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Cloning of Thermostable Cellulase Genes of *Clostridium thermocellum* and Their Secretive Expression in *Bacillus subtilis*

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Abstract Screening for the powerful cellulase genes with improved activities remains a challenge for the biorefinery research. In this study, five cellobiohydrolase genes and one endoglucanase gene sourced from *Clostridium thermocellum* DSM 1237, *cbhA*, *celK*, *celO*, *cel48Y*, *cel48S*, *and celA* were cloned into a newly established tool vector pP43JM2 and expressed in two *Bacillus subtilis* strains, *B. subtilis* WB600 and *B. subtilis* WB800, respectively. Most of the cellulases produced in the *B. subtilis* recombinants were efficiently secreted into the culture medium. These secreted soluble proteins showed distinct cellulase activities using phosphoric acid swollen cellulose (PASC) as the substrate and they also demonstrated strong synergistic effects for PASC, Avicel cellulose, and the dilute acid pretreated corn stover. The current work provided a quick secretive cloning method for screening cellulase genes and may provide a host strain for constructing a consolidated bioprocessing platform with the capacity of secretive expression of multiple cellulases.

Keywords *Clostridium thermocellum* · *Bacillus subtilis* · pP43JM2 · Cellulase genes · Synergy

Introduction

Cellulases responsible for the degradation of lignocellulose include endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase. Endoglucanase cuts accessible intramolecular β-1,4-glucosidic bonds of cellulose chain randomly, exoglucanase acts in a processive manner on the reducing or nonreducing ends to release cellobiose, and β-glucosidase hydrolyzes cellobiose to glucose [1]. These three cellulase enzymes often act synergistically during the degradation of cellulose. Screening for the powerful cellulase genes is important in the booming biorefinery industry, especially from the thermo-tolerant strains such as *Clostridium thermocellum* [2]. Various methods had been tested for the

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identification of functional cellulase genes but the expression assay in *Escherichia coli* was found not to be efficient due to the formation of intracellular proteins or even inclusion bodies [3, 4]. Cellulases used for the degradation of lignocellulose in biorefining use was generally in the form of secretive proteins thus the intracellular expression of cellulases do not behave the real performance of the cellulase genes.

Bacillus subtilis is frequently used for secretive expression of various heterogeneous proteins including cellulase enzymes [5–7]. *B. subtilis* is also a suitable host strain for constructing consolidated bioprocessing platform for its unique properties of strong protein secretive capacity, utilization of both hexose and pentose, fast growth rate and low nutrient need, etc. [8]. In this study, a new tool shuttle vector between *E. coli* and *B. subtilis*, pP43JM2, was constructed by adding a multi-cloning sites fragment in it and used for the secretive expression in *B. subtilis*, respectively. Six genes encoding the major cellulase enzymes of *C. thermocellum* DSM 1237, including five cellobiohydrolase genes, *cbhA*, *celK*, *celO*, *cel48Y*, *cel48S*, and one endoglucanase gene *celA*, were cloned. The secreted cellulase was assayed using phosphoric acid swollen cellulose (PASC) as the substrate, then the crystalline cellulose Avicel PH101 and the dilute acid pretreated corn stover (DACS) were used for the synergy assay. The results showed that most of the *C. thermocellum* genes were secretively expressed in the recombinant *B. subtilis* and demonstrated the significant synergistic effect. The synergistic effect illustrated the possibility for co-expression of multiple cellulase enzymes in one cell, which might mimic the composition of the mixed commercial cellulases for lignocelluloses degradation. This work provided a quick and efficient cloning method for screening heterogeneous cellulase genes in the secretive way using *B. subtilis* and may also provide a host strain for constructing a consolidated bioprocessing cell in the way of secretive expression of multiple cellulases.

Materials and Methods

Reagents and Chemicals

Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Vilnius, Lithuania). Kanamycin, ampicillin, isopropyl-β-D-thiogalacto-pyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were from Ameresco Chemical Inc. (Cleveland, OH, USA). Tryptone and yeast extract were from Oxoid (Cambridge, UK). Avicel microcrystalline cellulose PH101 NF was from FMC Biopolymer (Newark, NY, USA). All other chemicals used in this study were purchased from Sinopharm Chemical Reagent (Shanghai, China), unless otherwise noted.

Strains and Plasmids

The thermophilic anaerobic bacterium *C. thermocellum* DSM 1237 was purchased from DSMZ, Germany. The shuttle vector between *E. coli* and *B. subtilis*, pP43NMK, and its host strain *B. subtilis* WB800 were provided by Dr. Y.H.P. Zhang, Virginia Polytechnic Institute and State University. *B. subtilis* WB600 was provided by Dr. Sheng Yang, Chinese Academy of Sciences. *B. subtilis* WB600 and WB800 were deficient in six and eight extracellular protease genes, respectively.

C. thermocellum DSM 1237 was grown under strict anaerobic conditions at 60 °C in the pre-reduced GS-2 medium [9]. *E. coli* DH5α, *B. subtilis* WB600, and WB800 were cultured in Luria–Bertani (LB) medium aerobically at 37 °C [10]. Two antibiotics,

ampicillin (100 μg/ml) and kanamycin (50 μg/ml), were added into the LB medium as the selection markers.

DNA Manipulation

Plasmid extraction and gel purification were manipulated using the Omega plasmid mini kit and gel extraction kit (Omega Biotek, Norcross, GA, USA). The genomic DNA of *C. thermocellum* was prepared using Qiagen DNeasy Tissue Kit (Valencia, CA, USA). The methods of DNA preparation, cloning, restriction analysis, and transformation were routinely executed according to the established methods [11]. Transformation of *B. subtilis* with plasmids was carried out by chemical competence method [12]. The primers designed for DNA amplification were listed in Table 1 and synthesized by Shanghai Sangon Biotechnology Services (Shanghai, China).

Construction of Expression Plasmids

The original *mpd* gene fragment in the plasmid pP43NMK was replaced by a 1.7-kb fragment obtained from *C. thermocellum* genomic DNA using the primers 1 and 2 (Table 1) to generate a new shuttle vector pP43JM2 (Fig. 1a) [13]. The new fragment was located at the downstream of the P43 promoter and the NprB signal peptide-encoding fragments of pP43NMK and contained the multi-cloning sites of PstI, XbaI, XmaI, XhoI, and HindIII at the site of PstI and HindIII. The existence of multi-cloning sites in the new plasmid pP43JM2 greatly facilitated the cloning operation.

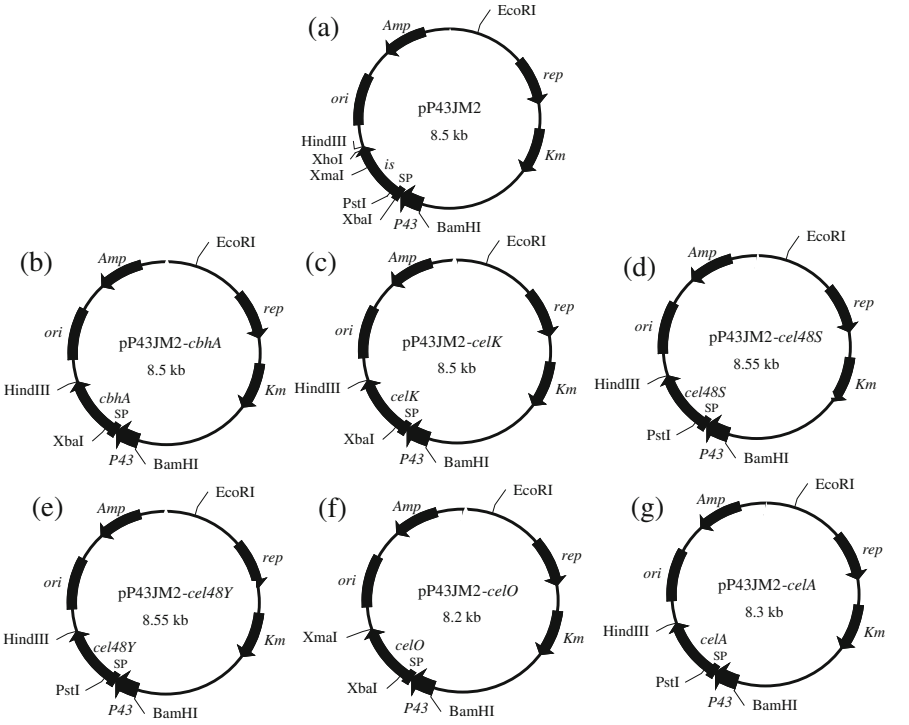
The genes *cbhA*, *celK*, *cel48S*, *cel48Y*, and *celO* encoding the cellobiohydrolases CbhA, CelK, Cel48S, Cel48Y, and CelO were amplified from the *C. thermocellum* genomic DNA, respectively, using LA Taq DNA polymerase (Takara Bio, Tokyo, Japan). The gene *celA* encoding the endoglucanase CelA was amplified from the *C. thermocellum* genomic DNA.

Table 1 Oligonucleotides used in this study

Primers	Target genes	Sequences 5′–3′
1	*is*-S	AACTGCAGCTCTAGACCGACAGGTTTATAGCTCTTTTT (PstI/XbaI)
2	*is*-A	CCCAAGCTTCTCGAGGTTATTCCGTTCTTTATTTCGG (HindIII/XhoI)
3	*cbhA*-S	GCTCTAGAAGCCTGTTGAATATATACTTCCGC (XbaI)
4	*cbhA*-A	CCCAAGCTTTTACTCGTCAAGATAAGCTGTAACC (HindIII)
5	*celK*-S	GCTCTAGAAGCCTGTTGAATATGTACTTCCGCAG (XbaI)
6	*celK*-A	CCCTCGAGTTAAGCTGTAACCCATGCAAACG (XhoI)
7	*celO*-S	AACTGCAGAACCGACACCTCCGCC (PstI)
8	*celO*-A	GATACCCGGGTTATCCTCCCAGCGGTATTACAT (XmaI)
9	*cel48S*-S	AACTGCAGATGGGACATCTTATAAGGATCTTT (PstI)
10	*cel48S*-A	CCAAGCTTTTAGAAGTATGTAGCCAATACACCC (HindIII)
11	*cel48Y*-S	AACTGCAGTCTATTCCGACAGGTTTATAGC (PstI)
12	*cel48Y*-A	CCCAAGCTTTTAAAAGAAAATGTGATACATGG (HindIII)
13	*celA*-S	GCTCTAGATGCAGGTGTGCCTTTTAACAC (XbaI)
14	*celA*-A	CCCAAGCTTCTAATAAGGTAGGTGGGGTATG (HindIII)

Underlined bases indicate introduced restriction enzyme site elucidated in the bracket

S sense primer, *A* anti-sense primer, *is* insert sequence



Genes	*MW (KDa)*	*Plasmids*	*Recombinant strains*
control		pP43JM2	WB600 (pP43JM2)
control		pP43JM2	WB800 (pP43JM2)
cbhA	68.3	pP43JM2-*cbhA*	WB600 (pP43JM2-*cbhA*)
cbhA	68.3	pP43JM2-*cbhA*	WB800 (pP43JM2-*cbhA*)
celK	68.8	pP43JM2-*celK*	WB600 (pP43JM2-*celK*)
celK	68.8	pP43JM2-*celK*	WB800 (pP43JM2-*celK*)
cel48S	71.1	pP43JM2-*cel48S*	WB600 (pP43JM2-*cel48S*)
cel48S	71.1	pP43JM2-*cel48S*	WB800 (pP43JM2-*cel48S*)
cel48Y	71.7	pP43JM2-*cel48Y*	WB600 (pP43JM2-*cel48Y*)
cel48Y	71.7	pP43JM2-*cel48Y*	WB800 (pP43JM2-*cel48Y*)
celO	44.4	pP43JM2-*celO*	WB600 (pP43JM2-*celO*)
celO	44.4	pP43JM2-*celO*	WB800 (pP43JM2-*celO*)
celA	52.5	pP43JM2-*celA*	WB600 (pP43JM2-*celA*)
celA	52.5	pP43JM2-*celA*	WB800 (pP43JM2-*celA*)

Fig. 1 The plasmids and the recombinant strains used in this work. **a** pP43JM2. **b** pP43JM2-*cbhA*. **c** pP43JM2-*celK*. **d** pP43JM2-*cel48S*. **e** pP43JM2-*cel48Y*. **f** pP43JM2-*celO*. **g** pP43JM2-*celA*

Only the catalytic domains of the genes were cloned without including the cellulose-binding domains (Fig. 2), except IgD of *cbhA* and *celK* because of the essential function of IgD-GH9 for keeping the activity [14]. The catalytic domains of the genes were cloned into the plasmid pP43JM2 to yield six recombinant plasmids, pP43JM2-*cbhA*, pP43JM2-*celK*, pP43JM2-*cel48S*, pP43JM2-*cel48Y*, pP43JM2-*celO*, and pP43JM2-*celA* harboring *cbhA*, *celK*, *cel48S*, *cel48Y*, *celO*, and *celA*, respectively (Fig. 1b–g). The 12 recombinant *B. subtilis* WB600 and WB800 strains harboring the above plasmids were shown in Fig. 1.

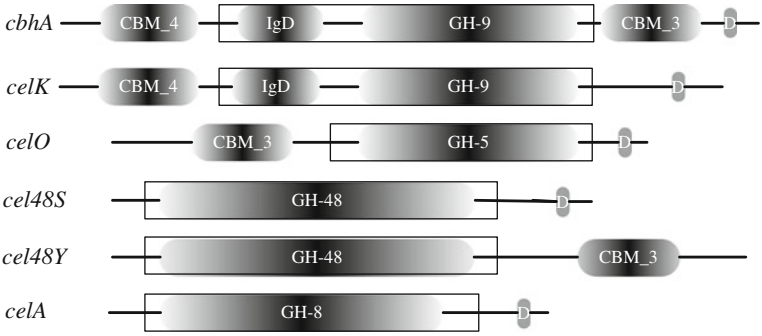


Fig. 2 Domain layouts of cellulases from *C. thermocellum*. The cloned gene fragments were marked in *squares*. CBM_4, carbohydrate-binding module family 4; *IgD*, immunoglobulin-like domain; *GH-9*, glycoside hydrolase family 9; *CBM_3*, carbohydrate-binding module family 3; *GH-5*, glycoside hydrolase family 5; *GH-48*, glycoside hydrolase family 48; *D*, dockerin domain

Cellulase Activity Assay

The recombinant *B. subtilis* strains were cultivated in 100 ml LB medium with 50 μg/ml of kanamycin and incubated at 37 °C at 250 rpm. The cell growth was measured by optical density at 600 nm. A 20-ml sample was withdrawn at 18, 24, and 28 h intervals, respectively. Each sample was centrifuged at 8,000×*g* for 10 min at 4 °C to remove cells and the supernatant was collected. The proteins in the supernatant were precipitated using ammonium sulfate at 40% (*w*/*v*) saturation and redissolved in 1 ml 50 mM citric acid buffer (pH 6.0) to give the final enzyme solution. This solution was mixed with 1 ml of 1% (*w*/*v*) PASC solution for the cellulase activity assay [15]. The mixture was incubated at 60 °C for 1 h and centrifuged at 10,000×*g* for 1 min. The dinitrosalicylic acid (DNS) method was used to measure liberated reducing sugars as the D-glucose equivalent [16].

For SDS-PAGE of the extracellular enzymes, a 20-ml culture medium of each strain cultivated for 24 h was centrifuged at 8,000×*g* for 10 min and the supernatant was collected and then pure ethanol was added gradually to precipitate the secreted proteins until the concentration of ethanol reaching 50% (*v*/*v*). The precipitated sample was then dissolved in pH 7.0 Tris–HCl buffer.

Assessment of Synergy

The degree of the synergistic effect was defined as the ratio of the observed activity of the combined enzymes to the sum of the individual activities [17]. Each cellobiohydrolase enzyme solution was prepared from the protein precipitate of the culture media of *B. subtilis* WB800 (pP43JM2-*cbhA*), WB800 (pP43JM2-*cel48Y*), and WB800 (pP43JM2-*cel48S*) after 24 h, then it was dissolved in 50 mM citric acid buffer (pH 6.0). Each 20 ml culture of the recombinant *B. subtilis* WB800 strains was concentrated and mixed to a total volume of 1 ml to keep the same protein quantity for activity assay using PASC and DNS method.

For the synergy between endoglucanase and cellobiohydrolase, CelA was prepared from 24 h culture of *B. subtilis* WB600 (pP43JM2-*celA*) and CbhA was from 24 h broth of *B. subtilis* WB800 (pP43JM2-*cbhA*). Besides PASC, Avicel PH101 and the DACS were used as the substrates and their concentrations were 2% (*w*/*v*) as a final [18]. The assay was conducted with 1 ml of the crude enzyme solution and 1 ml substrate suspension and incubated at 60 °C for 12 h to measure its released reducing sugars.

Results and Discussion

Secretive Expression of the *C. thermocellum* Cellulase Genes in *B. subtilis*

The six cellulase genes, including five cellobiohydrolases and one endoglucanase from *C. thermocellum* DSM 1237, were expressed in *B. subtilis* as shown in Fig. 3. The *B. subtilis* WB600 and WB800 strains harboring the empty vector plasmid pP43JM2 were used as the

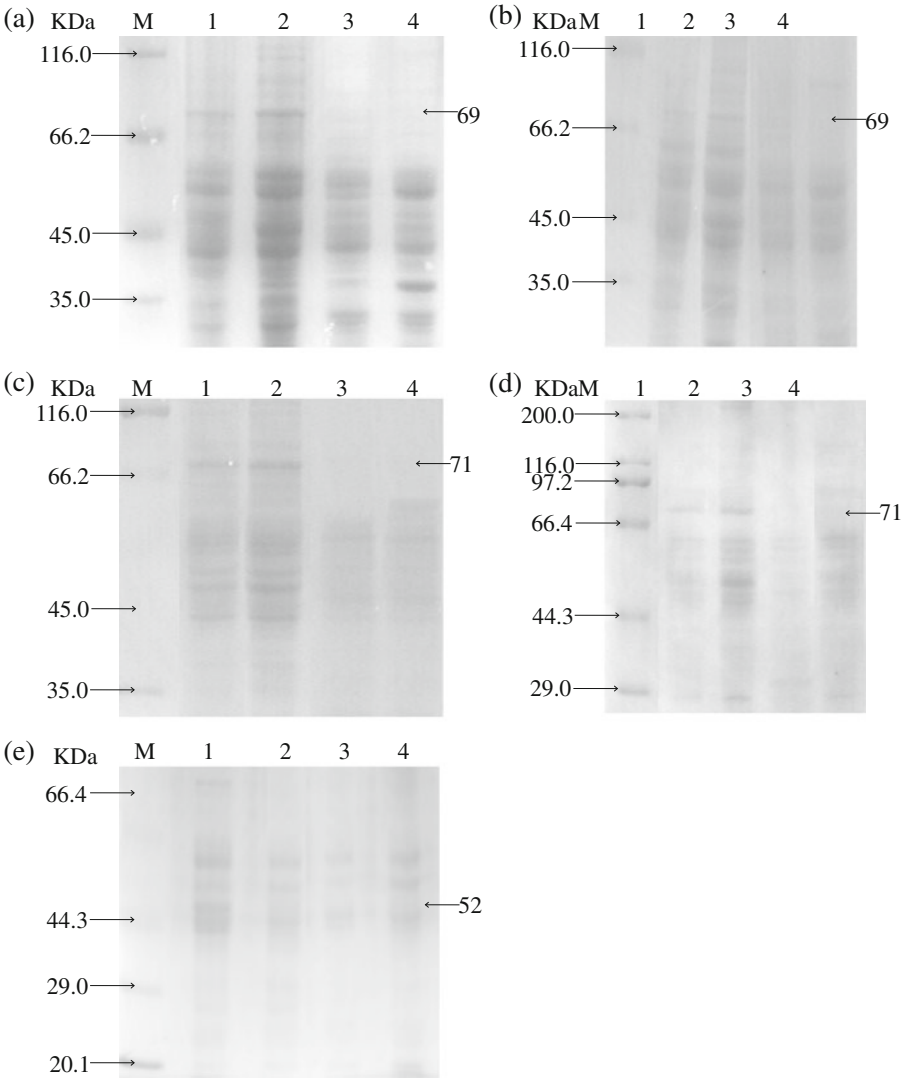


Fig. 3 SDS-PAGE of proteins secreted from the recombinant *B. subtilis* strains. In (**a**–**e**), *lane M*, protein molecular weight marker; *lane 3*, WB600 (pP43JM2) control; *lane 4*, WB800 (pP43JM2) control. (**a**) CbhA. *Lane 1*, WB600 (pP43JM2-*cbhA*); *lane 2*, WB800 (pP43JM2-*cbhA*). (**b**) CelK. *Lane 1*, WB600 (pP43JM2-*celK*); *lane 2*, WB800 (pP43JM2-*celK*). (**c**) Cel48S. *Lane 1*, WB600 (pP43JM2-*cel48S*); *lane 2*, WB800 (pP43JM2-*cel48S*). (**d**) Cel48Y. *Lane 1*, WB600 (pP43JM2-*cel48Y*); *lane 2*, WB800 (pP43JM2-*cel48Y*). **e** CelA. *Lane 1*, WB600 (pP43JM2-*celA*); *lane 2*, WB800 (pP43JM2-*celA*)

two controls. One single band close to the molecular weight (MW) of CbhA and CelK (69 kDa) was found in both the recombinant *B. subtilis* WB600 and WB800 strains in subpanels a and b of Fig. 3, respectively, indicating CbhA and CelK were well expressed and secreted into the medium. A specific band close to the MW of Cel48S and Cel48Y (71 kDa) found in both the recombinants *B. subtilis* WB600 and WB800 strains in subpanels c and d of Fig. 3, respectively, indicates that Cel48S and Cel48Y were also well expressed and secreted. Figure 3e shows that the specific protein band close to 53 kDa matched the deduced MW of CelA. Figure 4 shows the time courses of cell growth and cellulase activity expression of the ten recombinant *B. subtilis* strains (five in WB600

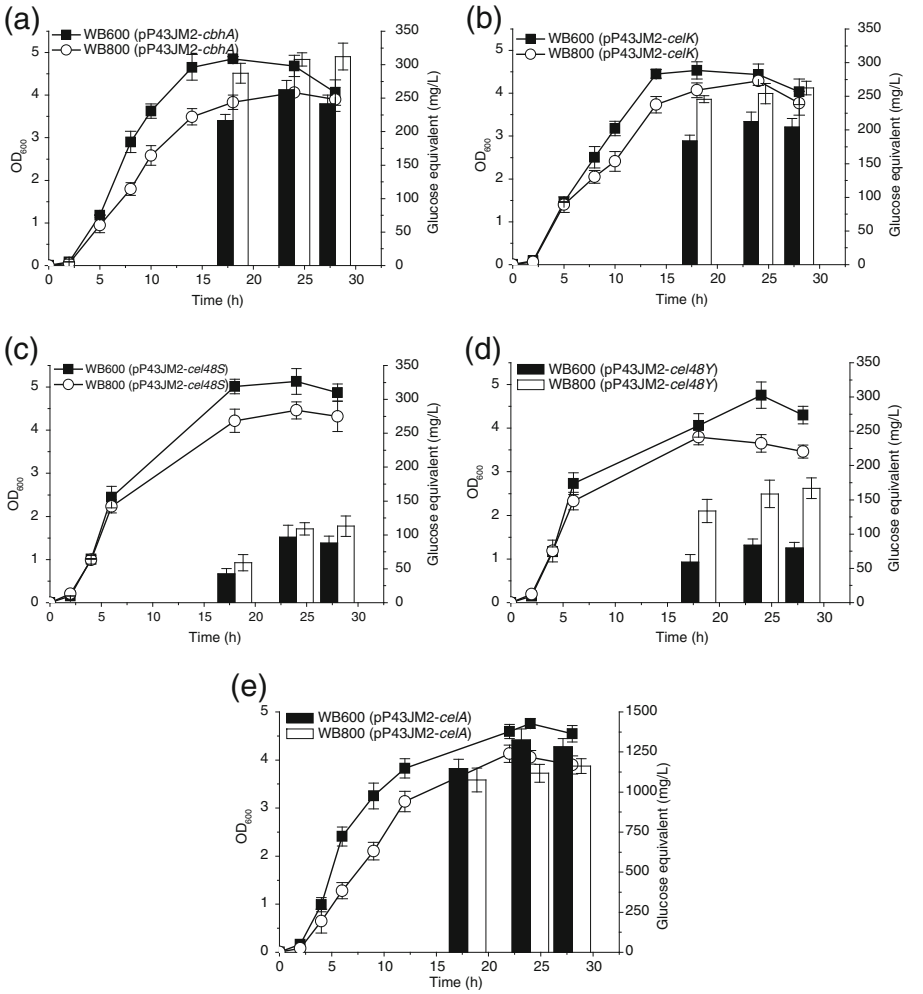


Fig. 4 Growth and enzymatic activities of the recombinant *B. subtilis* strains. Culture conditions, 37 °C, 250 rpm. The enzymatic activities were determined using PASC substrate (1%, *w*/*v*) at 60 °C for 1 h. *Filled bars* indicate the reducing sugars released by cellulase from WB600, while the *blank* represents the reducing sugars released by cellulase from WB800. (**a**) WB600 (pP43JM2-*cbhA*) and WB800 (pP43JM2-*cbhA*); (**b**) WB600 (pP43JM2-*celK*) and WB800 (pP43JM2-*celK*); (**c**) WB600 (pP43JM2-*cel48S*) and WB800 (pP43JM2-*cel48S*); (**d**) WB600 (pP43JM2-*cel48Y*) and WB800 (pP43JM2-*cel48Y*). (**e**) WB600 (pP43JM2-*celA*) and WB800 (pP43JM2-*celA*). Values are averages of two independent measurements

and five in WB800) during the culture. The *B. subtilis* WB600 and WB800 strains harboring pP43JM2 were taken as the two controls and no cellulase activities were detected with PASC as the substrate from the two controls. Figure 4 shows that the cell growth rates of all the *B. subtilis* WB600 recombinants were greater than those of the corresponding WB800 recombinants under the same culture conditions, and the enzymatic activities demonstrated approximately positive correlations with the cell growth for this specific recombinant strain. As shown in Fig. 4a–d, the extracellular cellobiohydrolase activity of the *B. subtilis* WB800 recombinants was greater than that of the WB600 recombinants, opposite to the growth behaviors of WB800 and WB600. For CbhA and CelK, the activities from the WB800 recombinants were approximately 30% greater than those from the WB600 recombinants; for Cel48Y, the activity of the WB800 recombinant was almost doubled than its corresponding WB600 recombinant; for Cel48S, the activity of the WB800 recombinant was also greater than that of the WB600. These results were in good agreement with Wong for the expression of EngB from *Clostridium cellulovorans* in *B. subtilis* WB700 and WB800: more protease silencing was helpful to obtain the higher cellulase activity [19]. However, as shown in Fig. 4e, the activity of the endoglucanase CelA from *B. subtilis* WB800 was not superior to that of WB600, which was different from the cellobiohydrolases. The reducing sugars hydrolyzed by CelA from *B. subtilis* WB600 and WB800 were approximately 1,300 and 1100 mg/L using PASC as the substrate, respectively, which were significantly greater than those released from the cellobiohydrolases above. The reason might be that two more deficient proteases in the *B. subtilis* WB800 were not efficient for degradation of CelA, thus the fast cell growth of WB600 resulted in the greater CelA secretion, then the greater activity.

No secreted protein band of CelO was found and no extracellular enzyme activity was detected when both WB600 and WB800 recombinants harboring *celO* gene (pP43JM2-*celO*) were cultured. The reason was perhaps that the fusion of CelO and the signal peptide NprB was unable to secrete into the culture medium. Zhang used the P43 promoter and the NprB signal peptide to secrete CpCel48 successfully in *B. subtilis*, but the same promoter and the signal peptide were not successful in secreting an endoglucanase of glycoside hydrolase family 9 from *Clostridium phytofermentans* in *B. subtilis* [20]. Many subtle differences may cause the significant difference in the expression and secretion of heterologous proteins.

Synergy of Secreted Cellulases from the Recombinant *B. subtilis* Strains

Synergy refers to the phenomenon that the observed action of the combination of enzymes is higher than the sum of actions of the individual enzyme [21]. The synergy of the secreted cellulases from the recombinant *B. subtilis* strains were examined in this study. The three combinations of cellobiohydrolases, CbhA-Cel48S, CbhA-Cel48Y, and CbhA-Cel48S-Cel48Y, were mixed with PASC, respectively, for measuring the synergistic effects among the cellobiohydrolases. Figure 5 shows that the cellobiohydrolases of CbhA, Cel48S, or Cel48Y only produced 304, 110, and 153 mg/L reducing sugars, respectively. When the combined CbhA-Cel48S and CbhA-Cel48Y were used at the same enzyme quantity with the separate enzymes in volume, the reducing sugars increased to 532 and 611 mg/L, respectively, almost fivefold greater than those by the separate enzymes. The triple mixture of CbhA, Cel48S, and Cel48Y did not show a clear increase and the synergy effect. The synergy may be due to that CbhA and CelK were active from the non-reducing side of the polymeric substrate, whereas Cel48S and

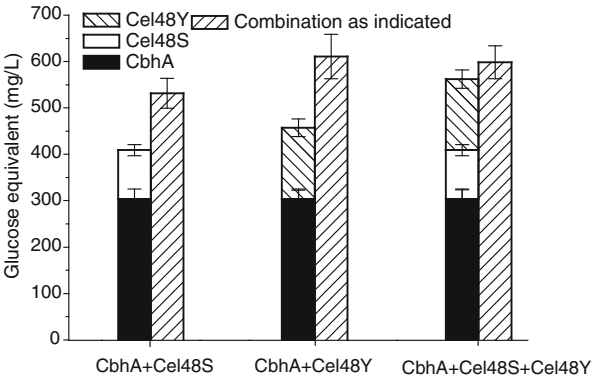


Fig. 5 Synergistic effect of cellobiohydrolases using PASC substrate. The *filled*, *blank*, and *obliqued bars* indicate the sugars released by individual CbhA, Cel48S, and Cel48Y. The *right oblique* represents the reducing sugars liberated by the combination of the enzymes as indicated. Values are averages of two independent measurements

Cel48Y exerted effects on the reducing ends; these two kinds of cellobiohydrolases would enable cellulase to hydrolyze cellulose in both directions and give synergism with each other [22].

The synergy between cellobiohydrolase and endoglucanase always played a crucial role in the degradation of crystalline cellulose. The synergistic action of CbhA and

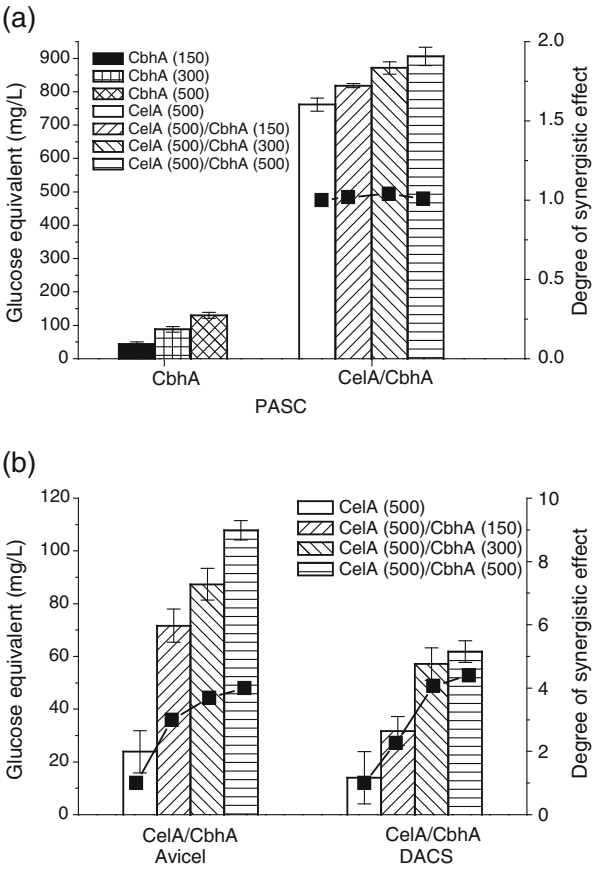


Fig. 6 Enzymatic activities and synergistic effect against different types of substrate. *PASC*, phosphoric acid swollen cellulose. *DACS*, dilute acid pretreated corn stover. (**a**) The activities and degree of synergistic effect with PASC as the substrate through the combination of endoglucanse CelA and exoglucanase CbhA. The *numbers* encircled indicate the volume (in microliters) of enzymes added to the mixture. (**b**) The activities and degree of synergistic effect with Avicel PH101 and DACS as the substrates. The cellobiohydrolases showed no activity against Avicel PH101 and DACS. Values are averages of two independent experiments

CelA, the secretive cellobiohydrolase and endoglucanase of the recombinant *B. subtilis*, were determined using different substrates in this study. Figure 6a shows that when CelA was mixed with different dosages of CbhA, the reducing sugars increased stepwise from 761 to 905 mg/L with PASC as the substrate. When the more rigid cellulose substrates, Avicel PH101 and the DACS, were used as shown in Fig. 6b, CelA only showed weak activities (24 mg/L for Avicel and 14 mg/L for DACS), whereas the cellobiohydrolases showed no activity on the two substrates. However, when CelA and CbhA were combined at the same enzyme quantity with the separate CelA and CbhA in volume, the reducing sugars increased significantly to 110 mg/L for Avicel PH101 and 60 mg/L for DACS from almost zero reduced sugar. Also, the reducing sugar increased with the increase of CbhA dosage. The activity of the enzyme mixture was about fourfold greater than that of the single enzyme CelA, exhibiting higher synergistic effects than PASC as the substrate.

Figure 6 indicates that the degree of synergistic effect was low against PASC but high for crystalline cellulose and DACS, which was in agreement with the previous reports [23, 24]. The basic synergistic mechanism between endoglucanase and exoglucanase was believed to create new sites for exoglucanase hydrolysis thus enhanced the collaboration between cellulases. These results indicate that the secretive *C. thermocellum* cellulases by the recombinant *B. subtilis* demonstrated strong synergistic effect on the real lignocellulose feedstocks, suggesting the *C. thermocellum* cellulase genes were synergistically functional in *B. subtilis* and could be used for developing the potential consolidated bioprocessing cells of secretive expression type, when two or more such genes were co-expressed in *B. subtilis*.

Conclusions

Six cellulase genes sourced from *C. thermocellum* DSM 1237, *cbhA*, *celK*, *celO*, *cel48Y*, *cel48S*, *and celA*, were cloned into pP43JM2 and expressed in *B. subtilis* WB600 and *B. subtilis* WB800. The cellulases produced in the *B. subtilis* recombinants were efficiently secreted into the culture broth, showed clear activities using PASC as the substrate, and demonstrated strong synergistic effects on PASC, Avicel cellulose, and DACS. Efficient secretion of active cellulase is the first step in the development of *B. subtilis* consolidated bioprocessing cells that can produce cellulase, hydrolyze cellulose, and ferment the soluble sugars to low-cost biocommodities.

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References

1. Sharrock, K. (1988). *Journal of Biochemical and Biophysical Methods, 17*, 81–105.
2. Percival Zhang, Y. H., Himmel, M. E., & Mielenz, J. R. (2006). *Biotechnology Advances, 24*, 452–481.
3. Maki, M., Leung, K. T., & Qin, W. (2009). *International Journal of Biological Sciences, 5*, 500–516.
4. Makrides, S. (1996). *Microbiology and Molecular Biology Reviews, 60*, 512–515.
5. Schumann, W. (2007). *Advances in Applied Microbiology, 62*, 137–189.

6. Arai, T., Matsuoka, S., Cho, H., Yukawa, H., Inui, M., Wong, S., et al. (2007). *Proceedings of the National Academy of Sciences of the United States of America, 104*, 1456–1460.
7. Cho, H. Y., Yukawa, H., Inui, M., Doi, R., & Wong, S. (2004). *Applied and Environmental Microbiology, 70*, 5704–5707.
8. Zhang, X. Z., & Zhang, Y. H. (2010). *Engineering in Life Sciences, 10*, 398–406.
9. Johnson, E. A., Madia, A., & Demain, A. L. (1981). *Applied and Environmental Microbiology, 41*, 1061–1062.
10. Luria, S. E., & Delbrick, M. (1943). *Genetics, 28*, 491–511.
11. Sambrook, J., Fritsch, E., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. NY: Cold Spring Harbor.
12. Spizizen, J. (1958). *Proceedings of the National Academy of Sciences of the United States of America, 44*, 1072.
13. Zhang, X. Z., Cui, Z. L., Hong, Q., & Li, S. P. (2005). *Applied and Environmental Microbiology, 71*, 4101–4103.
14. Kataeva, I., Uversky, V., Brewer, J., Schubot, F., Rose, J., Wang, B., et al. (2004). *Protein Engineering, Design and Selection, 17*, 759–769.
15. Zhang, Y. H., Cui, J., Lynd, L. R., & Kuang, L. R. (2006). *Biomacromolecules, 7*, 644–648.
16. Ghose, T. (1987). *Pure and Applied Chemistry, 59*, 257–268.
17. Riedel, K., Ritter, J., & Bronnenmeier, K. (1997). *FEMS Microbiology Letters, 147*, 239–243.
18. Zhang, J., Wang, X., Chu, D., He, Y., & Bao, J. (2011). *Bioresource Technology, 102*, 4480–4488.
19. Murashima, K., Chen, C. L., Kosugi, A., Tamaru, Y., Doi, R., & Wong, S. (2002). *Journal of Bacteriology, 184*, 76–81.
20. Zhang, X. Z., Sathitsuksanoh, N., & Zhang, Y. H. (2010). *Bioresource Technology, 101*, 5534–5538.
21. Irwin, D. C., Spezio, M., Walker, L. P., & Wilson, D. B. (1993). *Biotechnology and Bioengineering, 42*, 1002–1013.
22. Barr, B. K., Hsieh, Y. L., Ganem, B., & Wilson, D. B. (1996). *Biochemistry, 35*, 586–592.
23. Nidetzky, B., Hayn, M., Macarron, R., & Steiner, W. (1993). *Biotechnology Letters, 15*, 71–76.
24. Berger, E., Zhang, D., Zverlov, V. V., & Schwarz, W. H. (2007). *FEMS Microbiology Letters, 268*, 194–201.