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Enhancement of lipase r27RCL production in *Pichia pastoris* by regulating gene dosage and co-expression with chaperone protein disulfide isomerase

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ABSTRACT

Pichia pastoris has been successfully used in the production of many secreted and intracellular recombinant proteins, but there is still a large room of improvement for this expression system. Two factors drastically influence the lipase r27RCL production from *Rhizopus chinensis* CCTCC M201021, which are gene dosage and protein folding in the endoplasmic reticulum (ER). Regarding the effect of gene dosage, the enzyme activity for recombinant strain with three copies lipase gene was 1.95-fold higher than that for recombinant strain with only one copy lipase gene. In addition, the lipase production was further improved by co-expression with chaperone PDI involved in the disulfide bond formation in the ER. Overall, the maximum enzyme activity reached 355 U/mL by the recombinant strain with one copy chaperone gene *PDI* plus five copies lipase gene *proRCL* in shaking flasks, which was 2.74-fold higher than that for the control strain with only one copy lipase gene. Overall, co-expression with PDI vastly increased the capacity for processing proteins of ER in *P. pastoris*.

1. Introduction

In recent years, a great number of heterologous proteins have been produced in the methylotrophic yeast *Pichia pastoris* using the alcohol oxidase promoter (*PAOX1*), which contains a number of advantages, such as ease of manipulation and growth capacity, performing the post-translational processing of complex proteins, growing on minimal medium at very high cell densities and secreting the heterologous proteins to the media [1]. It has been reported that many factors can potentially affect heterogonous protein production in *P. pastoris* expression system, which include the properties of gene sequence, gene dosage, gene transcription, protein translation, protein folding in the endoplasmic reticulum (ER) and the translocation from ER to the Golgi apparatus.

Optimization of protein expression in *P. pastoris* commonly includes the isolation of multi-copy expression strains after transformation, which generally yield higher expression levels of recombinant proteins than do by single-copy strain. So, gene dosage can be critical for maximum expression. The expression level of IH protein (Interleukin-1 receptor antagonist through fusion with human serum albumin) was found to be higher in culture medium of the high copy strains than that of the low copy strains [2]. Through porcine insulin precursor (PIP) copy number optimization, the maximum protein expression level of 181 mg/L was achieved by a 12-copy strain [3]. Although many researches have shown varying degree of effects of gene copy number on the protein expression, the increasing of secretory foreign proteins is not correlated linearly with its gene dosage. It has been suggested that increasing gene copy number even possibly leads to a blockage of the secretory pathway, resulting in accumulation of target protein in the cell. Increasing gene copy number of *Necator americanus* secretory protein (Na-ASP1) in *P. pastoris* saturated secretory capacity and therefore, decreased the amount of secreted protein in strains harboring multiple copies of *Na-ASP1* gene [4].

Generally, the rate-limiting step of the secretory pathway has been considered to be the protein disulfide bond formation and the protein folding in the ER of eukaryotic cells, e.g., *P. pastoris* [5,6]. The ER contains folding assistants that help proteins achieve their correct disulfide arrangement [7]. In particular, protein disulfide isomerase (PDI) is a multi-functional protein from the thioredoxin superfamily that catalyzes the disulfide bond formation and help in the correct folding of protein [8]. It was shown that co-expression with PDI had significantly improved the yields of many target proteins [2,9–12].

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are well known hydrolases capable of hydrolyzing the ester bonds of water insoluble substrates at the interface between substrate and water and have various industrial applications in pulp and paper,

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food, detergent, textile industries and so on, which have recently been reviewed by Singh and Mulkhopadhyay [13]. In the previous research, a lipase gene *proRCL* from *Rhizopus chinensis* CCTCC M201021 was cloned and successfully expressed in *P. pastoris*, named as r27RCL (*R. chinensis* lipase) [14]. Furthermore, the recombinant strain in the Mut^s phenotype (methanol utilization slow) exhibited higher enzyme activity and specific activity than those of the recombinant strain in the Mut$^+$ phenotype (methanol utilization plus). We have also investigated the relationship between lipase gene copy number and lipase production of the recombinant strains in the Mut$^+$ phenotype, and the lipase expression level decreased by the recombinant strain with six copies lipase gene. However, the function of gene copy number on the lipase production in the Mut^s phenotype has not been systematically explored. It is well known that two key parameters in the production of the lipase r27RCL in *P. pastoris* are the gene dosage and the protein folding in the ER. Therefore, in this study, we discussed the effects of lipase gene copy number and co-expression with PDI on the lipase r27RCL secretion in the Mut^s phenotype.

2. Materials and methods

2.1. Enzyme and reagent

Restriction enzymes, T4 DNA ligase, polymerase chain reaction (PCR) reagent (TaKaRa Biotechnology (Dalian) Co., Ltd.), primers (SBS Gene Technology (Shanghai) Co., Ltd.), Gel Extraction Kit and Plasmid Mini Kit I (OMEGA BIO-TEK), PCR Purification Kit (Bioflux), Real-time quantitative PCR (RT-qPCR) reagent and material (Bio-Rad).

2.2. Strains, plasmids and medium

P. pastoris GS115, plasmids pPIC9K, and pPICZαA from Invitrogen BV were used as the host strain and the vector, respectively. Recombinant plasmid pPIC9K-*proRCL* was constructed before [14]. Yeast nutrient medium YPD, BMGY, BMMY, YPD-G418 and YPD-Zeocin are prepared by means of "*P. pastoris* expression Kit" (*Pichia* Multi-Copy Expression Kit, version A, Invitrogen BV, The Netherlands).

2.3. Construction of plasmid pPICZ-PDI

The *PDI* gene (GenBank accession number: AJ302014) was amplified from chromosomal DNA of *P. pastoris* GS115 using the forward primer 5′-GGGTTCGAAACGATGCAATTCAACTGGG-3′ (*Asu* II site underlined) and the reverse primer 5′-TTGCGGCCGCTTAAAGCTCGTCG TGAGCGTC-3′ (*Not* I site underlined). The *Asu* II/*Not* I amplicon was cloned into *Asu* II/*Not* I -digested pPICZαA resulting in pPICZ-*PDI* without the *Saccharomyces cerevisiae* α-mating factor secretion signal under the control of the *AOX1* promoter.

2.4. Transformation of P. pastoris cells

Competent cells were prepared under the way optimized by Wu et al. [15]. Instrument settings were 1.5 kV, 25 μF, and 200 Ω. After pulsing the mixture of competent cells and linearized plasmids, immediately added 1 mL of ice-cold 1 M sorbitol to the cuvette and transferred the cuvette contents to a sterile 1.5 mL EP tubes. After 1 h in 1 M sorbitol at 30 °C, then plated out all of the cells by spreading 100 μL on 150 mm plates containing antibiotics with different concentrations. Incubated plates for 2–5 days at 30 °C until colonies formed. The colonies were picked and the gene dosage was also identified by RT-qPCR established before [16].

2.5. Gene copy number determination

The method we used was constructed before [16]. The copy numbers of *PAOX1* (promoter of gene *AOX1* existed in both the genome of *P. pastoris* GS115 and the recombinant plasmids) equal the copy numbers of the inserted heterogenous genes.

2.6. Small scale fermentation

A single colony of recombinant strain was picked and inoculated into 25 mL BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer with pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) medium, and grew at 28–30 °C in a shaking incubator (250–300 rpm) until the culture reached an OD_{600} of 4.0. The cells were harvested, and was fully transferred into 100 mL BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.5% methanol) medium to obtain a cell suspension with OD_{600} = 1.0. The cells were grown for another 5 d and expression of lipase was induced by methanol at a final concentration of 1% added every 24 h. The culture supernatants were all checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. SDS-PAGE analysis of the expressed products

Denaturing SDS-PAGE was conducted in accordance with the method of Laemmli [17]. The supernatant samples (10 μL) were collected at 96 h and resolved on 12% SDS-PAGE under reducing conditions and visualized by Coomassie Blue R-250 staining.

2.8. Lipase activity determination

Lipase activity was measured on emulsified *p*-nitrophenyl palmitate (*p*NPP) according to Kordel et al. [18]. One volume of a 1.08-mM solution of *p*NPP in 2-propanol was mixed just before used with 9 volumes of 50 mM Tris–HCl buffer pH 8.0 containing 4 g/L Triton X-100 and 1 g/L arabic gum. The standard reaction was started by pre-equilibration of 2 mL of above mixture at 37 °C and addition of 0.1 mL of enzyme solution at an appropriate dilution in 50 mM pH 8.0 Tris–HCl buffer. The variation of the absorbance at 410 nm of the assay against a blank without enzyme was monitored for 2 min using a UV–vis spectrophotometer (UNICOUV-3102 PC, China). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 44,656 cm^{-1} M^{-1} for *p*-nitrophenol. One enzyme unit was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions.

2.9. RNA extraction and cDNA synthesis

During the fermentation of the recombinant strains, 1 mL fermentation broth was taken and centrifuged to get the cells at 96 h. Total RNA was extracted by Yeast RNAiso Kit (TaKaRa Bio Co., Ltd.). RNA integrity was tested in 1.2% agarose gels and its concentration measured by densitometry and by 260/280 nm absorbance ratio. Five hundred nanograms of total RNA was subjected to reverse transcription using PrimeScript® RT reagent Kit (TaKaRa Bio Co., Ltd.). The reaction was terminated by heating at 85 °C for 5 s.

2.10. Transcription levels of intracellular relevant genes

The transcription levels of gene *proRCL* or *PDI* in all constructed recombinant strains with different gene copy numbers were normalized using gene *GAPDH* as the endogenous control (housekeeping gene). In this case, the transcription levels of lipase gene and chaperone gene in SRCL-1 strain were set as control to normalize the data. RT-qPCRs were run in triplicate with biological replicates to allow for statistical confidence in differential gene expression.

3. Results

3.1. Construction of the recombinant strains carrying different gene copy numbers of lipase gene and chaperone gene

The plasmid pPIC9K-*proRCL* was digested by *Bgl* II and subsequently the big fragment was transformed into the host *P. pastoris* GS115, resulting in the strain SRCL-1. To construct the multi-copy lipase gene recombinant strains, strain SRCL-1 was transformed with *Sal* I linearized plasmid pPIC9K-*proRCL* and the transformants were selected in YPD-G418 plates with higher G418 concentration. The PDI-overexpressing strains PDI-1-SRCL-1, PDI-2-SRCL-1, PDI-3-SRCL-1, and PDI-4-SRCL-1 were the products of transformation of recombinant strain SRCL-1 with plasmid pPICZ-*PDI* linearized by *Sac* I, which were selected in the YPD-Zeocin plates. The recombinant strains PDI-1-SRCL-3, PDI-1-SRCL-5, PDI-2-SRCL-5, PDI-1-SRCL-7, PDI-2-SRCL-7, PDI-3-SRCL-7, and PDI-4-SRCL-7 were the results transforming SRCL-3, SRCL-5, and SRCL-7 with *Sac* I linearized pPICZ-*PDI* and the selection was also done in the YPD-Zeocin plates. The copy numbers of the lipase gene *proRCL* and the chaperone gene *PDI* in all these recombinant strains were identified by the method RT-qPCR constructed before [16]. The recombinant strains were also listed in Table 1.

3.2. Effect of lipase gene dosage on the lipase production

Recombinant strains SRCL-1, SRCL-3, SRCL-5 and SRCL-7 were selected to evaluate the effects of gene dosage on the lipase production in shaking flasks by inducing with methanol. As shown