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RESEARCH PAPER

Enhancing Functional Expression of β-glucosidase in *Pichia pastoris* by Co-expressing Protein Disulfide Isomerase

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Abstract The expression of heterologous proteins may exert severe stress on the host cells at different levels. Protein folding and disulfide bond formation were identified as rate-limited steps in recombinant protein secretion in yeast cells. For the production of β-glucosidase in *Pichia pastoris*, final β-glucosidase activity reached 1,749 U/mL after fermentation optimization in a 3 L bioreactor, while the specific activity decreased from 620 to 467 U/mg, indicating a potential protein misfolding. To solve this problem, protein disulfide isomerase, a chaperone protein which may effectively regulate disulfide bond formation and protein folding, was co-expressed with β-glucosidase. In the co-expression system, a β-glucosidase production level of 2,553 U/mL was achieved and the specific activity of the enzyme reached 721 U/mg, which is 1.54 fold that of the control.

Keywords: β-glucosidase, fermentation optimization, *Pichia pastoris*, protein disulfide isomerase, protein folding, specific activity

1. Introduction

The *P. pastoris* expression system is one of the most widely used eukaryotic expression systems. Compared to the prokaryotic system, this system can effectively overcome the defect of the formation of inclusion bodies [1]. Currently, large quantities of heterologous proteins are expressed in *P. pastoris* in both academic research and industrial settings [2,3]. However, upon expression in *P. pastoris*, the recombinant protein is sometimes mis-folded due to non-native, suboptimal cultivation conditions and/or overloading of the host's folding machinery [4]. The mis-folded protein can accumulate in the endoplasmic reticulum (ER) or in the cytoplasm and lead to lower expression levels [5].

Currently, the idea that co-expression of some molecular chaperones involved in protein folding and transportation may effectively improve foreign protein production has been supported by numerous experiments. Disulfide bond isomerase (PDI), an ER associated molecular chaperone which primarily catalyzes disulfide formation and protein folding in the ER lumen, has been co-expressed in *P. pastoris* to improve heterologous protein expression [6-8]. In addition, PDI is also involved in other processes such as ER associated degradation (ERAD) and trafficking of misfolded proteins [9].

β-glucosidase (β-D-glucoside glucohydrolase, BGL, EC 3.2.1.21) is a type of hydrolase which is capable of catalyzing the cleavage of non-reductive β-D-glucoside bonds at the end of the sugar chains [10], and has been widely used in cellulose degradation [11,12]. In addition, BGL also displays transglycosylation activity by catalyzing the transfer of sugar residues from one activated donor substrate to another accepter molecule [13,14]. In our previous study, BGL from *Aspergillus niger* was cloned and expressed in *P. pastoris*, and the recombinant enzyme was utilized for the enzymatic synthesis of gentio-oligosaccharide [15]. In the present study, we optimized the fermentation performance of engineered *P. pastoris*; however, the results showed that, with the improved expression of target protein, the specific activity of BGL in the culture media

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decreased. The protein misfolding issue was solved by co-expression of PDI. The effect of PDI on the expression of BGL is discussed in this paper.

2. Materials and Methods

2.1. Strains, vectors, and materials

Strain *P. pastoris* KM71/pPIC9K-BGL for expression of *A. niger* BGL was previously constructed in our lab [15]. Plasmid pPICZαA was obtained from Invitrogen. The EZ-10Spin Column Plasmid Mini-Preps kit, agarose gel DNA purification kit, restriction enzymes, PCR enzyme Primer STAR™, and T4 DNA ligase were obtained from TakaRa (Dalian, China). *p*-Nitrophenyl-α-D-glucopyranoside (*p*NPG) was purchased from Seebio Biotech. Inc. (Shanghai, china). Yeast Extract and Trypton were purchased from Oxoid. Ltd, and chemical reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. DNA sequencing was performed by Shanghai Generay. *E. coli* JM109 was grown in Low Salt Luria-Bertani (LB) containing the antibiotic Zeocin™ at a final concentration of 25 μg/mL. YPD, MD, BMGY, BMMY, and BSM were prepared according to the Easy-Select™ *Pichia* Expression Kit for the cultivation of yeast.

2.2. Fermentation optimization of *P. pastoris* KM71/pPIC9K-BGL

A 2-phase cultivation was utilized and further optimized for the production of BGL in *P. pastoris* KM71/pPIC9K-BGL in a 3 L bioreactor (BIOFLO 110, America). Fermentation inoculum (10% v/v) was inoculated into the 3 L bioreactor containing 1 L of basal salts medium (BSM). The pH was adjusted and maintained at 5.0 with ammonium hydroxide, and the temperature was maintained at 30°C. With a rapid increase of dissolved oxygen, exponential feeding of glycerol was conducted to achieve a high cell density. Different cell concentrations were produced as the OD_{600} level reached 120, 140, 160, 180, 200, 240, and 320 during the first glycerol feeding phase. Additionally, with the increase of dissolved oxygen, different volumes of methanol (0.5, 1, 2, 3, 4, and 5%, v/v), which is an inducer of alcohol oxidase (AOX) promoter as well as being a carbon source, were added at the beginning of the second methanol induction phase. Three different levels of dissolved oxygen were studied during the fermentation. The dissolved oxygen was maintained at different levels (< 10%, between 20 and 30%, and > 60%) by a cascaded control of agitation rate and aeration rate. The optimization processes were carried out step by step and the methanol concentration was controlled at 0.5% (v/v) by the methanol on-line control station (FC2002, East China University of Science and Technology) throughout the induction phase when the initial added methanol was depleted.

2.3. Cloning of *P. pastoris* PDI

The *pdi* gene (NCBI accession number AJ302014) was amplified using the genome of *P. pastoris* as a template The cloning primer sequences were designed as follows: P1: AG*TTCGAA*ATGCAATTCAACTGGGATATTAAAA-CTGTG (forward primer); P2: TT*GCGGCCGC*TTAAAG-CTCGTCGTGAGCGTC (reverse primer). The *BspT*104*I* and *NotI* restriction sites (italic and underline) were designed into P1 and P2, respectively.

The PCR was performed using 30 cycles as following: denaturation at 94°C for 45 sec, annealing at 56°C for 30 sec, and primer extension at 72°C for 2 min. The PCR product was digested with *BspT*104*I* and *NotI*, gel-purified, and then ligated into the expression vector pPICZαA, which was also digested with *BspT*104*I* and *NotI*. The recombinant plasmid, pPICZαA-PDI was used to transform chemically competent *E. coli* JM109 cells and identified by restriction analysis and sequencing. The signal peptide of the expression vector pPICZαA was removed by *BspT*104*I* and *NotI*, because of the intracellular function of PDI.

2.4. Expression of PDI in *P. pastoris* KM71/pPIC9K-BGL

The recombinant plasmid pPICZαA-PDI was linearized with *SacI* and electroporated into *P. pastoris* KM71/pPIC9K-BGL. The transformants were preliminarily selected at 30°C on the MD agar plates with G418 at a final concentration of 500 μg/mL for 2 ~ 4 days, and further screening on the YPD agar plates with an increase in Zeocin™ concentrations to 500 μg/mL, 1 and 2 mg/mL. The recombinant *P. pastoris* with multiple copies of *pdi* was obtained. The presence of the *pdi* gene in the transformants was confirmed by bacterial colony PCR. 5' *AOX1* primer (GACTGGTTCCAATTGACAAGC) paired with 3' *AOX1* primer (GCAAATGGCATTCTGACATCC) were used according to the EasySelect™ *Pichia* Expression Kit for pPICZαA.

For expression, the colonies were cultivated in 10 mL of YPD medium at 30°C for 24 h, and then inoculated into 50 mL BMGY medium at 30°C and shaken at 200 rpm. The cells were collected by centrifugation at 5,000 rpm for 5 min at 4°C when the OD_{600} reached 5 ~ 6. Cells were resuspended in 25 mL of BMMY with an initial methanol addition of 4% (v/v). Methanol was supplemented every 24 h with the addition of 0.5% (v/v) throughout the induction phase, and samples were collected.

The culture broth samples were centrifuged at 12,000

rpm for 10 min and the fermentation supernatants were used for determination of BGL activity. The cells were washed and resuspended in 50 mM phosphate buffer (pH 7.0), and disrupted by high pressure homogenization to produce cell-free extracts. The intracellular PDI expression level was detected by SDS-PAGE.

2.5. Cultivation of *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI in a 3 L bioreactor

The recombinant *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI was used to scale-up cultivation in the 3 L bioreactor. When the OD_{600} reached 160, with an increase of dissolved oxygen, 2% (v/v) of methanol was added into the bioreactor. The level of dissolved oxygen was maintained between 20 and 30%, and the concentration of methanol was maintained at 0.5% (v/v) throughout the induction phase.

2.6. β-glucosidase activity assay

The activity of BGL was determined by the amount of *p*-nitrophenol (*p*NP) generated from *p*NPG. The reaction mixture contained 0.96 mL of 200 mM sodium acetate buffer (pH 4.6), 0.02 mL of 100 mM *p*NPG, and 0.02 mL of enzyme appropriately diluted by sodium acetate buffer (pH 4.6). The reaction was incubated at 60°C for 10 min and terminated by 0.2 mL of 1 M sodium carbonate solution. One unit (U) of enzyme activity was defined as the amount of 1 umol *p*NP produced per min under the above conditions.

2.7. Miscellaneous methods

The concentration of the strain was determined by OD_{600}, which was tested using the UV-2450 ultraviolet-visible spectrophotometer from Shimadzu. Protein concentration was determined by the Bradford method. SDS-PAGE was performed on a 12% polyacrylamide gel [16]. The gel was visualized with 0.25% Coomassie Brilliant Blue R-250 stain.

3. Results and Discussion

3.1. Fermentation optimization of *P. pastoris* KM71/pPIC9K-BGL

In our previous study, *P. pastoris* KM71/pPIC9K-BGL was constructed and cultivated in a shake-flask to produce *A. niger* BGL. The BGL activity reached 115 U/mL and the specific activity was 620 U/mg [15]. In the present study, we attempted to cultivate the engineered *P. pastoris* in a 3 L bioreactor. Using the initial culture conditions, BGL activity reached 1,175 U/mL and the specific activity was 491 U/mg (Fig. 1A). To improve the enzyme production,

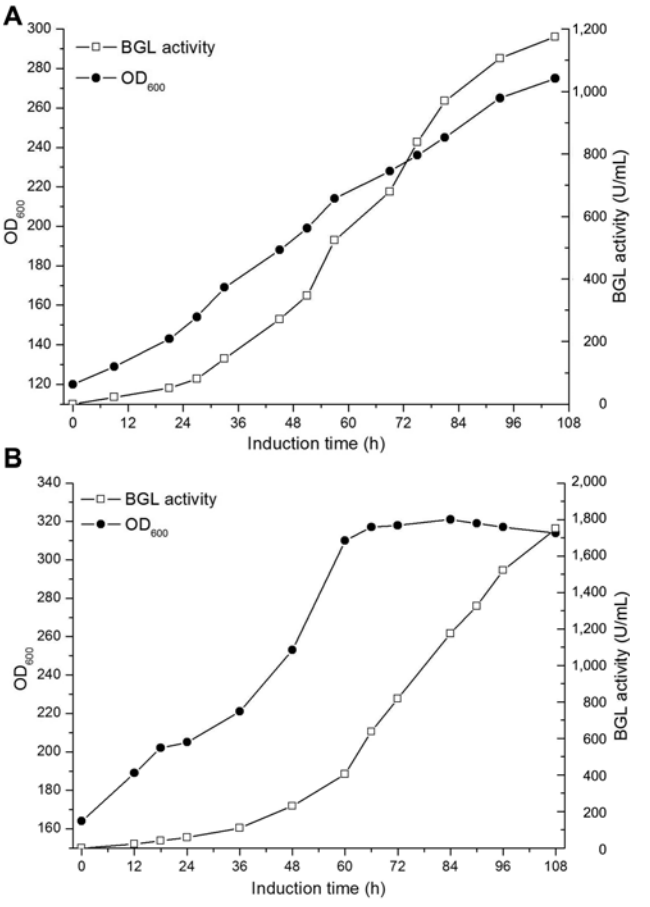

Fig. 1. Time courses of cell concentration and BGL activity during the induction phase by *P. pastoris* KM71/pPIC9K-BGL in a 3 L bioreactor. (A) Before fermentation optimization. (B) After fermentation optimization.

fermentation conditions such as initial cell concentration, methanol addition in the post-induction phase, and dissolved oxygen concentration during the induction phase were optimized in detail. The optimized condition was obtained as follows: when the initial cell concentration reached an OD_{600} of 160, 2% (v/v) of methanol was added, and the dissolved oxygen concentration was maintained between 20 and 30% throughout the induction phase. Using these conditions, BGL activity increased to 1,749 U/mL (Fig. 1B), which was 1.49-fold higher than before optimization; however, the specific activity of the enzyme decreased from 620 to 467 U/mg, indicating a potential misfolding of expressed protein. To solve this issue, we attempted to co-express PDI, a protein folding chaperone, to improve protein folding.

3.2. Construction of *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI

The gene of PDI was amplified from the genome of *P. pastoris* by PCR and inserted into the expression vector of

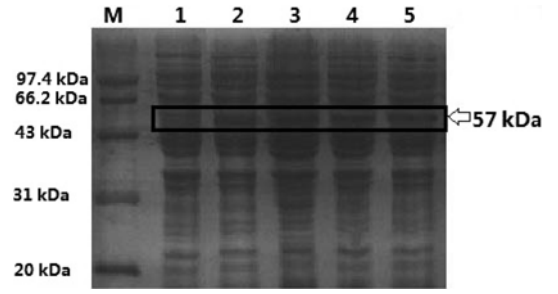

Fig. 2. SDS-PAGE analysis of expressed intracellular PDI after shake-flask cultivation for 96 h. M: protein marker. Lane 1: *P. pastoris* KM71/pPIC9K-BGL. Lanes 2 ~ 5: *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI. The target protein band is marked with an arrow and pane.

pPICZαA. The recombinant plasmid (pPICZαA-PDI) was multiplied by *E. coli* JM109, and then transformed into previously constructed *P. pastoris* KM71/pPIC9K-BGL cells. The transformants were preliminarily selected on the MD agar plates with G418 and further screened on the YPD agar plates with an increase in Zeocin™ concentration. Single colonies (34, 10, and 4) were obtained on the YPD agar plates with Zeocin™ at concentrations of 0.5, 1 and 2 mg/mL, respectively. The 14 colonies on plates with 1 and 2 mg/mL Zeocin™ were chosen for bacterial colony PCR. Finally, when the insertion of *pdi* was confirmed, only 4 colonies with the 2 mg/mL of Zeocin™ were chosen for further cultivation in shake-flasks. After cultivation for 96 h, SDS-PAGE was performed to analyze the expressed intracellular protein (Fig. 2). The results showed that, when compared to the cells before co-expression, a protein band, which corresponds to a molecular weight of 57 kDa, was effectively increased in all 4 strains (Fig. 2). This molecular weight was consistent with that of PDI. In addition, significant increase in extracellular BGL activity was observed in all 4 strains after 96 h cultivation (Fig. 3). Among them, strain 4 showed the highest BGL activity and was chosen for further investigations.

3.3. Fermentation of *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI in a 3 L bioreactor

Strain 4 was further cultivated in the 3 L bioreactor using the above optimized fermentation conditions for *P. pastoris* KM71/pPIC9K-BGL. The results showed that a BGL production level of 2,330 U/mL was achieved (Fig. 4) and the specific activity of BGL reached 733 U/mg, which was 1.54-fold that of *P. pastoris* KM71/pPIC9K-BGL grown in the same conditions, and 1.17-fold that of *P. pastoris* KM71/pPIC9K-BGL grown in shake-flask conditions. These results demonstrated enhanced functional expression of BGL upon co-expression of PDI.

It has been reported that PDI primarily catalyzes oxidation, reduction, and isomerization of disulfide bonds in the ER lumen [17]. BGL from *A. niger* contains 7 cysteines in the amino acid sequence and disulfide bonds are formed during the nascent chain folding and assembly [18]. Thus, the results suggest that PDI effectively improved disulfide bond formation in the production of BGL. Also, it has been reported that PDI can function as a catalyst in protein folding during BGL expression, and a 3-fold increase in the secretion of *Pyrococcus furiosus* β-glucosidase without disulfide bond was demonstrated [9].

Except for the increased BGL activity in the cells co-expressing PDI, it was found that the consumption of methanol and oxygen were increased during the cultivation, and cell growth was inhibited after a 60 h induction using methanol (Fig. 4). Studies showed that superfluous

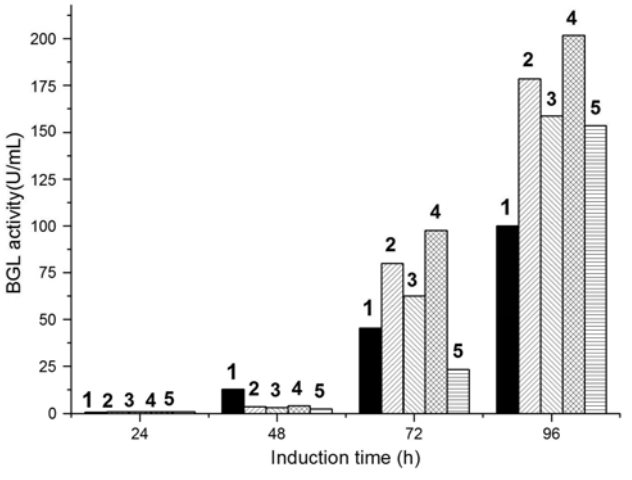

Fig. 3. The comparison of BGL activity in shake-flask cultivation. 1: BGL activity of *P. pastoris* KM71/pPIC9K-BGL. 2 ~ 5: BGL activity of *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI.

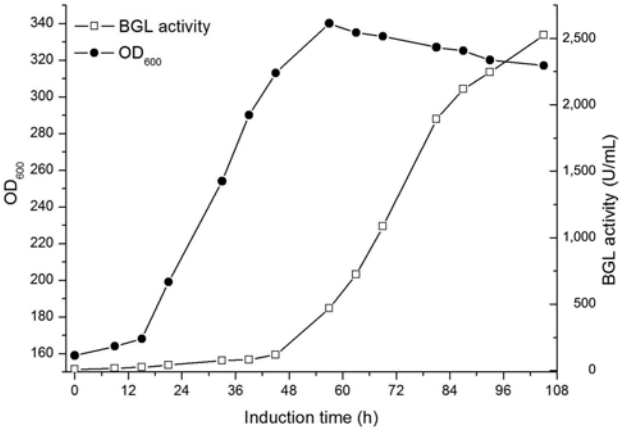

Fig. 4. Time courses of cell concentration and BGL activity during the induction phase of *P. pastoris* KM71/pPIC9K-BGL/pPICZαA-PDI in a 3 L bioreactor.

oxygen was consumed by yeasts by the partial functioning of several non-respiratory oxygen consuming pathways. As a result, reactive oxygen species (ROS) were produced [19], which would lead to lower cell activity, a delayed cell-division cycle, and even apoptotic death [20]. Additionally, some studies showed that co-feeding of non-repressing carbon sources such as alanine, sorbitol, and mannitol, with methanol could effectively reduce the consumption of methanol and oxygen [21,22]. For example, in the expression of alkaline polygalacturonate lyase in *P. pastoris*, cell mortality was effectively alleviated with reduced oxygen consumption and heat production when sorbitol was co-fed [21]. Therefore, these strategies can be important for our future work to improve cell growth and further enhance the production of BGL.

4. Conclusion

In this study, the specific activity of recombinant BGL was decreased upon the increase of enzyme production during fermentation optimization, indicating a potential protein misfolding issue. After co-expression of PDI with BGL in *P. pastoris*, BGL activity reached 2,553 U/mL, and the specific activity was increased to 721 U/mg, which was 1.54-fold that of the control without PDI co-expression. Our studies showed that PDI, an ER associated chaperone protein, could effectively improve BGL folding as well as enzyme production in *P. pastoris*, indicating a potential role for PDI in the fermentation optimization and industrial utilization of various recombinant proteins. These potential benefits require further investigation.

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