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Secretory pathway engineering enhances secretion of cellobiohydrolase I from *Trichoderma reesei* in *Saccharomyces cerevisiae*

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Improving the cellulase secretion is beneficial for *Saccharomyces cerevisiae* used in consolidated bioprocessing (CBP) of cellulosic ethanol. In this study, protein secretory pathway, including protein folding, disulfide bond formation, and protein trafficking and sorting, was modified in *S. cerevisiae*. The effects of these modifications on the secretion of cellobiohydrolase I (*Tr*-Cel7A) with its native signal peptide from *Trichoderma reesei* were investigated. The results showed that overexpression of the protein disulfide isomerase *Sc-PDI1* and the plasma membrane targeting soluble N-ethylmaleimide-sensitive factor attachment protein receptor *Sc-SSO1*, and disruption of the sorting receptor *Sc-VPS10* and a Ca^{2+}/Mn^{2+} ATPase *Sc-PMR1*, improved respectively the extracellular *Tr*-Cel7A activities. Among them, disruption of *Sc-PMR1* showed better improvement of 162% in the extracellular activity and decreased the glycosylation of *Tr*-Cel7A. Multiple modifications generally resulted in higher activities. The extracellular activities of the quadruple-modified strain (*vps10Δ/pmr1Δ/SSO1/PDI1/cel7AF*) using p-nitrophenyl-β-D-cellobioside (pNPC) and phosphoric acid swollen cellulose (PASC) as the substrates, respectively, were 3.9-fold and 1.3-fold higher than that of the reference strain *cel7AF*. The results indicated that engineering of the protein secretory pathway is an effective approach to improve the *Tr*-Cel7A secretion in *S. cerevisiae*.

[**Key words:** Cel7A; Protein secretion; *PMR1*; Consolidated bioprocessing; Cellulosic ethanol; Budding yeast]

Saccharomyces cerevisiae is widely studied as a cell factory for production of biofuels, fine chemicals, pharmaceutical proteins and so on. *S. cerevisiae* has many advantages, such as being generally regarded as safe, easy and fast to grow, suitability for genetic modification, capability to perform post-translational modification that occurs in higher eukaryotes, high ethanol production yield, large-scale fermentation performance, high ethanol and inhibitor tolerance and so on (1). However, for heterologous protein expression, there are some bottlenecks such as hyperglycosylation and low secretion efficiency in the native *S. cerevisiae*.

Many factors including target protein properties, codon usage, vector system, promoter, signal peptide and cultivation condition, and secretory pathway affect the protein production and secretion significantly (2). Especially, the posttranslational processing of secretory proteins is very complex. Proteins are folded and assembled in endoplasmic reticulum (ER) lumen where chaperones participate in the process, then transferred to Golgi apparatus and further modified such as glycosylation. Meanwhile, the proteins with correct modifications are trafficked to plasma membrane for secretion, the misfolded proteins are translocated through ER back to cytosol and degraded by ER-associated protein degradation (ERAD) (3), or transported to vacuole and degraded by proteolytic enzymes (4). Genetic modification of the ER protein folding process is beneficial for the production of secreted proteins. The extracellular activity of laccase from *Trametes* sp. AH28-2 was increased by overexpression of the ER chaperone *KAR2* in *S. cerevisiae* (5). Overexpression of protein disulfide isomerase (PDI), an ER-resident protein with both disulfide bonds formation and chaperone functions (6), enhanced the secretion yields of human platelet-derived growth factor B homodimer and *Schizosaccharomyces pombe* acid phosphatase tenfold and fourfold, respectively in *S. cerevisiae* (7). It has been reported that the extracellular activity of the human secretory leukocyte protease inhibitor (SLPI) in *Pichia pastoris* was increased by forming more disulfide bonds, resulted from PDI overexpression (8). Engineering of intracellular trafficking process, for example, ER-to-Golgi or Golgi-to-plasma membrane process, can also improve the secretion of heterologous protein in *S. cerevisiae*. Idiris et al. (9) found that partial intracellular retention of a human growth hormone mainly resulted from the mis-sorting process from Golgi to vacuole in *S. pombe*. Deletion of Pmr1p, a Ca^{2+}/Mn^{2+} ATPase required for Ca^{2+} and Mn^{2+} transport into the Golgi apparatus has been reported to increase the secretion yields of heterologous proteins in *S. cerevisiae* (10). Tatsumi et al. (11) reported that the immunogenicity of β-lactoglobulin secreted from *P. pastoris* was reduced with surface structure slightly changed by introducing site-specific glycosylation. The extracellular activity of a protein should include the secretion yields and the biological activity based on changes in the structure, both of these two aspects might be affected by genetic modifications.

Along with the recent development in cellulosic biofuel research, *S. cerevisiae*, as a traditional ethanol production microorganism, has

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been identified as a good potential host for consolidated bioprocessing (CBP) (12), in which cellulase production, substrate hydrolysis, and fermentation are accomplished in a single process step (13). So far, microorganisms with the properties required for CBP are not available, but many efforts have been taken to construct the CBP strains (14–16). A desired CBP strain should have good capabilities to produce and secrete cellulase with high efficiency, continuously hydrolyze and ferment cellulose to ethanol with high yield. An available CBP strain requires vigorous ability of not only ethanol fermentation but also at least partial hydrolysis of cellulose. Three kinds of enzymes are needed to degrade the cellulose, namely, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (17). Among them, the cellobiohydrolases are crucial for the cellulose degradation, but the expression levels of them in *S. cerevisiae* are relatively lower than the other two components. Therefore, relatively high productivity of cellobiohydrolase was required for the construction of the yeast strain that was capable to convert cellulose to ethanol. As one of the most powerful cellulase producers (18), the filamentous fungus *Trichoderma reesei* could produce two cellobiohydrolases (*Tr*-Cel7A and *Tr*-Cel6A), at least six endoglucanases and two β-glucosidases. Among them, *Tr*-Cel7A, which hydrolyzes the cellulose chain from the reducing end progressively (19), is the major component (~60%) of the total cellulolytic protein (20). The *Tr*-Cel7A and *Tr*-Cel6A from *T. reesei* were heterologously secreted in *S. cerevisiae*. The secretion level of recombinant *Tr*-Cel7A was much lower than *Tr*-Cel6A (14,21,22). Therefore, as the crucial enzyme in CBP, improving the *Tr*-Cel7A secretion in *S. cerevisiae* is necessary to enhance the hydrolysis capability. The native *Tr*-Cel7A from *T. reesei* with glycosylation usually exhibits a molecular weight (MW) near approximately 70 kDa in SDS-PAGE (14,23), which belongs to high molecular weight protein. Further, it has ten disulfide bonds in the catalytic domain (CD) and two additional disulfide bonds in the cellulose-binding domain (CBD) (24). In this study, the genes involved in the protein post-translational modification and secretion processes, including protein folding, intracellular protein trafficking and sorting were modified to improve the secretion of *Tr*-Cel7A from *T. reesei* in *S. cerevisiae*.

MATERIALS AND METHODS

Construction of plasmids and strains The *Tr-cel7A-FLAG* DNA fragment, which was the *Tr-cel7A* gene (GenBank accession no. E00389.1) containing its native signal peptide sequence as well as the FLAG-tag sequence in 3′ end, was artificially synthesized and inserted into the *Xba* I site between the stronger promoter *TEF1p* and *PGK1* terminator in plasmid pJFE3 (25), resulting in plasmid pJCF (Fig. 1A).

The *Sc-PDI1* and *Sc-SSO1* DNA fragments were respectively amplified from genomic DNA of the budding yeast and ligated into the *Bgl* II site between the *PGK1* promoter and terminator in plasmid pYMIKP (26), resulting in plasmids pYMIKPD and pYMIKPS (Fig. 1B). pYMIKPSD (Fig. 1C) contained both two expression cassettes *PGK1p-PDI1-PGKt* and *PGKp-SSO1-PGK1t*.

The *Sc-VPS10* and *Sc-PMR1* genes were disrupted using the Cre-*loxP* system as described previously (27). Two disruption cassettes VPS10F-*loxP-KanMX-loxP*-VPS10R and PMR1F-*loxP-KanMX-loxP*-PMR1R were obtained and transformed into *S. cerevisiae* CEN.PK102-3A (28) using the lithium acetate transformation method (29), and the *KanMX4* marker was removed through Cre-*loxP* recombination (30), resulting in strains *vps10Δ*, *pmr1Δ* and *vps10Δ*/*pmr1Δ*.

The *Apa* I linearized-pYMIKPD, pYMIKPS, and pYMIKPSD were respectively integrated into the genome of *S. cerevisiae* CEN.PK102-3A, *vps10Δ*, *pmr1Δ* and *vps10Δ*/*pmr1Δ*, as described previously (31,32), resulting in single- and multiple-modified strains.

Plasmid pJCF (Fig. 1A) was introduced into CEN.PK102-3A and all modified strains to express the *Tr-cel7A* gene. The host strain CEN.PK102-3A containing empty plasmid pJFE3 (25), indicated as BSX000, was used as the control.

All *S. cerevisiae* strains and plasmids used in this study are listed in Table 1, and the primers used for PCR amplification in this study are listed in Table S1.

Dry weight of yeast cells The cells were incubated in 40 mL SC-Ura medium (1.7 g L^{-1} yeast nitrogen base, 5 g L^{-1} ammonium sulfate, and 0.77 g L^{-1} amino acid mixture with omitted uracil) containing 20 g L^{-1} glucose or yeast extract peptone dextrose (YPD) medium (33) at 30°C in the shake flasks and agitated at 200 rpm. A known volume of the culture was collected through a pre-dried and pre-weighed nitrocellulose filter membrane (0.22 μm pore size) and then washed with deionized water. The filter membrane and the yeast cells were dried at 105°C and weighed using a moisture analyzer (Sartorius, Germany). The dry weight of yeast cells was calculated as the difference between filter membrane before and after use.

Assay for the extracellular *Tr*-Cel7A activity The cells were incubated in 40 mL SC-Ura medium containing 20 g L^{-1} glucose at 30°C in the shake flasks and agitated at 200 rpm. The culture supernatants were used as crude enzymes. Since the concentration of total protein in supernatants declined due to the cell growth in the pre-experiment, the ratio between the enzyme units and the dry weight of the yeast cells in grams was described as the extracellular *Tr*-Cel7A specific activity.

The *Tr*-Cel7A activity assay followed the method described previously (34). The reaction was performed in 50 mM sodium acetate buffer (pH 5.0) containing some crude enzymes and final concentration of 1.67 mM p-nitrophenyl-β-D-cellobioside (pNPC) (Sigma, USA) as the substrate. After appropriate incubation (about 10 h) at 50°C, the same volume of 10% Na_2CO_3 was added to stop the reaction. The absorbance at 405 nm for p-nitrophenol (pNP) was detected. One unit of *Tr*-Cel7A activity was defined as the amount of enzyme required to release 1 μmol of pNP from the pNPC substrate in 1 min at 50°C and pH 5.0.

The extracellular *Tr*-Cel7A activity was also detected using a water-insoluble substrate phosphoric acid swollen cellulose (PASC). The PASC was prepared as described by Zhang et al. (35). The reaction was performed in 50 mM sodium acetate buffer (pH 5.0) containing some crude enzymes, 10 μl β-glucosidase (Novozyme 188, Sigma) and final concentration of 5 g L^{-1} PASC as the substrate at 50°C for 10 h. The reducing sugar equivalents formed were determined using via Nelson-Somogyi method (36).

Assay for the extracellular invertase activity To measure the invertase activities secreted to the cell surface, the cells were incubated in SC-Ura medium containing 2% sucrose for invertase expression at 30°C for 12 h. The extracellular invertase activity was measured as described by Ruohonen et al. (37). The glucose released from sucrose by the invertase was determined using Glucose (GO) Assay kit (Sigma, USA). One unit of invertase activity was defined as the amount of enzyme required to release 1 μmol of glucose from sucrose substrate in 1 min at 30°C and pH 5.0.

Quantification of transcription level of genes using real-time quantitative PCR The transcription levels of *Tr-cel7A*, *Sc-SSO1* and *Sc-PDI1* were quantified using real-time reverse quantitative PCR (RT-qPCR). An overnight culture of each strain was transferred into a 100 mL of fresh SC-Ura medium with initial OD_{600} of 0.2, and harvested at mid-exponential phase (OD_{600} ~ 0.7). The total RNA was isolated using the UNIQ-10 spin column Trizol total RNA extraction kit (Sangon, China) and treated with RQ1 DNase (Promega, USA). The PrimeScript RT-PCR Kit (Takara, Japan) was employed for reverse transcription reaction. The reverse transcription reaction products were used for PCR amplification using the LightCycler PCR System (Roche Molecular Biochemicals, Germany), and SYBR Green Real-time

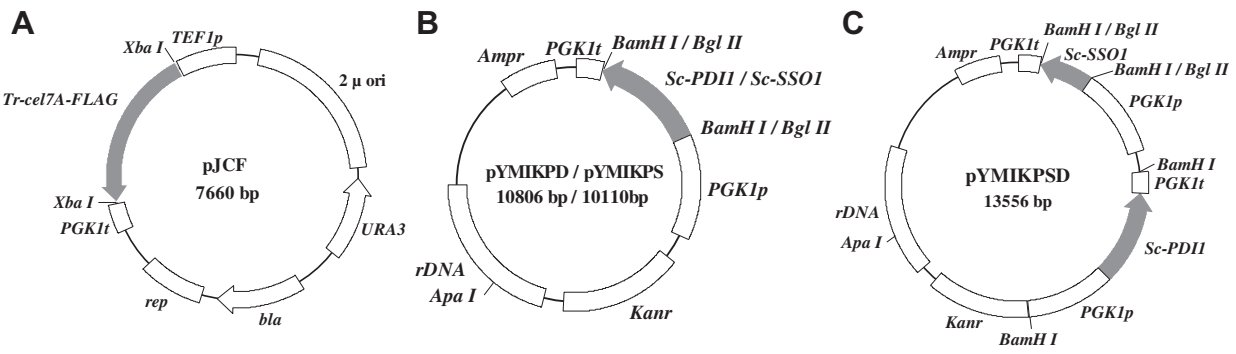

FIG. 1. The physical maps of vectors pJCF (A), pYMIKPD/pYMIKPS (B), and pYMIKPSD (C).