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SHORT COMMUNICATION

High expression of a neutral endo-β-glucanase gene from *Humicola insolens* in *Trichoderma reesei*

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Abstract The neutral endo-β-glucanase gene *cel5A* from *Humicola insolens* was cloned and connected with the cellobiohydrolase 1 promoter from *Trichoderma reesei* to construct a recombinant plasmid pCB-hEG with the hygromycin B resistance marker. The plasmid was introduced into conidia of *T. reesei* using the *Agrobacterium tumefaciens* mediated transformation method. Eight transformants were obtained on screening plates with sodium carboxymethyl cellulose as the sole carbon source. Stable integration of the *cel5A* gene into the chromosomal DNA of *T. reesei* was confirmed by PCR. An obvious protein band (approximately 52 kDa) was detected by SDS-PAGE from fermentation broth, which showed that the *cel5A* gene in recombinant *T. reesei* successfully fulfilled efficient expression and extracellular secretion. After 96 h shaking-flask fermentation, the endo-β-glucanase activity at pH 6.5 from recombinant *T. reesei* reached 3,068 U/ml, which was 11 times higher than that of the host strain. In a 2 m^3 fermenter, the endo-β-glucanase activity could be further increased to 8,012 U/ml after 96 h fermentation. The results showed a good prospect for application of neutral endo-β-glucanase in the textile industry.

Keywords Endo-β-glucanase · Neutral cellulase · *Trichoderma reesei* · Transformation · Gene expression

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Introduction

Endo-β-glucanase (EG), cellobiohydrolase (CBH) and cellobiase (CB) are three main types of cellulolytic enzymes produced by fungi and bacteria [1, 5, 20, 26]. They act synergistically in the hydrolysis process of crystalline cellulose [24, 25]. The EG hydrolyzes cellulose chains internally to produce new chain ends. The CBH hydrolyzes cellulose chains progressively from the chain ends to create mainly cellobiose. The CB hydrolyzes cellobiose and other cello-oligosaccharides to form glucose [27]. The major sources of cellulases are cellulolytic microorganisms, including fungi and bacteria, which are responsible for most of the cellulose degradation in soils. Cellulase gene has been recombinantly expressed in host organisms, such as *Escherichia coli*, yeast, and filamentous fungi [7, 23]. The filamentous fungus *Trichoderma reesei* produces a variety of extracellular cellulases and hemicellulases. It has enjoyed a long history of safe use for cellulase production and serves as a model for studying lignocellulose degradation. The readily available genome sequence of *T. reesei* provides a roadmap for constructing enhanced *T. reesei* strains for industrial applications [14]. The strong promoter of the CBH I gene has been widely used for the over-expression of homologous and heterologous proteins in *T. reesei* [19].

Cellulases are used in the textile industry for biofinishing of cellulose-containing fibers, e.g., biofinishing of indigo-dyed denim fabric to impart a stone-washed effect. *T. reesei* cellulases are used commercially in biofinishing of denim fabric due to their high activity in an acid environment (about pH value 4.8). But the utilization of *T. reesei* cellulases is sometimes restricted by the problem of backstaining, for the released indigo dyes would redeposit on denim fabrics in the acid conditions. Neutral cellulases

have superior biofinishing properties such as low backstaining [3]. Therefore, the construction of neutral cellulase overproducing strains is an available way to improve the effects of biofinishing.

Endo-β-glucanases are grouped into more than 11 glycoside hydrolase (GH) families based on sequence and structure [2]. The filamentous fungus *Humicola insolens* produces many different cellulases. HiCel5A is a GH family 5 EG [13]. The *H. insolens cel5A* gene has already been cloned and sequenced [4]. The amino acid sequence of the HiCel5A was found to be homologous with EG II from *T. reesei*. Enzymatic properties of HiCel5A have been characterized [13]. HiCel5A shows high catalytic activity under neutral conditions. The overall aim of our work is to create a recombinant *T. reesei* strain expressing *H. insolens* HiCel5A and improve biofinishing processes by reducing backstaining. In this study, the *H. insolens cel5A* gene was cloned. Over-expression of the *cel5A* gene and extracellular secretion of enzyme protein were successfully achieved by recombinant *T. reesei*. This made possible the use of the EG preparations produced by the transformants in the biofinishing application at neutral pH.

Materials and methods

Strains and media

Escherichia coli DH5a was used for the propagation of plasmids. *T. reesei* ZU-02, stored in our laboratory [29], was used as a recipient for fungal transformation and chromosomal DNA preparation. *H. insolens* ATCC 16454 was used for isolation of the *cel5A* gene. *Agrobacterium tumefaciens* strain AGL-1 [10] was used as a T-DNA donor for maintenance of constructs and for fungal transformation.

The seed medium for *H. insolens* and *T. reesei* had the following composition (g/L): glucose 20, corn steep liquor 9, KH_2PO_4 10, $(NH_4)_2SO_4$ 5, $MgSO_4{\cdot}7H_2O$ 1, $CaCl_2$ 0.5, $FeSO_4{\cdot}7H_2O$ 0.005, $MnSO_4{\cdot}H_2O$ 0.0016, $ZnSO_4{\cdot}7H_2O$ 0.0014 and $CoCl_2{\cdot}6H_2O$ 0.0037.

The composition of the fermentation medium for *T. reesei* was as follows (g/L): lactose 18, microcrystalline cellulose 20, corn steep liquor 12, KH_2PO_4 10, $(NH_4)_2SO_4$ 5, $MgSO_4{\cdot}7H_2O$ 1, $CaCl_2$ 0.5, $CaCO_3$ 1.8, wheat bran 2, Tween 80 0.2, $FeSO_4{\cdot}7H_2O$ 0.005, $MnSO_4{\cdot}H_2O$ 0.0016, $ZnSO_4{\cdot}7H_2O$ 0.0014 and $CoCl_2{\cdot}6H_2O$ 0.0037. The initial pH value of the medium was adjusted to 4.8.

The composition of the liquid induction medium (IM) for *A. tumefaciens* was as follows (per litre): 1 ml potassium-buffer pH 7.0 (200 g/L K_2HPO_4, 145 g/L KH_2PO_4), 20 ml magnesium-sodium solution (30 g/L $MgSO_4{\cdot}7H_2O$, 15 g/L NaCl), 1 ml 1 % $CaCl_2{\cdot}2H_2O$ (w/v), 1 ml 0.1 % $FeSO_4$ (w/v), 5 ml trace elements (100 mg/L $ZnSO_4{\cdot}7H_2O$, 100 mg/L $CuSO_4{\cdot}5H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4{\cdot}H_2O$, 100 mg/L $Na_2MoO_4{\cdot}2H_2O$), 2.5 ml 20 % NH_4NO_3 (w/v), 10 ml 50 % glycerol (w/v), 40 ml 21 % 2-(*N*-morpholino) ethanesulfonic acid (w/v) pH 5.3, 10 ml 20 % glucose (w/v) and 200 µmol acetosyringone (AS).

Cloning of *H. insolens cel5A* gene

Conidia of *H. insolens* were inoculated into the seed medium and cultured at 30 °C, 200 rpm for 3 days. The mycelia were then frozen in liquid nitrogen and ground to fine powder with mortar and pestle. Total RNA was then isolated from this powder using the TRNzol reagent (Takara, Japan). Synthesis of cDNA from the total RNA was carried out using the reverse transcriptase-polymerase chain reaction kit (Takara, Japan). The DNA amplification was performed with a forward primer (5′-TTACTCGAGC AGGGCGGTGCATGGCAGCAG-3′, containing XhoI site) and a reverse primer (5′-GCGTCTAGACTATG GCACGTATTTCTTGAG-3′, containing XbaI site) using the obtained cDNA as template. The resulting PCR product was cloned into a pMD18-T simple vector (Takara, Japan).

Construction of vector with cellobiohydrolase 1 promoter and terminator

Conidia of *T. reesei* ZU-02 were inoculated into the seed medium and cultured at 30 °C, 200 rpm for 3 days. The mycelia were then frozen in liquid nitrogen and ground to fine powder with mortar and pestle. Fungal genomic DNA was prepared from this powder by the method of cetyltrimethylammonium bromide (CTAB) [21].

The PCR amplification of CBH 1 promoter (*Pcbh1*) with its signal sequence (*ss*) and CBH 1 terminator (*Tcbh1*) from *T. reesei* was carried out with the primers P1 (5′-G TAGGATCCAAGCTTCCATTTGGCGGCT-3′), P2 (5′-C CGCTCGAGAGCTCGAGCAGTAGCCAAG-3′), and T1 (5′-CGCTCTAGATGAACCCTTACTACTCTCAGT-3′), T2 (5′-ATTAAGCTTACTAGTGTCCTCGGCACGTTGT CATC-3′) using the chromosomal DNA of *T. reesei* as template.

The vector pPT containing the *Pcbh1-ss* and *Tcbh1* was constructed with PUC18 as a vector backbone. The *cel5A* gene was inserted into the plasmid pPT under the control of *Pcbh1* and its signal peptide to create the *Pcbh1-ss-cel5A-Tcbh1* expression cassette.

Construction of recombinant vector containing hygromycin B resistance gene

The 1.4 kb *PtrpC-hph* expression cassette (the hygromycin B phosphotransferase gene *hygB*, under the control of the *PtrpC* gene promoter) was amplified with the primers H1

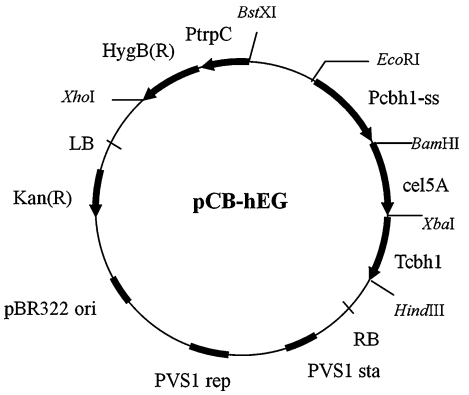

Fig. 1 Illustration of recombinant vector pCB-hEG with *H. insolens cel5A* gene

(5′-CGCCACCATGTTGGGACGTTAACTGATATTGAAGG-3′) and H2 (5′-GCCTCGAGCGTTAACTGGTTCCCGGTCGGC-3′) using the plasmid pDESTR (Genebank: AB218275.1) as template. The PCR products were cloned into the vector pCAMBIA1300 to create recombinant vector pPCB. The *Pcbh1-ss-cel5A-Tcbh1* expression cassette was inserted into the pPCB vector to obtain recombinant plasmid pCB-hEG (Fig. 1).

Agrobacterium-mediated fungal transformation

Agrobacterium tumefaciens AGL1 was electroporated with pCB-hEG as previously described [17]. The strain of *A. tumefaciens* AGL-1 containing pCB-hEG was cultured at 28 °C for 36 h in LB medium supplemented with 50 μg/ml kanamycin and 25 μg/ml rifampicin. The cells of *A. tumefaciens* were collected and diluted to an optical density of 0.15 at 660 nm (OD_{660}) in liquid IM with 200 μM AS, and then grown at 28 °C for 8 h.

The strain of *T. reesei* was cultured at 30 °C for 5 days on a plate with potato dextrose agar (PDA). The conidia were obtained by washing the plate gently with physiological salt solution, and re-suspended in liquid IM.

For co-cultivation, 50 μl of the *T. reesei* conidial suspension (10^7 conidia per milliliter) was mixed with an equal volume of *A. tumefaciens* cells (OD_{660} between 0.4 and 1.0), spread on the surface of nitrocellulose filters placed on solid IM plates (the same as liquid IM except that it contains 5 mM glucose and 18 g agar per litre). After incubation at different temperatures (20–30 °C) and pH (5.0–6.2) for 48 h, the nitrocellulose filter was transferred to solid PDA plates containing 150 μg/ml hygromycin B as selection agent for fungal transformants and 200 μM cefotaxime to kill the cells of *A. tumefaciens*. The transformants were subsequently transferred to PDA plates containing 150 μg/ml hygromycin B.

All the transformants were inoculated onto PDA plates and cultured for 3 days. Three hyphal clumps (4 mm in diameter) were cut from the edge of each colony and transferred onto screening plates containing (g/L): sodium carboxymethyl cellulose (CMC) 20, KH_2PO_4 2.0, $(NH_4)_2SO_4$ 1.4, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2$ 0.3, $FeSO_4{\cdot}7H_2O$ 0.005, $MnSO_4{\cdot}H_2O$ 0.0016, $ZnSO_4{\cdot}7H_2O$ 0.0014, $CoCl_2{\cdot}6H_2O$ 0.0037 and agar 18. After cultivation for 3 days, fast-growing transformants were selected for further analysis.

Detection of target gene in recombinant *T. reesei*

Fungal genomic DNA of the transformant was prepared by the method of CTAB [21]. The PCR amplification of *H. insolens cel5A* gene was carried out using genomic DNA of *T. reesei* transformant and the host strain as template with the upstream and downstream primer of *H. insolens cel5A* gene. The PCR products were assayed by agarose gel electrophoresis.

Fermentation test in Erlenmeyer flask

Production of EG from *T. reesei* transformant was carried out in a 250 ml Erlenmeyer flask with 50 ml of fermentation medium. The inoculum ratio was 10 % (v/v), and the broth was cultured at 30 °C, 180 rpm for 168 h. Three replicated experiments were performed for every strain.

Production of endo-β-glucanase in a 2 m^3 fermenter

A 2 m^3 stirred-tank fermenter with a working volume of 1.5 m^3 was used for production of EG. The inoculum ratio was 10 % (v/v). The fermentation temperature was 28–30 °C, initial pH value was 4.8, the agitation speed and air flow rate were kept at 160 rpm and 90 m^3/h, respectively. During the whole fermentation process, samples were periodically withdrawn for analysis of enzyme activities.

Analysis methods

Measurement of EG activity was based on a previously published protocol [8] with modifications. The CMC (Sigma, USA) solution in sodium acetate buffer (pH 6.5, 1 %, 1.0 ml) was used as substrate, and was mixed with diluted crude enzyme solution (0.5 ml) to react at 50 °C for 15 min. The reducing sugar formed was estimated by a 3,5-dinitrosalicilic acid colormetric assay (DNS) method. One unit of EG activity is the amount of enzyme that produces 1 mg of glucose (reducing sugars as glucose) per hour during the hydrolysis reaction.

The molecular mass of HiCel5A in the crude fermentation broth produced by recombinant *T. reesei* was determined by SDS-PAGE (12 % polyacrylamide). The gel was stained with Coomassie Brilliant Blue R-250, and destained overnight in 20 % methanol and 10 % acetic acid.

Results

Construction of expression plasmid

The *cel5A* gene was cloned from *H. insolens*. Comparison of the sequence with that registered in GenBank (accession no. X76046) showed two nucleotide differences at positions 363 (G-C) and 723 (C-T), and there is no difference in the deduced amino acid sequence.

The recombinant vector pCB-hEG containing the hygromycin B resistance gene and the *cel5A* gene expression cassette was used throughout the study to obtain independent *cel5A* integrated transformants by the AMT procedure. The strong promoter of *Pcbh1* has been widely used for overexpression of homologous and heterologous proteins in *T. reesei* [19]. Therefore, the *cel5A* gene from *H. insolens* could be efficiently expressed and its products secreted under the control of *Pcbh1* and the signal peptide from *T. reesei*. In addition, the hygromycin B resistance marker simplified the screening procedure for positive transformants. It suggested that the expression system of *T. reesei* had been successfully established, which could be used in overexpression of heterologous proteins in *T. reesei*.

Main factors effecting transformation efficiency

Co-cultivation conditions of *T. reesei* conidia with *A. tumefaciens* cells have an important influence on transformation efficiency. Increasing the density of *A. tumefaciens* cells led to an increase in the transformation efficiency. However, when the density of *A. tumefaciens* cells was up to OD_{660} of 0.8, the addition of too many *A. tumefaciens* cells did not improve the transformation efficiency (Fig. 2a). The optimal co-cultivation temperature leading to the highest transformation frequency was 25 °C (Fig. 2b). This result was consistent with the observations made in AMT of other fungi, where similar temperatures were found to be optimal for transformation [6, 16]. The effect of pH was also assessed. It was found that the optimal pH was 5.4 (Fig. 2c). Co-cultivation pH higher than 5.8 was less favourable and no transformant was obtained at pH 6.4.

In the AMT experiment, 351 transformants were obtained and then cultured on screening plates with CMC as the sole carbon source. Eight fast-growing ones (named H1-H8), as indicated by their larger colonies, were selected for further analysis. The AMT method is an effective and suitable technique for fungal transformation [11, 15]. Cells of *Agrobacterium* can transform intact cells, conidia and protoplasts. By this method, a large number of stable transformants with integrated DNA were generated, indicating that the AMT method was an efficient tool for molecular manipulation.

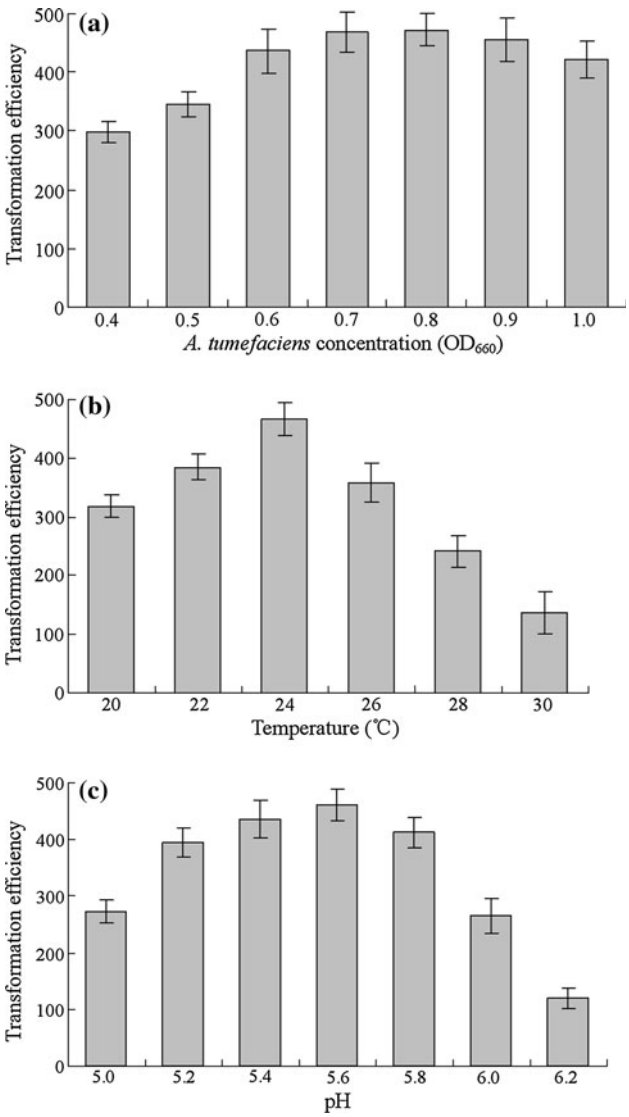

Fig. 2 Effect of co-cultivation conditions on the efficiency of *Agrobacterium*-mediated *T. reesei* transformation. **a** Effect of the density of *A. tumefaciens* cells, **b** Effect of co-cultivation temperature, **c** Effect of co-cultivation pH. Transformation efficiency is evaluated as the number of transformants per 10^7 conidia. Values are means of three determinations and *error bars* are standard deviations

Verification of the chromosomal DNA of recombinant *T. reesei*

To confirm that the *H. insolens cel5A* gene was effectively integrated into the host genome, PCR experiments were

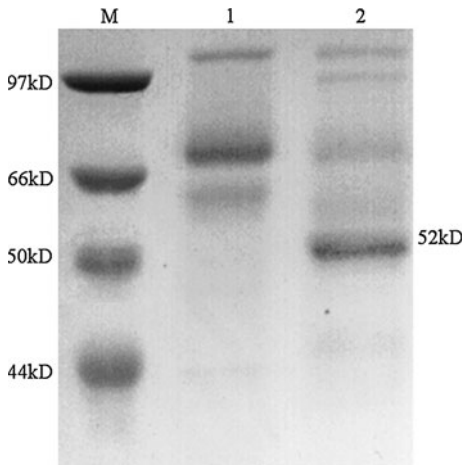

Fig. 3 SDS-PAGE analysis of recombinant HiCel5A from *T. reesei* fermentation broth. *Lane M* protein standard markers, *Lane 1* proteins secreted by the host strain, *Lane 2* proteins secreted by *T. reesei* transformant

carried out using genomic DNA of *T. reesei* transformant and the host strain as template with the upstream and downstream primer of *H. insolens cel5A* gene. A PCR product of 1.1 kb DNA fragment was amplified from the genomic DNA of the recombinant *T. reesei*, while no PCR product was amplified from the genomic DNA of the host strain. The PCR product was cloned into the pMD18-T cloning vector and then sequenced. The results were in complete agreement with the nucleotide sequence of *H. insolens cel5A* gene. It demonstrated that the *H. insolens cel5A* gene had been integrated stably into the chromosomal DNA of recombinant *T. reesei*.

The host strain and the transformants were cultured for 24 h to investigate the production of the heterologous HiCel5A. The molecular weight of HiCel5A in the crude fermentation broth was determined by 12 % SDS-PAGE analysis. The results (Fig. 3) revealed that the transformants secreted the recombinant HiCel5A of about 52 kD, a molecular weight expected for the expressed *H. insolens* HiCel5A. The data further verified the effective expression of the *cel5A* gene in the *T. reesei* transformants and the successful secretion of the enzyme protein from the cell.

Endo-β-glucanase production in shaking-flask

After 96 h shaking-flask fermentation, the culture supernatants of the eight transformants were collected for enzyme activity assay. All the transformants possessed EG activity higher than 2,000 U/ml, with the maximum value of 3,068 U/ml from transformant H5, which was 11 times as high as that of the host strain (278 U/ml). Conidial isolates derived after ten generations from the primary transformant H5 showed the same resistance level to hygromycin as the primary transformant. Moreover, these isolates exhibited EG activity similar to that of the primary transformant. Although the strain of *T. reesei* was widely used for the production of homologous and heterologous proteins, the yield of heterologous proteins was usually low [9]. In this study, the *H. insolens cel5A* gene was fused to the CBH 1 signal sequence from *T. reesei* to enhance the secretion of heterologous protein. The experimental results showed the gene fusion strategy was very successful in expression of *H. insolens cel5A* gene. The N-terminal fungal fusion partner has been suggested to stabilize the recombinant mRNA, so fusion strategies are used to facilitate translocation of foreign proteins in the secretion pathway and to protect the heterologous protein from degradation [18, 22].

Endo-β-glucanase production in a 2 m^3 fermenter

The time course of EG production in a 2 m^3 fermenter is shown in Fig. 4. The maximal EG activities reached 8,012 U/ml on the 4th day of incubation. The higher EG activities in the fermenter were considered to have resulted from the higher level of dissolved oxygen and the more efficient mass transfer as compared to shake-flask cultivation. Recently, some studies on the production of neutral EG were reported, but the production levels were relatively low [12, 28]. The results indicated that *H. insolens cel5A* gene was efficiently expressed and the recombinant HiCel5A with high enzyme activity was achieved.

Trichoderma reesei is an industrially important filamentous fungus due to its effective production of hydrolytic enzymes. The cellulolytic enzyme system of *T. reesei* is composed of two CBHs (Cel6A and Cel7A), at least five EGs (Cel7B, Cel5A, Cel12A, Cel61A Cel45A) and two CBs (Cel3A and Cel1A). For total hydrolysis of cellulose into glucose, a combination of all cellulases (CBHs, EGs

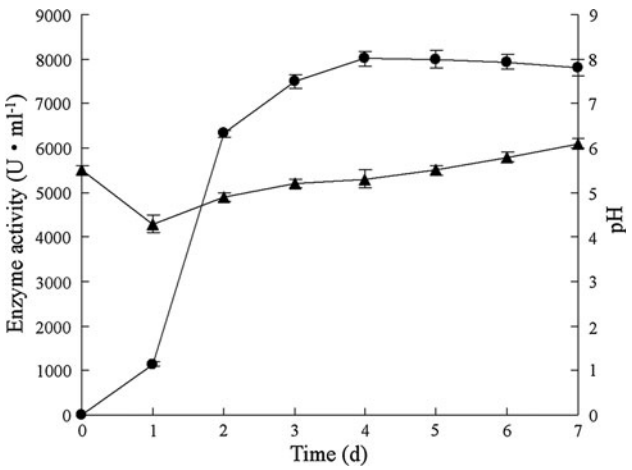

Fig. 4 The time course of endo-β-glucanase production in 2 m^3 fermenter by recombinant *T. reesei* H5. *Filled circles* enzyme activity, *filed triangles* pH. Values are means of three determinations and *error bars* are standard deviations

and CBs) will be the optimal choice. However, for a biofinishing application, only EGs are required. The EGs derived from *T. reesei* are acid cellulases and exhibit low enzyme activity under near-neutral conditions. In this study, EG preparation derived from the HiCel5A-producing *T. reesei* H5 exhibits high catalytic activity at neutral pH. Up to now, most biotechnological processes are based on the use of crude enzymes, and high activity of the enzyme is crucial. Therefore, the results suggest that EG production by recombinant *T. reesei* at large scale had industrial application potential.

Conclusion

A recombinant plasmid pCB-hEG with the hygromycin B resistance marker was successfully constructed, in which the *cel5A* gene from *H. insolens* was inserted between the strong promoter *Pcbh1* and the terminator *Tcbh1* from *T. reesei*. The plasmid was introduced into the conidia of *T. reesei* by the AMT method. *T. reesei* transformants with high neutral EG activity were obtained. The results of this investigation showed a good prospect for application of neutral cellulase from *T. reesei* transformants.

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