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Construction of a promoter collection for genes co-expression in filamentous fungus *Trichoderma reesei*

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Abstract *Trichoderma reesei* is the preferred organism for producing industrial cellulases. However, cellulases derived from *T. reesei* have their highest activity at acidic pH. When the pH value increased above 7, the enzyme activities almost disappeared, thereby limiting the application of fungal cellulases under neutral or alkaline conditions. A lot of heterologous alkaline cellulases have been successfully expressed in *T. reesei* to improve its cellulolytic profile. To our knowledge, there are few reports describing the co-expression of two or more heterologous cellulases in *T. reesei*. We designed and constructed a promoter collection for gene expression and co-expression in *T. reesei*. Taking alkaline cellulase as a reporter gene, we assessed our promoters with strengths ranging from 4 to 106 % as compared to the pWEF31 expression vector (Lv D, Wang W, Wei D (2012) Construction of two vectors for gene expression in *Trichoderma reesei*. Plasmid 67(1):67–71). The promoter collection was used in a proof-of-principle approach to achieve the co-expression of an alkaline endoglucanase and an alkaline cellobiohydrolase. We observed higher activities of both cellulose degradation and biostoning by the co-expression of an endoglucanase and a cellobiohydrolase than the activities obtained by the expression of only endoglucanase or cellobiohydrolase. This study makes the process of engineering expression of multiple genes easier in *T. reesei*.

Keywords *Trichoderma reesei* · Promoter · Alkaline cellulase · Co-expression · Biostoning

Wei Wang and Fanju Meng have contributed equally to the work.

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Introduction

Trichoderma reesei is a fungus of noteworthy industrial importance, mainly because of its employment both in production of native extracellular enzymes and heterologous protein production [1–4]. Glycoside hydrolases, including cellulases and xylanases, secreted by this fungus are used in broad areas of applications [1–4], e.g., in pulp and paper, food and feed, and textile industries. The set of hydrolytic enzymes produced by *T. reesei* comprises, e.g. cellobiohydrolases, CBHs (EC 3.2.1.91); endoglucanases, EGs (EC 3.2.1.4); β-glucosidases, BGLs (EC 3.2.1.21); endoxylanases, XYNs (EC 3.2.1.8); and β-xylosidases, BXLs (EC 3.2.1.37) [1, 5–7].

T. reesei has an excellent extracellular secretion capability for a large amount of proteins as compared to other microbial production/secretion systems such as *Escherichia coli* and *Saccharomyces cerevisiae* [1]. The total amount of secreted proteins of *T. reesei* has been reported to reach the levels of 40 g/l [3] to 100 g/l [1]. Only four major cellulases are usually secreted in notable quantities by *T. reesei*: CBHI (Cel7A), CBHII (Cel6A), EGLI (Cel7B), and EGLII (Cel5A) [8]. These typically represent up to 90–95 % of the total secreted protein, CBHI making up to 50–60 % and CBH2 ~20 % of the extracellular protein [6]. The most

Table 1 Transformants of *T. reesei* RUT-C30 constructed in this study

Strain	Relevant features	Promoter	Reporter gene	Source
RUT-C30	parent strain	–	–	ATCC
pWEF31-V1	RUT-C30 harboring vector pWEF31-V1	*cbh*1	*egv*1	This study
pWEF31-V2	RUT-C30 harboring vector pWEF31-V2	*cbh*1	*egv*2	This study
pWEF31-V3	RUT-C30 harboring vector pWEF31-V3	*cbh*1	*egv*3	This study
pWEF31-H2	RUT-C30 harboring vector pWEF31-H2	*cbh*1	*H. cbh*2	This study
s-pSB101-V3	RUT-C30 harboring vector s-pSB101-V3	*cbh*1	*egv*3	This study
s-pSB401-V3	RUT-C30 harboring vector s-pSB401-V3	*cbh*2	*egv*3	This study
s-pSBe1-V3	RUT-C30 harboring vector s-pSBe1-V3	*egl*1	*egv*3	This study
s-pSBe2-V3	RUT-C30 harboring vector s-pSBe2-V3	*egl*2	*egv*3	This study
s-pSB601-V3	RUT-C30 harboring vector s-pSB601-V3	*xyn*1	*egv*3	This study
s-pSB901-V3	RUT-C30 harboring vector s-pSB901-V3	*xyn*2	*egv*3	This study
s-pSB101-rfp	RUT-C30 harboring vector s-pSB101-rfp	*cbh*1	*rfp*	This study
s-pSB101-gfp	RUT-C30 harboring vector s-pSB101-gfp	*cbh*1	*gfp*	This study
s-pSB401-gfp	RUT-C30 harboring vector s-pSB401-gfp	*cbh*2	*gfp*	This study
s-pSB101-rfp-pSB101-gfp	RUT-C30 harboring vector s-pSB101-rfp-pSB101-gfp	*cbh*1, *cbh*1	*rfp*, *gfp*	This study
s-pSB101-rfp-pSB401-gfp	RUT-C30 harboring vector s-pSB101-rfp-pSB401-gfp	*cbh*1, *cbh*2	*rfp*, *gfp*	This study
s-pSB101-V3-pSB101-H2	RUT-C30 harboring vector s-pSB101-V3-pSB101-H2	*cbh*1, *cbh*1	*egv*3, *H. cbh*2	This study
s-pSB101-V3-pSB401-H2	RUT-C30 harboring vector s-pSB101-V3-pSB401-H2	*cbh*1, *cbh*2	*egv*3, *H. cbh*2	This study

abundantly secreted and industrially important xylanases are XYNI and XYNII [5]. The glycoside hydrolase system is a rational choice for industrial production of homologous or heterologous proteins in *T. reesei*.

Cellulases derived from *T. reesei* have their highest activity at acidic pH. When the pH value increases above 7, the enzyme activities almost disappear, thereby limiting the application of fungal cellulases under neutral or alkaline conditions [9]. Alkaline cellulases, with activities in the pH range 7–9, are less aggressive against cotton than acid cellulases and do not readily compromise the strength of the fabric [10]. Some heterologous alkaline cellulases have been successfully expressed in *T. reesei* to improve its cellulolytic profile [11, 12]. The set of cellulases (including cellobiohydrolases, endoglucanases and β-glucosidases) is synergistically working together to attain complete degradation of cellulose [2]. However, to our knowledge, there are few reports describing the co-expression of two or more heterologous cellulases in *T. reesei*. Usually, co-expression can be conducted using either (1) a single plasmid in which it is required to select compatible assembly sites to build a huge vector or (2) multiple plasmids in which case multiple markers and repeated transformations are required. Nowadays, standards for physical composition of BioBrick parts (http://parts.igem.org/Main_Page) are widely used [13].

In the present study, we designed and constructed a standardized promoter collection for gene expression and co-expression in *T. reesei*. We evaluated the ability of these promoters by determining alkaline cellulase levels. The co-expression constructs were further characterized by biostoning of denims. Our multi-promoter co-expression vectors will be used industrially, as already shown in our initial attempt to express alkaline cellulases.

Materials and methods

Strains and media

Escherichia coli DH5α was used as the host strain for the recombinant DNA manipulations. *T. reesei* RUT-C30 (ATCC 56765), less sensitive to glucose repression, was used as the host for gene expression. The plasmids used in this study are shown in Fig. 2. The *T. reesei* strains constructed in this study are summarized in Table 1. Luria–Bertani (LB) medium was used to cultivate *E. coli* and *Agrobacterium tumefaciens*. Basal fermentation medium (BFM) (supplemental Data File) was used for the fungal cultures and production of alkaline cellulases.

Standardization of gene expression devices in *T. reesei*

In this study, we modified pPK2 [12, 14] by introducing the promoter, terminator, and cloning sites (Fig. 1). The standardized gene expression devices were built by exploiting compatibility of cohesive ends generated by *Xba*I and *Spe*I in pPK2-derived BioBrick base vector, as described in Fig. 1. The devices carried two special cloning sites for

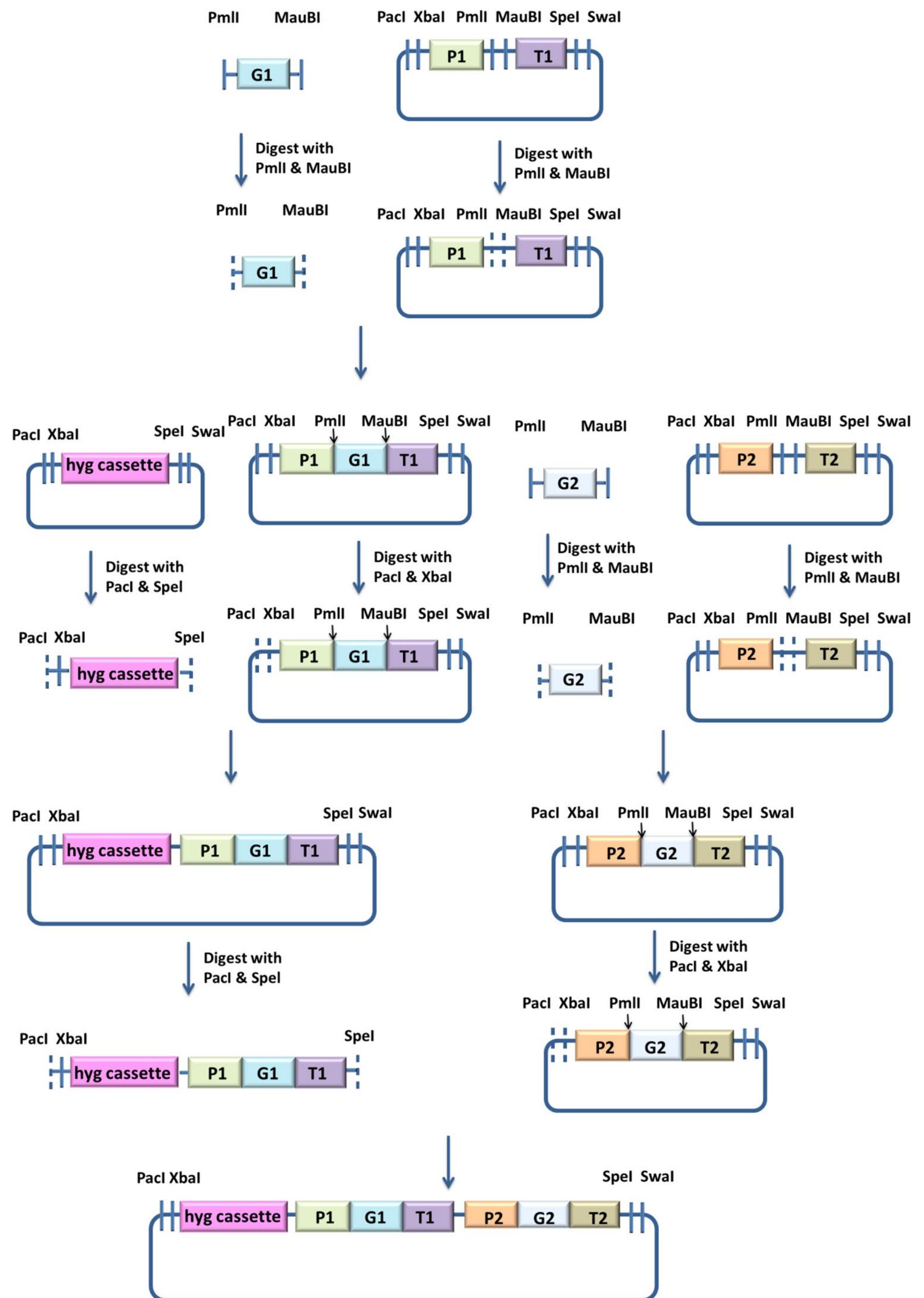

Fig. 1 Construction of co-expression vectors with pPK2-derived BioBrick base vectors. *P1* and *P2* promoters, *G1* and *G2* genes of interest, *T1* and *T2* terminators, *hyg cassette* hygromycin-resistant gene cassette

gene expression—*Pml*I (blunt-end restriction fragment) and *MauB*I (rare recognition site)—and optional C-terminal 6 × His Tag sequence.

The gene expression devices pSB101, pSB401, pSBe1, pSBe2, pSB601, and pSB901 were constructed with the core promoter regions, native signal peptides, and terminators of the genes *cbh*1, *cbh*2, *egl*1, *egl*2, *xyn*1, and *xyn*2, respectively (supplemental Data File).

Cloning of reporter genes

Alkaline endoglucanases ghf45eg-1 (KF056333) and ghf45eg-2 (KF056334): ghf45eg-1 and ghf45eg-2, two alkaline endoglucanases of *Phaeosphaeria* sp. LH21 from deep-sea mud [15], belong to glycoside hydrolase family 45. They are optimally active at pH 8 and 60–65 °C. The 639- and 633-bp genes, ghf45eg-1 (abbreviated *egv*1;

protein, V1) and ghf45eg-2 (abbreviated *egv*2; protein, V2), respectively, were synthesized by GENEray.

Chimeric fusion construct *egv*3: *egv*3 was designed via linking the DNA sequence of core enzyme EGV (PDB: 4ENG) from *Humicola insolens* [16] and a carbohydrate-binding module (CBM) (GenBank: BAA74956.1; residues 235–305) from *Humicola grisea var. thermoidea*. Chimeric fusion construct *egv*3 (protein V3) belongs to the glycosyl hydrolase family 45 encoding an alkaline endoglucanase with pH optima in the range of 7–9. The DNA sequence of V3 was synthesized by GENEray.

Alkaline cellulase *Humicola cbh*2: *Humicola cbh*2 (abbreviated *H. cbh*2), an alkaline cellobiohydrolase from *H. insolens* [16], belongs to the glycoside hydrolase family 6. *Humicola* CBH2 has the highest activity at pH 9, about 3 pH units higher than the homologous CBH2 from *Trichoderma*. This 1394-bp gene (protein, H2) was synthesized.

Reporter gene expression vectors

Red fluorescent protein expression vectors s-pSB101-rfp: The 0.68-kb *rfp* was subcloned into the *Pml*I/*MauB*I site of pSB101 to generate precursor vector pSB101-rfp. And the 2.7-kb hygromycin-resistant part (smaller fragment from pPK1s [17] digested with *Pac*I/*Spe*I) was subcloned into the *Pac*I/*Xba*I site of precursor vector to generate s-pSB101-rfp (Fig. 2).

Green fluorescent protein expression vectors s-pSB101-gfp and s-pSB401-gfp: The 0.72-kb *gfp* was subcloned into the *Pml*I/*MauB*I site of pSB101 and pSB401 to generate precursor vectors pSB101-gfp and pSB401-gfp, respectively, and the 2.7-kb hygromycin-resistant part was subcloned into the *Pac*I/*Xba*I site of precursor vectors to generate s-pSB101-gfp and s-pSB401-gfp, respectively (Fig. 2).

Fluorescent proteins coexpression vectors s-pSB101-rfp-pSB101-gfp and s-pSB101-rfp-pSB401-gfp: We ligated

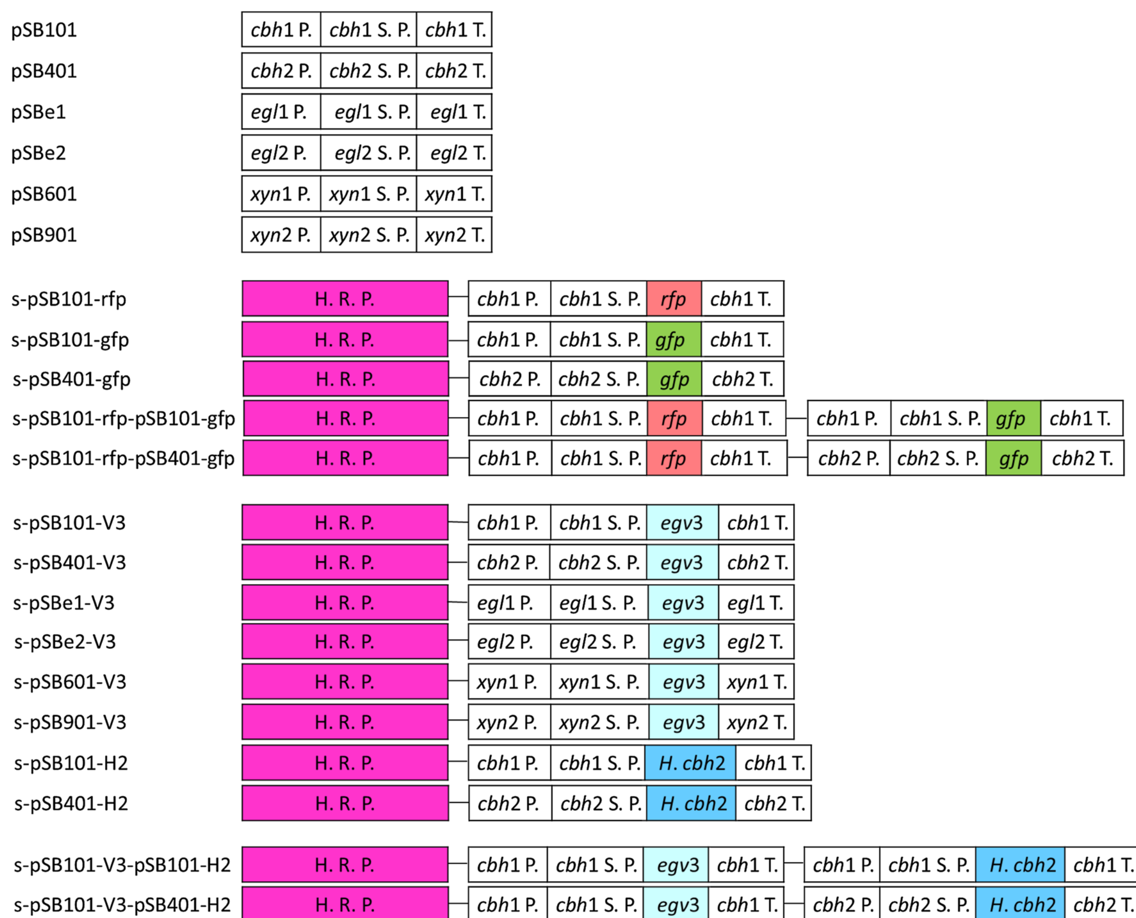

Fig. 2 Vectors constructed in the work. *P* promoters, *T* terminator, *SP* signal peptide, *HRP* hygromycin-resistant part, *rfp* red fluorescent protein, *gfp* green fluorescent protein

the purified smaller fragment from s-pSB101-rfp, digested with *Pac*I/*Spe*I, into *Pac*I/*Xba*I sites of pSB101-gfp and pSB401-gfp to generate s-pSB101-rfp-pSB101-gfp and s-pSB101-rfp-pSB401-gfp, respectively (Fig. 2).

Alkaline endoglucanase expression vectors pWEF31-V1, pWEF31-V2, pWEF31-V3 and pWEF31-H2: The 0.64-kb *egv*1, 0.64-kb *egv*2, 0.87-kb *egv*3, and 1.4-kb *H. cbh*2 were subcloned into the *Stu*I/*Pac*I site of pWEF31 [18] to generate the expression vectors pWEF31-V1, pWEF31-V2, pWEF31-V3, and pWEF-H2, respectively (see supplemental Data File Fig. S-1).

Alkaline endoglucanase expression vectors s-pSB101-V3, s-pSB401-V3, s-pSBe1-V3, s-pSBe2-V3, s-pSB601-V3, and s-pSB901-V3: The 0.87-kb *egv*3 was subcloned into the *Pml*I/*MauB*I sites of pSB101, pSB401, pSBe1, pSBe1, pSB601, and pSB901 to generate precursor vectors pSB101-V3, pSB401-V3, pSBe1-V3, pSBe2-V3, pSB601-V3, and pSB901-V3, respectively, and the 2.7-kb hygromycin-resistant part was subcloned into the *Pac*I/*Xba*I sites of the precursor vectors to generate s-pSB101-V3, s-pSB401-V3, s-pSBe1-V3, s-pSBe2-V3, s-pSB601-V3, and s-pSB901-V3, respectively (Fig. 2).

Alkaline cellobiohydrolase expression vectors s-pSB101-H2 and s-pSB401-H2: The 1.4-kb *H. cbh*2 was subcloned into the *Pml*I/*MauB*I sites of pSB101 and pSB401 to generate pSB101-H2 and pSB401-H2, respectively, and the 2.7-kb hygromycin-resistant part was subcloned into the *Pac*I/*Xba*I sites of pSB101-H2 and pSB401-H2 to generate s-pSB101-H2 and s-pSB401-H2, respectively (Fig. 2).

Alkaline endoglucanase *egv*3 and cellobiohydrolase *H. cbh*2 coexpression vectors s-pSB101-V3-pSB101-H2 and s-pSB101-V3-pSB401-H2: We ligated the purified smaller fragment from s-pSB101-V3, digested with *Pac*I/*Spe*I, into the *Pac*I/*Xba*I sites of pSB101-H2 and pSB401-H2 to generate s-pSB101-V3-pSB101-H2 and s-pSB101-V3-pSB401-H2, respectively (Fig. 2).

All primers used in this section are listed in Table S-1. Transformation of *T. reesei* RUT C30 was carried out as described in the studies by previous researches [18–20]. For each expression vector transformation, 12–24 transformants were collected, and we verified that the expression cassettes were successfully integrated into the genome with single copy. The confirmed transformants were further subcultured for 2–4 generations and examined for reporter gene production.

Reporter gene production in flask

Conidia of strains (10^6 conidia) were inoculated into 25 mL of BFM containing 30 g/l of microcrystalline cellulose (for RUT-C30, and pWEF31, pSB101, pSB401, pSBe1, pSBe2 and pSB901 tranformants), or xylose (for pSB601 tranformants) in a 250-mL flask and were cultivated by rotation (200 rpm) at 28 °C for 5 days. The pH was controlled within the range of 5.0–6.0. The alkaline cellulases were analyzed by carboxymethyl-cellulose hydrolyzing/filter-paper hydrolyzing (CMCase/FPAase) activity as described in section "Enzyme assays." The expression of *rfp*/*gfp* was examined under a fluorescence microscope (Olympus BX50, Japan).

Alkaline cellulase production in a jar

Alkaline cellulase production by transformants was accomplished in a 7-L jar fermenter (BIOTECH-5BG-7000, Baoxing BIO-ENGINEERING EQUIPMENT, shanghai, China) with a final working volume of 4 L. Seed cultivation was performed as follows: conidia of strains (10^7 conidia) were inoculated into 200 mL of BFM supplying 20 g/L of glucose in a 1,000-mL flask and were cultivated by rotation (200 rpm) at 28 °C for 2 days. This culture was poured into 3.8 L of fresh BFM containing 10 g/L of microcrystalline cellulose and 10 g/L of a fiber material in a 7-L jar fermentor. The cultivation was carried out at 28 °C with 25 % dissolved oxygen and 2 vvm (volumes of air per volume of liquid per minute) of aeration for 5 days. Microcrystalline cellulose (30 g/L) was supplied to the culture at 48 h of cultivation time. The pH was controlled within the range of 4.0–4.3 for the first 2 days and 5.5–5.8 thereafter.

Enzyme assays

The culture supernatant of fermentation broth ($7{,}000 \times g$ for 10 min at 4 °C) was subjected to the enzyme activity assay. The CMCase activity was determined in 50 mM sodium phosphate buffer (pH 5, 6, 7, 8, 9) at 50 °C for 30 min. The FPAase activity was determined in 50 mM sodium phosphate buffer (pH 5, 6, 7, 8, 9) at 50 °C for 60 min. The released reducing sugar was estimated using the 3,5-dinitrosalicylic acid (DNS) method [21]. One unit of CMCase/FPAase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar from the substrate per minute under standard assay conditions.

Biostoning of denims

In the machine industrially used for biostoning (HangXin Washes Machinery, Taizhou, China), 0.5 kg blue jeans (108 × 56 rope/inch) was loaded and 50 l of water was added. The pH was set to 8. According to Pederson [22], endoglucanase was the most important enzyme in removing the indigo dye from the fabric surface. The dosages of enzymes were added to 0.33 IU/mL (CMCase activity). The temperature was increased to 40 °C and the drum was set in alternate rotation for 90 min. The enzymes used for

biostoning were commercial neutral cellulase preparations from Youtell (China), Genencor (U.S.A.), and fermentation broth of *T. reesei* strains.

Results and discussion

Generation of standard expression vectors

An underlying goal of synthetic biology is to make the process of engineering biological systems easier and more reliable. In support of this goal, we developed an assembly standard for the gene expression device to enable the construction of coexpression vectors from modularized parts in filamentous fungus *T. reesei*.

The binary vector pPK2 [12] was used as a backbone to construct the expression devices of filamentous fungus *T. reesei*. We chose a very simple but powerful assembly standard for our expression devices, based on six restriction sites, *Pac*I, *Xba*I, *Spe*I, *Swa*I, *Pml*I, and *MauB*I (Fig. 1). The optional C-terminal His-Tag sequence was useful for protein purification. *Pac*I, *Swa*I, and *MauB*I enzymes are restriction endonucleases that recognize octanucleotide (8 bp) targets. Because of their specificity (recognizing a site that occurs once per 65 kb, in average), they were used for assembly of the vectors in our study. *Pml*I restriction enzyme cleavage produces blunt ends, which is always compatible with other blunt-end generating restriction enzymes, such as *EcoR*V and *Stu*I. If *Pml*I restriction site was located in the gene, we could choose other blunt end sites instead of mutation. Two special restriction sites *Pml*I and *MauB*I were enough for most of homologous and heterologous gene inserts.

Each device may be cut in four distinct ways yielding four fragments as follows (Fig. 1): (1) cutting with *Pac*I/*Spe*I creates a front insert, (2) cutting with *Xba*I/*Swa*I creates a back insert, (3) cutting with *Pac*I/*Xba*I creates a front vector, and (4) cutting with *Spe*I/*Swa*I creates a back vector. Because of the compatible overhangs of the *Xba*I and *Spe*I recognition sequences, back inserts can ligate with back vectors to add components to the back of existing constructs. Similarly, front inserts can ligate with front vectors to add components to the front of existing constructs (Fig. 1). In the ligation process, a mixed *Spe*I-*Xba*I site with a sequence ACTAGA is formed at the junction of the insertions. Since this is not a recognition site for any of the enzymes, it cannot be further cut. All parts must not contain the recognition sequences for *Xba*I and *Spe*I restriction enzymes. If the *Xba*I and *Spe*I sequences are present in a sequence, the sequence is recommended to be mutated.

Some homologous and heterologous promoters and terminators were cloned for the construction of standard expression devices. Our purpose was to build short but powerful devices. The sequences of promoters and terminators we selected were as short as possible based on the previous researches [23–27].

Initial attempts to select appropriate reporter gene

We transfered pWEF31-V1, pWEF31-V2, pWEF31-V3, and pWEF31-H2 into the *T. reesei* RUT-C30 cell. The CMCase activities of each transformant were assayed after 5 days' incubation (Fig. 3). The transformants of pWEF31-H2, pWEF31-V1, and pWEF31-V2 exhibited low activities at pH 8 than that of pWEF31-V3. In contrast, the CMCase activity was not detected in wild strains (Fig. 3a). The CMCase activities of pWEF31-H2, pWEF31-V1, and pWEF31-V2 transformants reached 3.16 ± 0.2, 3.67 ± 0.5, and 6.33 ± 0.56 IU/mL, respectively, at pH 8. The activities

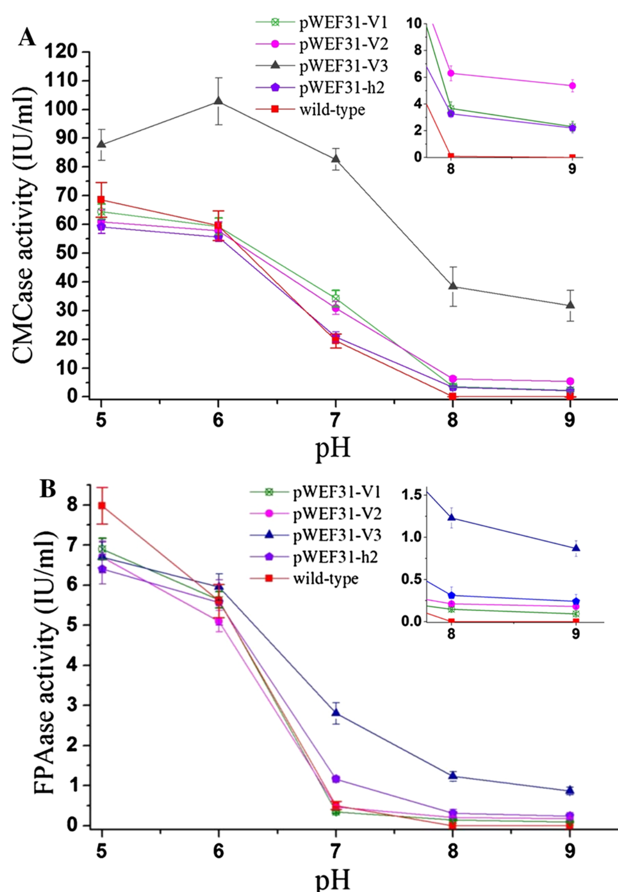

Fig. 3 Effect of pH on the CMCase and FPAase activitives of pWEF31-V1, pWEF31-V2, pWEF31-V3, pWEF31-H2 and parent strain. The effect of pH on the CMCase and FPAase activities were determined in the buffers of different pH (pH 5–9) at 50 °C. A, the CMCase activities of pWEF31-V1, pWEF31-V2, pWEF31-V3, pWEF31-H2, and parent strain; B, the FPAase activities of pWEF31-V1, pWEF31-V2, pWEF31-V3, pWEF31-H2, and parent strain. Averages from three parallel cultivations of each strain are shown with standard deviations

of the pWEF31-V3 transformants reached 38.3 ± 6.8 IU/mL at pH 8, which retained about 37.2 % of the maximal activity at pH 6.0. The enzymatic properties of pWEF31-V3 transformants exhibited above 80 % residual relative activities at pH 5–7. The FPAase activities of pWEF31-H2, pWEF31-V1, pWEF31-V2, and pWEF31-V3 transformants reached 0.21 ± 0.06, 0.26 ± 0.08, 0.37 ± 0.11, and 1.24 ± 0.16 IU/mL at pH 8, respectively (Fig. 3b).

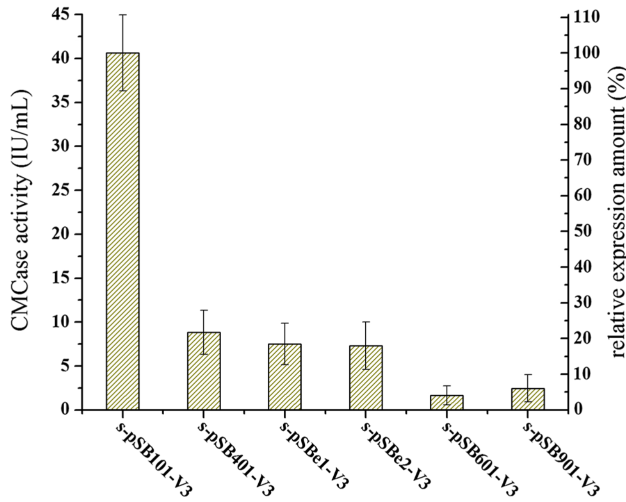

Fig. 4 Comparison of alkaline endoglucanase activities in transformants. The activity was determined at pH 8 and 50 °C. *Error bars* represent standard deviations. The activities of s-pSB101-V3 transformants were assigned a value of 1. Averages from twelve parallel cultivations of each strain are shown with standard deviations

Considering the textile industry, neutral (operate in the pH range 6–8) and alkaline (in the pH range 8–9) cellulases used in biostoning have been characterized to have less aggressive action on cotton than acid cellulases (in the range of pH 4.5–6), and they result in little or no back staining [28, 29]. We selected the chimeric fusion construct, alkaline endoglucanase *egv*3, as a reporter gene and for biostoning. Reyes-Ortiz [30] reported that cellulases (without CBM) are limited to surface degradation, while physical linkage to CBM permits penetration into the bulk of the substrate. Hydrolysis within the bulk creates sites for water coupling and enhances enzymatic access to additional substrates, resulting in swelling. V3 was designed via linking the core enzyme sequence of the *Humicola* EGV and a CBM sequence from *H. grisea var. thermoidea.*

Characterisation of synthetic expression devices using alkaline cellulase *egv*3

To evaluate protein production, we attempted to express *egv*3 under the control of our standard gene expression devices. We transfered s-pSB101-V3, s-pSB401-V3, s-pSBe1-V3, s-pSBe2-V3, s-pSB601-V3 and s-pSB901-V3 into the *T. reesei* RUT-C30 cell. The CMCase activities of each transformant at pH 8 were assayed after 5 days' incubation in a 250-mL flask (Fig. 4). The CMCase activity was highest under the control of pSB101 device, resulting into 40.6 ± 4.3 U/mL (pH 8). The relative activities expressed by the pSB401, pSBe1, and pSBe2 devices were about 21.7, 18.5, and 17.9 %, respectively, of that expressed by the pSB101 device. The pSB601 and pSB901 devices had a lower activity, about

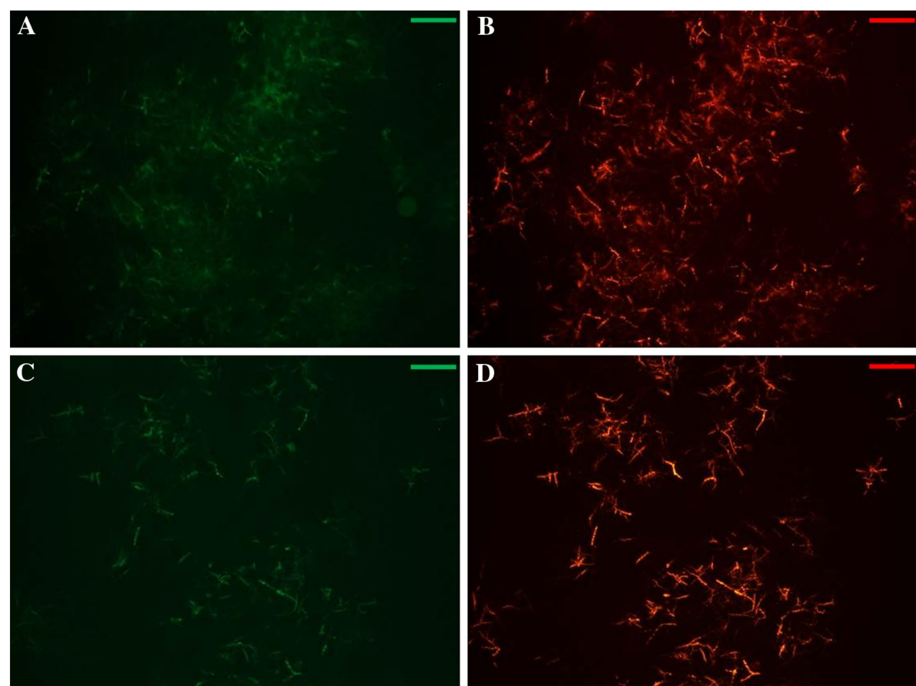

Fig. 5 Coexpression of *rfp* and *gfp* in s-pSB101-rfp-pSB101-gfp and s-pSB101-rfp-pSB401-gfp transformants. **a**, **b** s-pSB101-rfp-pSB101-gfp transformants; **c**, **d**, s-pSB101-rfp-pSB401-gfp transformants. *Bars* represent 500 µm (**a**, **b**, **c**, **d**)

4 and 6 %, respectively, of the pSB101 device. The CMCase activities (pH 8) standed for the abundance of alkaline cellulase V3, which was produced by respective promoters (*cbh*1, *cbh*2, *eg*1, *eg*2, *xyn*1 and *xyn*2). The data accorded with the protein (CBHI, CBHII, EGI, EGII, XYNI and XYNII) production reported earlier [7, 26, 27].

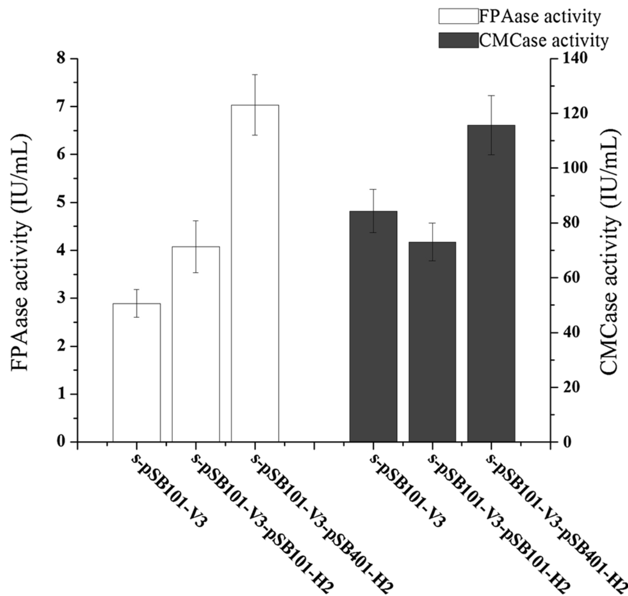

Fig. 6 The CMCase and FPAase activitives of s-pSB101-V3, s-pSB101-V3-pSB101-H2, and s-pSB101-V3-pSB401-H2 transformants. The activity was determined in pH 8 at 50 °C. Averages from three parallel cultivations of each strain are shown with standard deviations

Characterization of co-expressed devices for alkaline cellulose production

To evaluate our standard co-expressed devices, we transferred s-pSB101-rfp-pSB101-gfp, s-pSB101-rfp-pSB401-gfp, s-pSB101-V3-pSB101-H2, and s-pSB101-V3-pSB401-H2 into the RUT-C30 cell. The incubation of s-pSB101-rfp-pSB401-gfp transformants resulted in the appearance of marked fluorescence from *rfp* and *gfp* (Fig. 5), and the two proteins can be simultaneously purified by Ni-nitrilotriacetic acid chromatography (data not shown). The CMCase and FPAase activities of s-pSB101-V3, s-pSB101-V3-pSB101-H2, and s-pSB101-V3-pSB401-H2 transformants at pH 8 were assayed after 5 days' incubation in 7-L jar (Fig. 6). As shown in Fig. 6, the s-pSB101-V3-pSB401-H2 culture reached the highest CMCase and FPAase activities. This result also showed that the set of cellulases were synergistically working together, which accorded with data reported earlier [2] and also observed in the study of mixture of alkaline cellulase *egv*3 and *H*. *cbh*2 in vitro (Table S-2).

Biostoning of denims

The fermentation broth of s-pSB101-V3 and s-pSB101-V3-pSB401-H2 transformants incubated in a 7-L jar for 5 days were selected for biostoning of denims. The actions of s-pSB101-V3 and s-pSB101-V3-pSB401-H2 were as compared to the actions of commercial neutral cellulases.

The goal of the biostoning process is to obtain a distinct but a more uniform aged look for denim garments

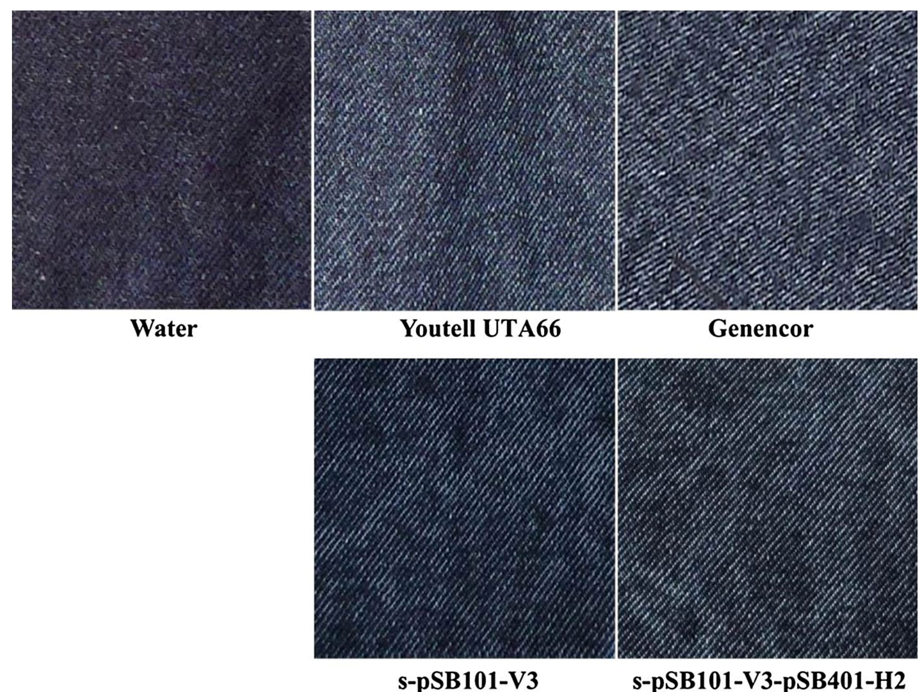

Fig. 7 Comparison between biostoning by commercial neutral cellulases and our cellulases broth

[8]. Figure 7 clearly showed that cellulases gave better results than the water washing in which some streaks are observed. Figure 7 also showed that when the same quantity of endo-type cellulases was loaded, the results obtained with s-pSB101-V3-pSB401-H2 were similar and even better than obtained with commercial cellulases at pH 8; the results obtained with s-pSB101-V3-pSB401-H2 were better than those with s-pSB101-V3. These results showed that the alkaline endoglucanase and cellobiohydrolase co-expressing *T. reesei* strains constructed in this study produced the more efficient enzyme system for biostoning, which relied on the action of two enzymes synergistically working together to modify the fabric surface. This research work is also meaningful in the conversion and utilization of renewable biomass and has good social and economical significance.

Conclusions

We designed and constructed a promoter collection and a multi-promoter construct to make the process of multiple engineering gene expression easier and more reliable. We observed higher activities of both cellulose degrading and biostoning by the co-expression of alkaline endoglucanase V3 and cellobiohydrolase H2. Our goal was to improve cellulolytic profile of *T. reesei* for specific industrial applications not only in the textile industry but also in the fuel, pulp, paper, food, and feed industries.

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