletion strain Δ*cbh1*-P as previously described by Gruber *et al.* (1990). To select for the excision of the marker gene *pyrG*, the Δ*cbh1*-P strain was grown in the presence of 5-FOA, which is toxic for the fungus with the *pyrG* gene product. Finally, 5-FOA resistant colonies, hereafter called Δ*cbh1*, were obtained after 3–4 days of incubation and used for the further genetic manipulations.

To construct the *cbh2* deletion strain, the Δ*cbh2*::*pyrG* cassette was designed as follows (Fig. 1B): the 5′ region of *cbh2*, the *pyrG* expression cassette, the direct repeat sequence (DR2), and the 3′ region of *cbh2* were firstly amplified using primer pairs cbh2-5F/cbh2-5R, pyrG-S/pyrG-A, pyrGd-S/pyrGd-A, and cbh2-3F/cbh2-3R, respectively. Then these fragments were fused together and the Δ*cbh2*::*pyrG* cassette was obtained by using the nested primer pair cbh2-L-N-S and cbh2-L-N-A. Fungal transformation and excision of the *pyrG* marker gene were performed as described above to acquire the Δ*cbh2* deletion strain.

For the double gene deletion, a recyclable marker based on the *pyrG* gene was used. The *cbh2* deletion cassette (Δ*cbh2*::*pyrG*) was further used to transform the protoplasts of the Δ*cbh1* strain to form the Δ*cbh1*Δ*cbh2*-P strain, which restored uracil/uridine prototrophy. Finally, the double deletion strain Δ*cbh1*Δ*cbh2* was acquired by growing in the presence of 5-FOA to excise the *pyrG* marker (Fig. 1C).

Southern blot analysis

Transformants were subjected to Southern blot analysis to dissect the integration types of deletion cassettes. Probe 1 and probe 2 located upstream of *cbh1* and *cbh2*, respectively, were amplified with the corresponding primer pairs listed in Supplementary data Table S1. The genomic DNA was digested by different restriction enzymes as shown in Fig. 1. Then the digested DNA fragments were separated on a 0.8% (w/v) agarose gel and transferred to Hybond N^+ membrane (Amersham Pharmacia Biotech). Probe labelling, hybridization, and detection were performed according to the manufacturer's recommendations for the use of the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche Applied Science).

Fungal growth on different carbon sources

The conidia (10^8/ml) of *T. reesei* strains were point-inoculated onto the centre of plates with minimal medium (0.5% NH_4SO_4, 0.06% $MgSO_4$, 1.5% KH_2PO_4, 0.06% $CaCl_2$, 0.00005% $FeSO_4{\cdot}7H_2O$, 0.00016% $MnSO_4{\cdot}H_2O$, 0.00014% $ZnSO_4{\cdot}7H_2O$, and 0.00002% $CoCl_2$) supplemented with 2% glycerol. After incubationat 30°C for 2 days, equal squares of agar plug from fungal colonies were transferred onto the MM plates containing glycerol, glucose, lactose, or cellobiose as carbon sources. The diameters of colonies were measured every 12 h and morphological phenotypes were observed at 3 days. Photographs of colonies were taken using a Digimax S500 camera (Samsung).

The double-layer microcrystalline cellulose (MCC) plates were prepared by casting a minimal medium containing 0.5% MCC onto a 2% agarbottom layer. Since crystalline cellulose,

such as MCC, is degraded at very slow rates, the more easily degradable soluble cellulose derivative, CMC-Na, was also used as the substrate for double-layer plate assay. The upper layer contained 1% CMC-Na while the bottom layer was composed of only 2% agar. Strains were pre-grown in medium containing 2% glycerol and equal squares of agar plug from fungal colonies were transferred onto the double-layer plates. The MCC and CMC-Na plates were incubated at 30°C for 7 days and 4 days, respectively. The CMC-Na plates were stained further with 0.1% Congo red solution. Diameter of the colonies and surrounding transparent zones were measured and morphological phenotypes were recorded using the camera described above. All assays were carried out in triplicate.

Enzyme activity assay and protein measurement

Filter paper activities in the supernatants were determined by Whatman No.1 filter paper (Sigma-Aldrich), using the method as previously described (Qian *et al.*, 2017). One filter paper unit was defined as the amount of enzyme required to reduce 1 μmol of sugar per min. Cellobiohydrolase activity was measured as described by Murray *et al.* (2004) with p-nitrophenyl-β-D-cellobioside (pNPC) as the substrate. β-Glucosidase activity was determined by p-nitrophenyl-β-D-glucoside (pNPG) (Qian *et al.*, 2017). One unit of activity was defined as the amount of enzyme required to release 1 μg of p-nitrophenyl per min. The supernatant proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad) and the predicted CBH1 and CBH2 bands were excised for MS-MS identification (BGI Tech. Solutions [Beijing Liuhe] Co., Ltd.). Three replicates were measured on each supernatant.

Saccharification of the cellulosic substrates

Five different cellulose-rich substrates, namely, commercial MCC, filter paper, acid-pretreated corncob residue (ACR), delignified corncob residue (DCR), and acid pre-treated corn stover (PCS) were applied to enzymatic saccharification. The components of ACR and DCR were reported by Liu *et al.* (2010) and Gao *et al.* (2017). The components of PCS were described by Ye *et al.* (2017). The reactions were carried out in 100 ml conical flasks, which contained 50 mM sodium phosphate buffer (pH 4.8) and 0.1% NaN_3 as bacteriostatic agent. Substrate concentrations were 5.0% (w/v) dry mass. The enzyme loading was at 10 FPU/g of dry biomass for the KU70 strain. For the other strains, the enzyme amount was normalised according to the β-glucosidase activity of KU70. The flasks were incubated at 50°C for 6 days at 150 rpm. Samples were collected at regular interval and analysed for released glucose content, which was measured with the SBA-40C biological sensor analyser (BISAS). The results shown were the mean of three independent experiments.

Results and Discussion

Deletion of cellobiohydrolase-encoding genes in *T. reesei*

To gain insight in the influence of the cellulases CBH1 and CBH2 on growth in *T. reesei*, the cellobiohydrolase-encoding genes, *cbh1* and/or *cbh2*, were deleted (Fig. 1) and deletion was confirmed by Southern blot analysis. Hybridization of *Nde*I-digested genomic DNA with probe 1 resulted in a 6.1 kb fragment in KU70, while a 6.7 kb fragment in Δ*cbh1*, and a 3.9 kb fragment in Δ*cbh1-P* (Fig. 1A and D). Similarly, Southern hybridization of *Sal*I-digested genomic DNA with probe 2 yielded a 3.7 kb fragment in KU70 and a 4.0 kb fragment in Δ*cbh2* (Fig. 1B and E). Furthermore, the integration

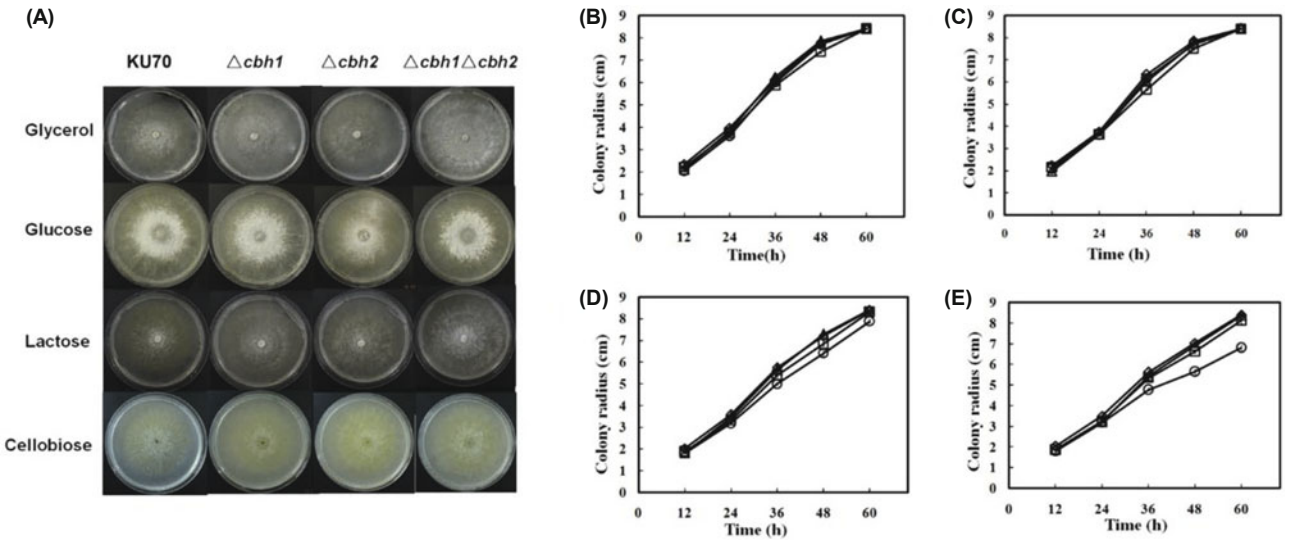

Fig. 2. Colonial phenotypes and growth rate of *T. reesei* KU70 and the knockouts. (A) Morphological phenotypes of *T. reesei* KU70 and the knockouts on different carbon sources. Photos were taken at 72 h after incubation on minimal medium supplemented with 2% (w/v) glucose, glycerol, lactose, or cellobiose. Growth rate of hyphae were measured after cultivation on the following carbon sources: (B) glucose, (C) glycerol, (D) lactose, and (E) cellobiose at 30°C for 4 days. Diamonds, Δ*cbh1*; squares, Δ*cbh2*; triangles, Δ*cbh1*Δ*cbh2*; circles, KU70. Each value represents the mean of three replicates and the error bars indicate standard deviation.

of the *pyrG* cassette into *cbh2* locus in the Δ*cbh1*-P genome using *Xba*I-digested genomic DNA with probe 2 yielded a 3.3 kb band in Δ*cbh1*-P and an 8.5 kb band in Δ*cbh1*Δ*cbh2* (Fig. 1C and F). These results show that the native *cbh1* and/or *cbh2* had been successfully knocked out and that there was no ectopic integration of the replacement cassette into the corresponding genomes.

Growth and morphology of Δ*cbh1*, Δ*cbh2*, and Δ*cbh1*Δ*cbh2* on different carbon sources

To investigate the deletion of *cbh1* or *cbh2* on growth with different carbon sources, a series of growth experiments were conducted in solid media. In detail, the deletion strains Δ*cbh1*, Δ*cbh2*, and Δ*cbh1*Δ*cbh2* were cultured on MM plates containing glucose, glycerol, lactose or cellobiose for 3 days, respectively. The results were shown in Fig. 2. No dramatical variation in morphology was observed for all the knockouts on different carbon sources (Fig. 2A), demonstrating that the morphology of *T. reesei* is not influenced by the deletion of cellobiohydrolases no matter what carbon source is used in the medium. Moreover, when grown on easily metabolizable glucose and glycerol, all knockouts showed normal colony sizes compared to the KU70 strain (Fig. 2B and C). It has been reported that preferentially utilisable carbon sources including glucose and fructose would not promote expression of cellulases (Ahamed and Vermette, 2008). Therefore, it is supposed that cellulase-negative strains should grow normally on glucose-containing media. However, disruption of single or both of the cellobiohydrolases resulted in slightly faster growth than KU70 when using lactose as the sole carbon source (Fig. 2D), and the growth promotion was much more obvious when these knockouts were culturedon cellobiose (Fig. 2E). These results were in accordance with the study of Ilmen *et al.* (1997) that cellulase expression occurred during the rapid growth is lower than the poor growth. In addition, it has been reported that the absence of cellulose result in lower growth rate on lactose than the parental strain (Nevalainen and Palva, 1978). As cellobiose and lactose can induce cellulase expression, it is supposed that synthesis and secretion of cellobiohydrolases under the induction condition consumes a large amount of energy and exerts pressure on growth. These results demonstrate that the role of cellobiohydrolases biosynthesis on fungal growth was carbon source-dependent.

Effect of *cbh1/2* gene deletion on the capacity of *T. reesei* to utilise cellulose substrates

The ability of the *T. reesei* strains to digest cellulose was investigated on solid media with MCC or CMC-Na as the sole carbon source (Fig. 3). When the strains were cultivated on MCC-containing plates, a considerably large and clear zone was observed around the colony of KU70, followed by Δ*cbh2* and Δ*cbh1* (Fig. 3A). The transparent zone radius of the strains was shown as Fig. 3B. Compared to that of Δ*cbh2*, Δ*cbh1* showed a much smaller clear zone, suggesting that CBH1 plays a more dominant role in the degradation of MCC. This was consistent with a previous report that deletion of *cbh2* has a much smaller effect on growth than *cbh1* for the Avicel-cultured *Neurospora crassa* (Tian *et al.*, 2009). When both cellobiohydrolase genes were deleted, neither clear zone nor mycelial growth could be observed under the same condition. These results validate that the cellulase system lacking cellobiohydrolases could not efficiently hydrolyse the crystal-

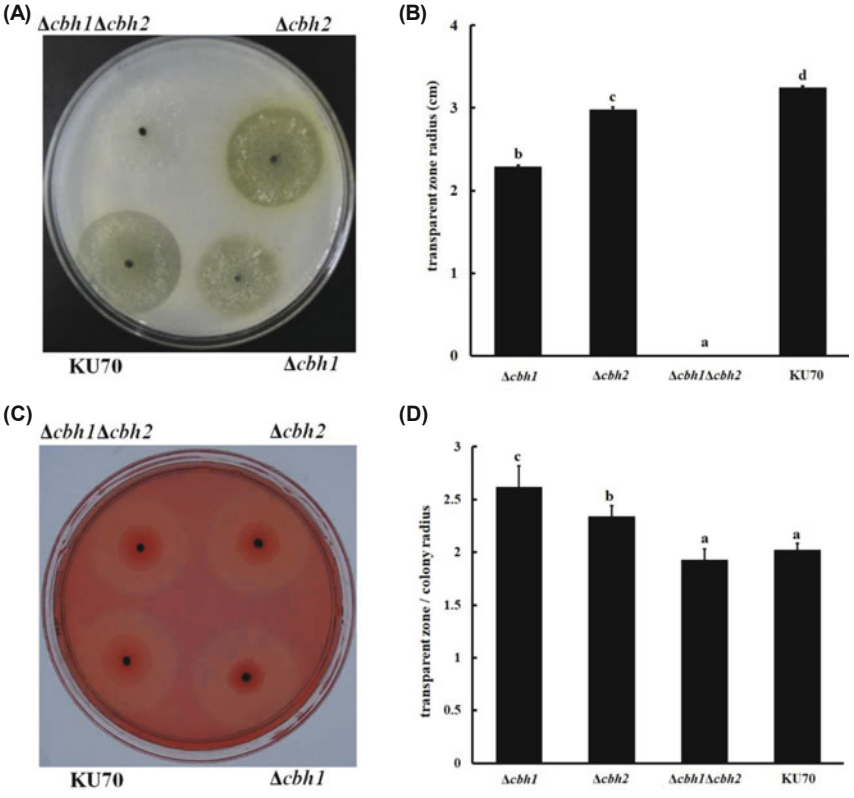

Fig. 3. Cellulase production of knockout strains on medium containing MCC and CMC-Na. *T. reesei* KU70 and knockout strains were cultured on medium containing MCC (A) and CMC-Na (C) at 30°C for 7 days and 4 days, respectively. The clear zone radius of strains on medium containing MCC (B) and ratios between the radius of the transparent zones and colonies (D) on medium containing CMC-Na. Different lowercase letters above the bars indicate significant differences at $P < 0.05$.

line cellulose into available carbon sources, which is vital for the survival of *T. reesei*. Furthermore, the amorphous ionic-substituted carboxymethyl cellulose (CMC-Na) was used as the sole carbon source to investigate the effects of cellobiohydrolase deletion on the ability of the fungus to utilize cellulose substrates. Colonies of the *cbh1* and *cbh2* knockouts formed similar transparent zones after 4 days of incubation compared with those of KU70 (Fig. 3C), suggesting that the absence of cellobiohydrolasedid not deprive KU70 of the ability to degrade the amorphous cellulose substrate. The ratios between the radius of the clear zone and the colony were also compared among the strains (Fig. 3D). The double deletion strain had a similar ratio to KU70 while the single deletion strains had slightly higher ratios. Interestingly, Δ*cbh1* showed the highest ratio after a 4-day incubation, which could be an indication of improved CMC-Na digestion. While the double deletion strain Δ*cbh1*Δ*cbh2* could hardly grow and the single deletion strains grew poorly using MCC as the sole carbon source, this effect was not found on CMC-Na substrate and was similar to the report of Seiboth *et al.* (1992) that the *cbh2* deletion caused a delayed growth on crystalline cellulose. Taken together, these results provide quantitative evidence demonstrating that the influence of CBH1/CBH2 secretion during fungal growth was carbon source-dependent.

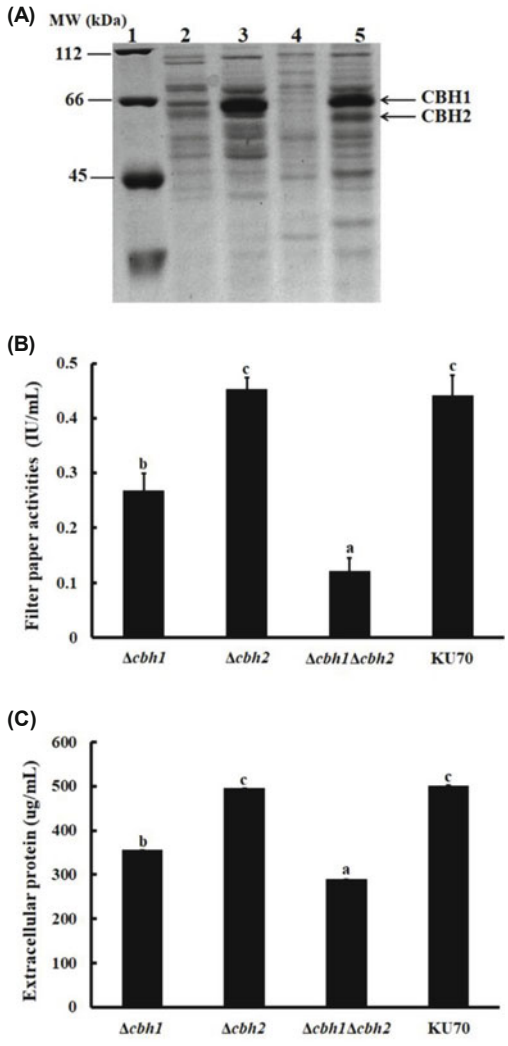

Fig. 4. SDS-PAGE analysis (A), filter paper activities (B), and extracellular protein concentration (C) of *T. reesei* KU70 and respective knockouts. In (A), proteins secreted by Δ*cbh1* (lane 2), Δ*cbh2* (lane 3), Δ*cbh1*Δ*cbh2* (lane 4), and KU70 (lane 5) were separated by SDS-PAGE alongside a protein ladder (lane 1). Protein bands representing CBH1 and CBH2 are marked. In (B) and (C), data indicate the mean of three independent experiments and error bars express the standard deviation. Different lowercase letters above the bars indicate significant differences at $P < 0.05$.

Cellulase production by the *cbh1/2* deletion strains

Cultivation of the double knockout strains on MCC substrate led to an absent clear cellulolytic zone prompted us to examine the cellulase production in liquid medium containing another carbon source, lactose. It is known that lactose is an excellent inducer for cellulase production in *T. reesei* and is used as the carbon source for fungal growth (Xiong *et al.*, 2004). Therefore, lactose instead of cellulose was chosen as the sole carbon source to avoid possible effects on growth of the knockouts lacking cellobiohydrolases. All strains were grown to exponential phase in minimal medium, thus keeping the growth rate at the same level, and then equal amounts of mycelia were transferred to the lactose medium and induced for cellulase expression. Analysis of the pNPC (the substrate for CBH1) activity demonstrated that Δ*cbh1* and Δ*cbh1*Δ*cbh2* lost nearly all extracellular pNPC activities, although Δ*cbh2* showed higher pNPC activity than KU70 (Supplementary data Fig. S1). Analysis by SDS-PAGE confirmed that the CBH1 bands in Δ*cbh1* and Δ*cbh1*Δ*cbh2* are absent while a stronger band was found in Δ*cbh2* (Fig. 4A). And the predicted CBH1 and CBH2 bands were also determined by MS/MS identification. Moreover, both extracellular protein production and filter paper activity were dramatically reduced by 41% and 63%, respectively, in Δ*cbh1*Δ*cbh2* compared with KU70 (Fig. 4B and C). While the absence of CBH2 alone led to slightly reduced extracellular protein production and filter paper activity, Δ*cbh1* showed a marked decrease and a ~20% loss of capacity to filter paper activity (Fig. 4B and C). These results indicate that cellobiohydrolase deficiencies had a significant impact on the total cellulase activity produced by *T. reesei*.

Influence of cellobiohydrolase deficiencies on enzymatic hydrolysis of different cellulose substrates

MCC and filter paper, which are important reference substrates for the study of cellulase function, were first used to investigate the ability of the *T. reesei* knockouts to hydrolyse pure cellulose substrates (Fig. 5A and B). For each enzyme investigated, the conversion rate of filter paper was higher than that of MCC, which could be explained by the higher degree of crystallinity in MCC (Zhang *et al.*, 2006).

Previous reports described that higher enzyme loads were required for hydrolysis of crystalline cellulose compared with amorphous substrate (Szijártó *et al.*, 2008) and that BGL activity is barely affected by the disruption of the other cellulases (Nevalainen and Palva, 1978; Kawai *et al.*, 2013). Therefore, the cellulase concentrations for biomass saccharification were normalised to BGL activity. Δ*cbh1* displayed a pronounced decrease in hydrolysis of both MCC and filter paper with cel-

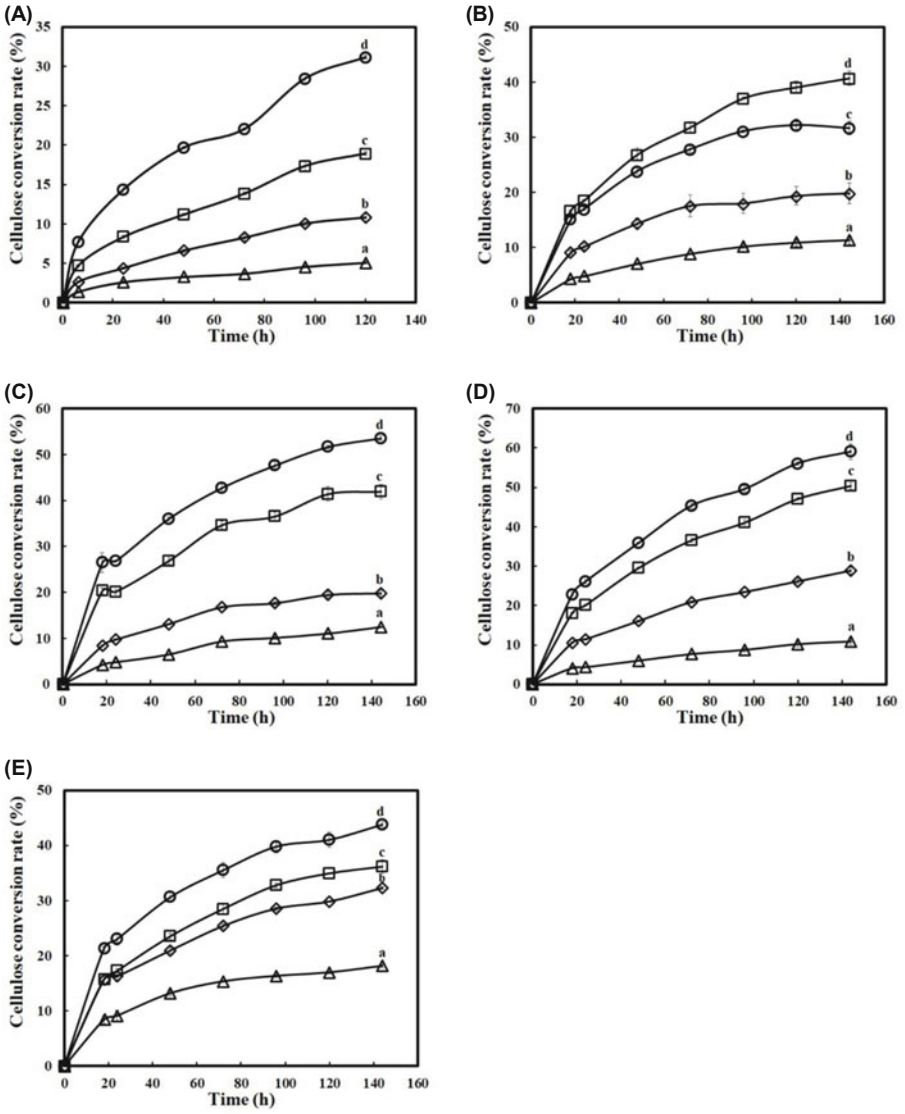

Fig. 5. Enzymatic hydrolysis over time of MCC (A), filter paper (B), acid pre-treated corncob residue (C), delignified corncob residue (D), and acid pre-treated corn stover (E). Enzymes were loaded at a concentration of 8.7 IU, 8 IU, 6.5 IU, 8.4 IU, or 3.7 IU cellobiose, respectively. Diamonds, Δ*cbh1*; squares, Δ*cbh2*; triangles, Δ*cbh1*Δ*cbh2*; circles, KU70. Different lowercase letters above the bars at the last time point of the reactions indicate significant differences at $P < 0.05$.

lulose conversion rates of 11.3% and 20.1%, respectively, compared with KU70 (31.2% and 32.5%, respectively). As expected, Δ*cbh1*Δ*cbh2* had the lowest ability to hydrolyse MCC and filter paper where only 5.2% and 10.8% cellulose conversion was achieved, respectively. Interestingly, Δ*cbh2* cellulase showed a reduced hydrolysis rate for MCC (19.8%), but exhibited an increased capacity to hydrolyse the filter paper (40.6%). Meanwhile, the activity of CBH1 in Δ*cbh2* was higher than that in KU70 (Supplementary data Fig. S1). These results indicate that CBH2 was ineffective for filter paper digestion and the improved hydrolysis ability of Δ*cbh2* could be explained by increased CBH1 activity. However, the significant decrease in the saccharification rate of MCC by Δ*cbh2* suggested that CBH2 might occupy a dominant position in hydrolysis of crystalline cellulose. This conclusion is in agreement with CBH2 being the main cellulase produced by *T. reesei* grown on MCC (Peciulyte *et al.*, 2014).

Corncobs and corn stover are abundantly available lignocellulosic materials and considered as a primary source for bioethanol production (Torney *et al.*, 2007). Here, ACR, DCR, and PCS were selected as substrates to investigate the role of cellobiohydrolases on saccharification rate. The results using ACR and DCR as substrates were shown in Fig. 5C and D. The cellulose conversion rate using ACR as substrate was found to be 53.2% in KU70, whereas the conversion rate with DCR was as high as 61.3%. Δ*cbh1*Δ*cbh2* exhibited a significant decrease of more than 85% in conversion rate on all substrates. The saccharification rate in Δ*cbh1* cellulase was reduced more significantly than in Δ*cbh2* cellulase (63% vs 25%) on ACR substrate. Even more pronounced results were obtained using DCR as substrate (51% vs 10%). These results indicated that although CBHs were the dominant cellulases in hydrolysing corn residues, the effect of the absence of CBH1 and CBH2 on cellulose conversion varied notably across the types of substrates and pre-treatment methods. Furthermore, the similar saccharification experiment was carried out with PCS as substrate (Fig. 5E). The cellulose conversion rates of the cellulases Δ*cbh1* and Δ*cbh2* were reduce by only 24.4% and 18.3%, respectively, compared with KU70. Meanwhile, double deletion of *cbh1* and *cbh2* led to a 59.6%

reduction in the cellulose conversion rate. Hence, the absence of CBH1 and CBH2 had a lesser effect on the hydrolysation of PCS compared with ACR and DCR, indicating that the contribution of CBHs on cellulose conversion rate depended on pre-treatment type as well as biomass origin and was in concordance with the report by Kawai *et al.* (2013).

Taken together, this study genetically investigated the effects of absence of CBH(s) on fungal growth and cellulolytic ability of *T. reesei*. The effect of cellobiohydrolase synthesis on fungal growth was found to be carbon source-dependent as synthesis consumes a large amount of energy on the induction conditions and exerts pressure on fungal growth. CBH1 and CBH2 are also key enzymes for cellulose degradation in the *T. reesei* cellulolytic system, thus the deficiency of cellobiohydrolases had a significant impact on the total cellulase activity. In particular, the double deletion knockout had more than 60% decrease in the hydrolysis rate of all tested substrates. In addition, CBH2 appeared to occupy a dominant position in the hydrolysis of crystalline cellulose by improving CBH1 activity compared to filter paper suggesting that CBHs can affect the expression of other cellulases as well as decomposition of different biomass materials. Thus, these results indicate that the genetic reconstitution of cellulase composition requires a specific harmony of cellulases for more efficient saccharification of each substrate, which will lead to lower costs for industrial cellulose-derived biofuel production.

Acknowledgments

This work was supported by grants from the National Key R&D Program of China (Grant no. 2018YFA0900503), the National Natural Science Foundation of China (Grant no. 31970070), and the Shandong Key Research and Development Program (Grant no. ZR2019ZD19 and 2016GGH3103).

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