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High efficient expression of cellobiase gene from *Aspergillus niger* in the cells of *Trichoderma reesei*

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ABSTRACT

The cellobiase gene from *Aspergillus niger* was cloned and connected with the strong promoter Pcbh1 from *Trichoderma reesei* to construct a recombinant plasmid pHB9 with the hygromycin B resistance marker. The plasmid was transformed into conidia of *T. reesei* using the modified PEG–$CaCl_2$ method. Main factors effecting the transformation were discussed and about 99–113 transformants/μg DNA could be obtained under optimal conditions. It was found that the molecular mass of the recombinant cellobiase was about 120 kDa by SDS–PAGE analysis. The activity of cellobiase could reach 5.3 IU/ml after 48 h fermentation, which was as high as 106 times compared with that of the host strain. Meanwhile, the filter paper activity of recombinant *T. reesei* was 1.44-fold of the host strain. Saccharification of corncob residue with the crude enzyme showed that the hydrolysis yield (84.2%) of recombinant *T. reesei* was 21% higher than that (69.5%) of the host strain.

1. Introduction

Cellulose is the major component of plant cell walls and the most abundant renewable resource that can be used to produce environment-friendly biofuels and chemicals (Sánchez, 2009). Conversion of cellulosic materials to glucose mainly depends on the degradation capacity of cellulase produced by cellulolytic microorganisms (Kovács et al., 2009). *Trichoderma reesei* is well known as one of the most important filamentous fungi for cellulase production (Martinez et al., 2008). The cellulase system of *T. reesei* is composed of endoglucanases (EG), cellobiohydrolases (CBH) and cellobiases (CB) (Barnett et al., 1991; Bower et al., 1998; Foreman et al., 2003; Saloheimo et al., 2002; Takashima et al., 1999; Ward et al., 1993). The cellulases from *T. reesei* act synergistically on the hydrolysis of crystalline cellulose (Singhania et al., 2010; Sipos et al., 2010). The endoglucanases mainly hydrolyze the cellulose chains internally, producing new chain ends for cellobiohydrolases. The cellobiohydrolases, also known as exoglucanases, act progressively from the chain ends to create mainly cellobiose. The cellobiase hydrolyze cellobiose and cello-oligosaccharides to form glucose (Teeri, 1997).

The major cellobiohydrolase component of *T. reesei* is CBHI, which accounts for 50–60% of the total secreted protein of *T. reesei* (Uusitalo et al., 1991). As the CBHI is encoded by a single gene, the promoter of the cellobiohydrolase 1 (cbh1) gene is regarded as a strong promoter, which has been widely used for the over-expression of homologous and heterologous proteins in *T. reesei* (Rahman et al., 2009).

The cellobiase only accounts for 1% of the total secreted protein of *T. reesei* (Uusitalo et al., 1991). Low cellobiase restricts the conversion of cellobiose and cello-oligosaccharides to glucose, and the accumulation of cellobiose and cello-oligosaccharides will cause feedback inhibition to the cellulase reaction (Saloheimo et al., 2002; Sukumaran et al., 2009). Thus, the cellobiase plays a critical role in the cellulose degradation process, and improving the activity of cellobiase in the cellulase system is the key to raise the saccharification yield of cellulose. The results in the previous work suggest that supplementing cellulase preparations from *T. reesei* with cellobiase from *Aspergillus niger* can significantly increase the cellulose hydrolysis yield (Chen et al., 2008). Therefore, the construction of cellobiase overproducing strains is an important way to enhance the efficiency of cellulose hydrolysis.

Many microbial cellobiase have been heterologously expressed (Bhatia et al., 2002; Hong et al., 2007). The cellobiase gene from *A. niger* has been already characterized and expressed in bacteria and yeast (Dan et al., 2000). In this work, the cellobiase gene from *A. niger* ZU-07 (Shen and Xia, 2004) was cloned, and the extracellular secretion and over-expression of cellobiase were successfully achieved by recombinant *T. reesei*.

2. Methods

2.1. Strains and medium

T. reesei ZU-02 (Xia and Shen, 2004) was used as recipients for transformation and chromosomal DNA preparation. *A. niger* ZU-07 (Shen and Xia, 2004) was used for isolation of cellobiase

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gene. *Escherichia coli* strain DH5α was used for propagation of plasmids.

The seed medium for *A. niger* and *T. reesei* was composed of 2.0% glucose, 0.9% corn steep liquor, 1.0% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.05% $CaCl_2$, 0.0005% $FeSO_4{\cdot}7H_2O$, 0.00016% $MnSO_4{\cdot}H_2O$, 0.00014% $ZnSO_4{\cdot}7H_2O$, and 0.00037% $CoCl_2{\cdot}6H_2O$.

The fermentation medium for *T. reesei* had the following composition: 2.0% lactose, 1.2% corn steep liquor, 1.0% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.05% $CaCl_2$, 0.18% $CaCO_3$, 0.2% wheat bran, 0.02% Tween 80, 0.0005% $FeSO_4{\cdot}7H_2O$, 0.00016% $MnSO_4{\cdot}H_2O$, 0.00014% $ZnSO_4{\cdot}7H_2O$, and 0.00037% $CoCl_2{\cdot}6H_2O$. The initial pH value was 4.8.

2.2. Cloning of A. niger cellobiase gene

Spores of *A. niger* ZU-07 were inoculated into the seed medium (containing 2% cellobiose instead of 2% glucose) and grown for 2 days at 30 °C with shaking at 200 rpm. The mycelium was then frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Total RNA was then produced from this powder by the TRNzol reagent (takara). Reverse transcriptase reaction using the total RNA as the template was carried out with the reverse transcriptase-polymerase chain reaction kit (takara) to obtain the cDNA. DNA amplification was carried out with the primers (5′-TTACTCGAGCTGATGAATTGGCCTACT-3′, containing XhoI site) and (5′-GCGTCTAGATTAGTGAACAGTAGGCAGA-3′, containing XbaI site) using the cDNA as the template. The resulting gene fragment was cloned into pMD18-T cloning vector (takara) and sent to be sequenced.

2.3. Construction of expression vector with Pcbh1 and Tcbh1 from T. reesei

Spores of *T. reesei* ZU-02 were inoculated into the seed medium and grown for 2 days at 30 °C with shaking at 200 rpm for chromosomal DNA preparation. The mycelium was then frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. The chromosomal DNA was prepared from this powder by the method of CTAB (Sambrook and Russell, 2001).

The PCR amplification of cellobiohydrolase 1 promoter (Pcbh1) with its signal peptide and cellobiohydrolase 1 terminator Tcbh1 from *T. reesei* was carried out with the primers (5′-GTAGGATCCAAGCTTCCATTTGGCGGCT-3′, 5′-CTATCTAGAAGCTCGAGCAGTAGCCAAG-3′) and (5′-CGCTCTAGATGAACCCTTACTACTCTCAGT-3′, 5′-ATTGCATGCA-CTAGTGTCCTCGGCACGTTGTCATC-3′) using the chromosomal DNA of *T. reesei* as the template. The recombinant expression vector pPT5 containing the Pcbh1 and Tcbh1 region of 1.4 kb and 0.6 kb was constructed with PUC18 as a vector backbone.

2.4. Construction of recombinant vector containing the hygromycin B resistant maker

The PCR amplification of pyruvate kinase promoter (Ppki1) from *T. reesei* was carried out with the primers (5′-CATAGATCTGCAGCACGAGATAACGGTG-3′) and (5′-ATAGAGCTC-GGTTAAGAGGGTTCTTCCG-3′) using the chromosomal DNA of *T. reesei* as the template.

The hygromycin B phosphotransferase (hph) gene was amplified with the primers (5′-CTAGAGCTCATGAAAAAGCCTGAACTCACCGCGACG-3′) and (5′-GTAGGATCCAC-GCGTCTATTTCTTTGCCCTCGGAC-3′) using the plasmid pCAMBIA1301 containing the *E. coli* hygromycin B phosphotransferase (hph) gene as the template.

The recombinant vector pPH4 containing a *T. reesei* Ppki1 region of 0.7 kb and a 1.0 kb *E. coli* hph gene was constructed with PUC18 as a vector backbone.

2.5. Preparation of protoplasts of T. reesei

Plates containing PDA medium were inoculated with 1×10^7 spores and incubated at 30 °C for 3–6 days. The spores collected from the plate were used to inoculate 50 ml of YPD medium and incubated in an orbital shaker at 200 rpm and 30 °C for 6–14 h. Most of the spores had germinated after this incubation period. The germinated spores were then washed once with 0.9% NaCl and twice with PM (Protoplast buffer: 10 mM sodium phosphate buffer, pH 5.8; 1.2 M magnesium sulfate). Then the germinated spores were resuspended in 20 ml of PM containing snailase at concentration of 10 mg/ml and incubated at 30 °C for 0.5–3 h with shaking at 100 rpm to allow the release of the protoplasts.

The protoplasts were collected and diluted 1:1 with TS buffer (10 mM Tris–HCl, pH 7.5; 1 M sorbitol). This protoplast suspension was centrifuged for 5 min at 3000 rpm, and the protoplasts were washed once with TSC (10 mM Tris–HCl, pH 7.5; 1 M sorbitol; 20 mM $CaCl_2$). Finally, the protoplasts were resuspended in TSC plus 1/10 of the final volume of TPC (10 mM Tris–HCl, pH 7.5; 20 mM $CaCl_2$; 60% polyethylene glycol 6000 or 25% polyethylene glycol 6000), at a concentration between 1×10^7 and 1×10^9 protoplasts/ml.

2.6. Protoplasts transformation

Hundred microliters of the protoplast suspension, obtained as previously described, were mixed with 2 μg of circular plasmid or linearized plasmid. The plasmid and the protoplast suspension were maintained on ice for 20 min. Then, 500 μl of TPC were added and the mixture was incubated at room temperature for another 20 min. Finally, the mixture was diluted with 600 μl of TSC and poured as an overlay on regeneration PDASH (PDA; 1 M sorbitol; 100 μg/ml hygromycin B) plates. The plates were maintained at room temperature until the medium had solidified, and subsequently incubated at 30 °C for 4–6 days, in order to allow the regeneration of the protoplasts and growth of the colonies.

2.7. Detection of chromosomal DNA of recombinant T. reesei by PCR

The chromosomal DNA of recombinant *T. reesei* transformants was prepared as above. The PCR amplification of *A. niger* cellobiase gene was carried out with upstream and downstream primer of *A. niger* cellobiase gene in method 2.2 using chromosomal DNA of recombinant *T. reesei* and the host strain as the template. The result was assayed by agarose gel electrophoresis.

2.8. Cellulase production

For inoculum preparation, 1.0 ml of a spore suspension (containing 10^7 conidia/ml) of the host strain and recombinant *T. reesei* transformants were inoculated into 50 ml of the seed medium in 250 ml Erlenmeyer flasks, and cultured at 30 °C, 180 rpm for 48 h.

The enzyme production experiments were carried out in 250 ml Erlenmeyer flasks with 50 ml of fermentation medium. The inoculum ratio was 10% (v/v), and the flasks were shaken at 30 °C, 180 rpm for 48 h.

2.9. Corncob pretreatment and enzymic hydrolysis process

Corncob was air-dried and ground to particles of about 3 mm diameter. One percent H_2SO_4 was added with a solid and liquid ratio of 1:8. The mixtures were reacted at 110 °C for 3 h and filtered. The residue was washed to pH 5.0 and dried, and had the following composition: cellulose 57.82%, hemicellulose 5.01%, lignin 21.20%, and ash 15.97%.

The hydrolysis experiments were performed with 10% corncob residue as substrate and 20 IU FPA of host strain and recombinant *T. reesei* at 50 °C, pH 4.8 for 48 h.

2.10. Analysis methods

Filter paper activity (FPA) and cellobiase activity were assayed according to standard IUPAC procedures and expressed as international units (IU) (Ghose, 1987). One international unit of cellulase activity is the amount of enzyme that forms 1 μmol of glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. One international unit of cellobiase activity is the amount of enzyme that forms 2 μmol of glucose/min from cellobiose.

The reducing sugar was determined using a 3,5-dinitrosalicilic acid (DNS) colorimetric assay method (Ghose, 1987). Glucose, cellobiose and xylose were analyzed by higher-performance liquid chromatography (HPLC) (Waters HPX-87P ion exclusion column).The yield of enzymic hydrolysis was calculated as follows:

$$\text{Yield } (\%) = (\text{reducing sugar} \times 0.9 \times 100)/(\text{carbohydrate in substrate})$$

The molecular mass of the crude cellobiase in the culture broth produced by recombinant *T. reesei* was determined by using SDS–PAGE (8% polyacrylamide). The electrophoresis was carried out at 10 mA for 1 h and 20 mA for 2 h. The gel was stained with 0.27% Coomassie Brilliant Blue R-250, and destained by washing overnight with a mixture of acetic acid–methanol–water (10:20:70, v/v/v).

3. Results and discussion

3.1. Construction of the expression plasmid with A. niger cellobiase gene

The cellobiase gene (CB) of 2.5 kb was inserted into the plasmid pPT5 under the control of Pcbh1 and its signal peptide to obtain the Pcbh1–CB gene–Tcbh1 expression cassette. This cassette was then inserted into the plasmid pPH4 containing the hygromycin B resistance marker to construct recombinant plasmid pHB9 (Fig. 1).

The strong Pcbh1 of *T. reesei* has been widely used for overexpression of homologous and heterologous proteins in *T. reesei* (Rahman et al., 2009). Therefore, through the plasmid pHB9 including the Pcbh1–CB gene–Tcbh1 expression cassette, the cellobiase from *A. niger* could be efficiently expressed and secreted under the control of the strong Pcbh1 and its signal peptide from *T. reesei*. In addition, comparing with the method reported before (Penttilä et al., 1987), the transformation can be carried out with plasmid pHB9 containing hygromycin B resistant maker instead of two plasmids of the expression plasmid with gene interested and the plasmid with resistance maker. It could be much more efficient for screening of positive transformants.

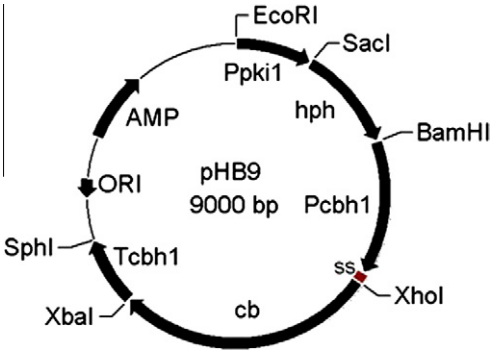

Fig. 1. Recombinant plasmid pHB9 with Pcbh1–CB gene–Tcbh1 expression cassette and hygromycin B resistant maker.

By means of the plasmid pHB9, the various kinds of homologous and heterologous proteins can be efficiently expressed and secreted instead of cellobiase. It suggested that the expression system of *T. reesei* had been successfully established, which was meaningful in the directed evolution of cellulase producing strain *T. reesei*.

3.2. Main factors effecting transformation efficiency

3.2.1. Concentration of polyethylene glycol (PEG)6000

According to the literature reports (Cardoza et al., 2006; Karhunen et al., 1993; Penttilä et al., 1987), all known protocols for protoplast transformation of filamentous fungi are based on the utilization of PEG (mainly containing 25% PEG6000 and 60% PEG6000). After 8 h incubation and 1 h snailase treatment of conidia, 5×10^8 protoplasts/ml was obtained. The transformation was carried out by 25% PEG6000 and 60% PEG6000 with the circular plasmid and linearized plasmid (Table 1). The transformation efficiency (101–110 transformants/μg DNA) with circular plasmid of pHB9 by 60% PEG6000 was about 5-fold of that (21–29 transformants/μg DNA) by 25% PEG6000. Nevertheless, with the linearized plasmid of pHB9, the transformation efficiency by 25% PEG6000 was 40–47 transformants/μg DNA, which was 8–13 times compared with that (3–6 transformants/μg DNA) by 60% PEG6000. This result showed that the 60% PEG6000 was beneficial for the transformation with circular plasmid. However, the 25% of PEG6000 was suitable for transformation with linearized plasmid.

3.2.2. Incubation time

The protoplast of filamentous fungus was always prepared by germinated conidia (Cardoza et al., 2006; Penttilä et al., 1987). The effect of the incubation time of conidia on transformation efficiency was investigated. The transformation efficiency reached 101–112 transformants/μg of DNA when the incubation time of conidia was 8 h. Most of the conidia had germinated after this incubation period, giving rise to a mycelium without closed pellets. However, the transformation efficiency was almost zero with 6 h incubation of conidia, and when the incubation time of conidia was longer than 8 h, the transformation efficiency also reduced sharply. This result showed that the incubation time of conidia was critical for the process of *T. reesei* transformation.

3.2.3. Enzymatic treatment time

The protoplast preparation is a critical process in the protoplast transformation experiment. The enzymatic treatment of conidia is the most important procedure in the process of protoplast preparation. The enzymatic treatment time of germinated conidia by snailase instead of expensive Novozym 234 and lysing enzymes (Cardoza et al., 2006; Penttilä et al., 1987) which were used frequently in the process of protoplast preparation was investigated. The optimal enzymatic treatment time was 1 h, and the transformation efficiency reached 99–113 transformants/μg of DNA. The

Table 1
Effect of concentration of PEG6000 on the transformation efficiency.

PEG6000	Transforming DNA	Transformants/μg DNA
25% PEG6000	pHB9, circular plasmid	21–29
	pHB9, linearized plasmid	40–47
60% PEG6000	pHB9, circular plasmid	101–110
	pHB9, linearized plasmid	3–6

result proved that the snailase can replace the expensive Novozym 234 and lysing enzymes to efficiently prepare protoplast.

3.2.4. Concentration of protoplasts

During the transformation of bacteria and yeast, the number of transformed cells is always an important factor. The effect of this factor on the transformation efficiency of *T. reesei* was also investigated. By increasing the concentration of protoplasts to about 5×10^8/ml, the transformation efficiency can be raised to 100–105 transformants/μg DNA. However, when the concentration of protoplasts was higher than 5×10^9/ml, a sharp decrease in the transformation efficiency occurred.

3.3. Verification of chromosomal DNA of recombinant T. reesei by PCR

Ten fast growth transformants on the culture plates containing hygromycin B as the screening maker were obtained. The host strain and 10 fast growth transformants were detected by PCR. There was no PCR product in the host strain. However, all of the transformants had the PCR products of 2.5 kb *A. niger* cellobiase gene. It demonstrated that the *A. niger* cellobiase gene had been integrated into the chromosomal DNA of recombinant *T. reesei*.

3.4. Cellobiase and cellulase productions in recombinant T. reesei transformants

After 48 h fermentation of the host strain and 10 fast growth transformants, the supernatants were detected by enzyme activity assay and SDS–PAGE. The SDS–PAGE (Fig. 2) revealed that the positive transformants secreted the recombinant cellobiase of about 120 kD which was similar to that of the native cellobiase of *A. niger* (Dan et al., 2000).

Fig. 3 shows that all of the transformants possessed the cellobiase activities of 2.43–5.3 IU/ml. The highest cellobiase activity (5.3 IU/ml) was as high as 106 times compared with that (0.05 IU/ml) of the host strain. It demonstrated that the cellobiase gene had been efficiently expressed under the control of strong Pcbh1 and the enzyme was continuously secreted into the culture through the cbh1 signal peptide sequence. In the study of Zhang et al. (2009), the cellobiase gene of *T. reesei* was cloned and expressed under the control of the modified four-copy Pcbh1, the cellobiase activity of recombinant *T. reesei* was 3.7-fold higher than that of host strain. Although the expression efficiency can be increased under the control of the modified Pcbh1, because the cellobiase gene from *T. reesei* was not as efficient as that from *A. niger*. The expression level will be still relatively low.

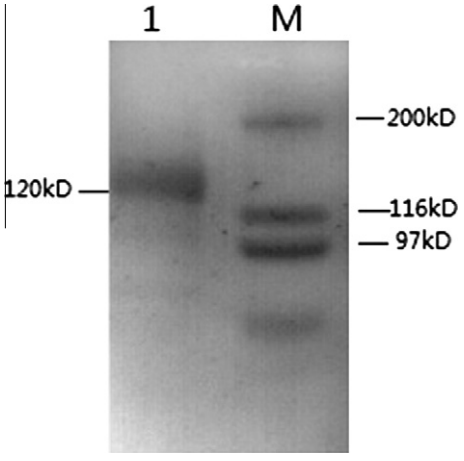

Fig. 2. SDS–PAGE analysis of recombinant cellobiase from culture supernatants. Lane 1, recombinant cellobiase from culture supernatants and lane M, high molecular mass marker.

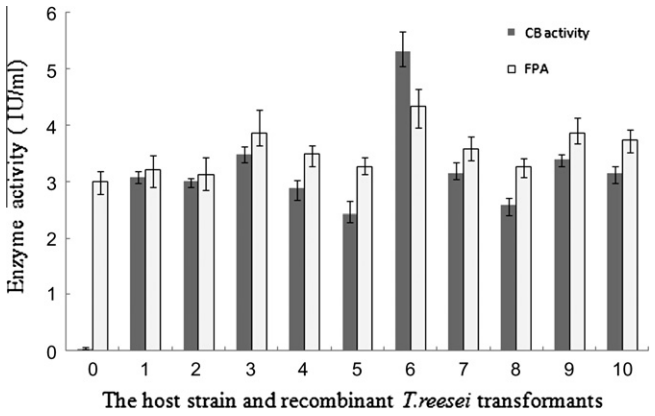

Fig. 3. Results of cellobiase and cellulase productions using recombinant transformants of *T. reesei* and the host strain. The enzyme production experiments were carried out in 250 ml Erlenmeyer flasks with 50 ml of fermentation medium. The inoculum ratio was 10% (v/v), and the flasks were shaken at 30 °C, 180 rpm for 48 h.

The FPA in culture broth produced by recombinant *T. reesei* can reach 4.33 IU/ml, which was about 1.44-fold of that (3.00 IU/ml) of the host strain. The filter paper activity (FPA) represents the total activity of cellulase. One international unit of FPA is the amount of enzyme that forms 1 μmol reducing sugar per minute during the hydrolysis reaction. The cellobiase mainly hydrolyze cellobiose to form glucose, the increasing of cellobiase activity can enhance the concentration of glucose in the hydrolysate, yet it cannot remarkably improve the concentration of reducing sugar. Therefore, although the raising of cellobiase activity eliminates feedback inhibition of cellobiose to the cellulase reaction, it cannot remarkably increase the filter paper activity (Breuil et al., 1992). The ratio of CB activity to FPA in recombinant *T. reesei* can reach 1.22 which was much higher than that (0.02) in the host strain. According to the previous reports (Chen et al., 2008), during the process of hydrolysis of cellulosic materials, when the ratio of CB activity to FPA reached above 0.3, the cellobiose will be efficiently converted to glucose. Therefore, the cellulase preparation of the recombinant *T. reesei* can be directly applied for hydrolysis of lignocellulosic biomass without supplementing cellobiase of *A. niger*. The result is significant in efficient utilization of lignocellulosic biomass.

3.5. Hydrolysis of corncob residue by cellulase of the host strain and recombinant T. reesei

Table 2 shows that the concentration of reducing sugar in the hydrolysate was only 48.50 g/l and the hydrolysis yield was 69.5% for the host strain. The concentration of glucose was relatively low, while that of cellobiose and other oligosaccharides was relatively high, showing the lack of cellobiase in the reaction system. However, for the recombinant *T. reesei*, the cellobiose was quickly hydrolyzed and the feedback inhibition caused by the cellobiose accumulation disappeared sharply. Therefore, the concentrations of reducing sugar and glucose in the hydrolysate were both clearly increased. The hydrolysis yield of the corncob residue was improved to 84.2%, which was 21% higher than that of the host strain. And the total reducing sugar reached 58.78 g/l. These results showed that the recombinant *T. reesei* produced the more efficient enzyme system to saccharify the cellulosic substrate. This research work is meaningful in the conversion and utilization of renewable biomass and has good social and economical significance.

Table 2
Saccharification of corncob residue by the host strain and *recombinant T. reesei* with equal FPA of 20 IU for displayed data representing averages of three independent experiments.

Strain	FPA (IU)	CB activity (IU)	Reducing sugar (g/l)	Glucose (g/l)	Cellobiose (g/l)	Xylose (g/l)	Others (g/l)	Hydrolysis yield (%)
Host strain	20	0.35	48.50	26.0	9.36	3.59	9.55	69.5
Recombinant *T. reesei*	20	25.4	58.78	52.6	0	4.88	1.29	84.2

4. Conclusions

A recombinant plasmid pHB9 with the hygromycin B resistance marker was successfully constructed, in which the cellobiase gene from *A. niger* was inserted between the strong promoter Pcbh1 (including secreting signal peptide sequence) and the terminator Tcbh1 from *T. reesei*. The plasmid was transformed into the conidia of *T. reesei* by modified PEG–$CaCl_2$ method, and fast growth transformants were isolated on the culture plates containing hygromycin B. High activity of cellobiase was achieved by the recombinant *T. reesei*. Meanwhile, the improvement of FPA was obtained in CB-overexpressing strains. Moreover, the crude cellulase preparations produced by the transformants can be used for saccharification of biomass with considerable efficiency.

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