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Overexpression of *Candida rugosa* lipase Lip1 via combined strategies in *Pichia pastoris*

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ABSTRACT

In this study, combined strategies were employed to heterologously overexpress *Candida rugosa* lipase Lip1 (CRL1) in a *Pichia pastoris* system. The *LIP1* gene was systematically codon-optimized and synthesized in vitro. The Lip1 activity of a recombinant strain harboring three copies of the codon-optimized *LIP1* gene reached 1200 U/mL in a shake flask culture. Higher lipase activity, 1450 U/mL, was obtained using a five copy number construct. Co-expressing one copy of the ERO1p and BiP chaperones with Lip1p, the CRL1 lipase yield further reached 1758 U/mL, which was significantly higher than that achieved by expressing Lip1p alone or only co-expressing one molecular chaperone. When cultivated in a 3 L fermenter under optimal conditions, the recombinant strain GS115/87-ZA-ERO1p-BiP #7, expressing the molecular chaperones Ero1p and BiP, produced 13,490 U/mL of lipase activity at 130 h, which was greater than the 11,400 U/mL of activity for the recombinant strain GS115/pAO815-α-mCRL1 #87, which did not express a molecular chaperone. This study indicates that a strategy of combining codon optimization with co-expression of molecular chaperones has great potential for the industrial-scale production of pure CRL1.

1. Introduction

Commercial crude *Candida rugosa* (formerly *Candida cylindracea*) lipase (CRL) is the most widely used traditional biocatalyst to catalyze the hydrolysis and synthesis of ester compounds for industrial applications, including the manufacturing of pharmaceuticals, foods, and cosmetics, owing to its stereo-specificity and generally regarded as safe (GRAS) status [1–4]. However, the crude enzyme is a mixture of eight isoenzymes encoded by the high-identity *lip* gene family that is localized on chromosome 1 and whose members exhibit remarkable variations in catalytic efficiency, regioselectivity, and stereospecificity [5,6]. Among the eight isoforms, *C. rugosa* lipase Lip1 (CRL1) has the highest level of constitutive expression (over 60% in the commercial enzymes), and it shows good enantioselectivity toward 2 arylpropionic acid non-steroidal anti-inflammatory drugs, such as ibuprofen, fenoprofen, flurbiprofen, and ketoprofen [7]. Therefore, pure CRL1 is very important for basic research, as well as for its commercial uses.

As is well known, the *Pichia pastoris* expression system is very suitable for expressing heterologous proteins at high levels because it has many advantages over other expression systems. It possesses a strong promoter that can strictly regulate the expression of exogenous gens; the target gene is integrated into the yeast genome, so it can be expressed stably; and *P. pastoris* can be fermented at high densities owing to its rapid growth and low nutritional requirements. Moreover, the highly expressed proteins can be easily purified. Therefore, *P. pastoris* has been widely used in many studies, and it was also chosen as the host expression system in this study.

Until now, active recombinant CRL1 has been expressed in a *Pichia* system either by multiple mutagenesis of non-universal serine codons or by directly synthesizing a codon-optimized version of the *LIP1* gene [8,9]. However, the expression level of recombinant CRL produced by wild-type strains or conventionally engineered strains is still fairly low, and it is not sufficient to meet the demands of diverse industrial applications. The wild-type strain only produced a lipase activity of 12.6 U/mL in a shake flask culture [10]. When Lip1p was heterologously expressed in the *Saccharomyces cerevisiae*, the detected lipase activity was only 7 U/mL [9]. The obtained lipase activities were still relatively low, only 100–300 U/mL, when 19 non-universal serine codons were replaced and *LIP1* was expressed in a *P. pastoris* system [8,9].

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In addition to fermentation, many other factors can potentially affect heterogonous protein production in *P. pastoris*, including the gene sequence, gene dosage, gene transcription, protein translation, protein folding in the endoplasmic reticulum (ER), and protein translocation from the ER to the Golgi apparatus [11]. In addition, different codon usage frequencies between the host and gene donor can also influence heterologous gene expression. *Pichia* species display a non-random pattern of synonymous codon usage and show general bias toward a subset of codons, leading to different heterologous expression efficiencies in *P. pastoris* [12–16]. Thus, codon optimization has to be performed by replacing rarely used codons with frequently used ones, as well as by considering the effect of codon sequences on mRNA secondary structure.

The non-spore-forming yeast *C. rugosa* obeys a non-universal codon usage: CTG, a universal codon for leucine, is read as serine. In the CRL lipase genes, CTG encodes most of the serine residues, including the catalytic Ser-209 [8,17]. Therefore, codon optimization and gene synthesis in vitro must be adapted when heterologously expressing *LIP1* in *P. pastoris.*

Gene copy number can significantly affect the outcome of heterologous expression in *P. pastoris*. In most cases, higher gene dosage may greatly enhance recombinant protein expression. However, too much gene dosage will lead to a plateau in expression, and may even be detrimental [18–22]. This is because high-level overexpression of heterologous proteins in *P. pastoris* saturates or overloads the secretory pathway, or even triggers the unfolded protein response, a process that involves many secretion helpers, such as the transcription factor Hac1, the protein folding factors endoplasmic reticulum oxidoreduction 1 (ERO1) and protein disulfide isomerase (PDI), and the translocation or other secretion helper factors Cog6p, Ssa4p, Sse1p, Sso2p [23]. Usually, the rate-limiting step in the eukaryotic secretion pathway is protein folding in the ER [24]. Two ER resident proteins are directly involved in this process: Ero1p and PDI. Ero1p oxidizes PDI, which then catalyzes the formation of disulfide bonds [25]. Additionally, immunoglobulin heavy chain binding protein (BiP), an ER-located member of the Hsp70 chaperone family, hydrolyzes ATP to provide energy for proper protein folding. It can also identify the hydrophobic structure of the newly produced protein to prevent aggregation of misfolded proteins [26]. Thus, their overexpression may enhance heterologous protein expression in *P. pastoris*.

Based on the above analyses, in this study, combined strategies were employed to enhance the heterologous expression of CRL1 in the *P. pastoris* GS115 strain. First, *P. pastoris* GS115 was electrotransformed with the codon-optimized and in vitro synthesized *LIP1* gene. Then, a moderate copy number vector was used to further increase *LIP1* gene expression. Consequently, the molecular chaperones Ero1p and/or BiP were co-expressed with the *LIP1* gene to further improve the translation efficiency. Moreover, the best recombinant strains were fermented under optimized conditions in a 3 L fermenter to confirm the highest CRL1 expression level.

2. Materials and methods

2.1. Strains, plasmids, and media

P. pastoris GS115 (*his4*) (Invitrogen, Carlsbad, CA, USA) was used as the host strain. Yeast nutrient media minimal dextrose (MD), yeast extract-peptone-dextrose (YPD), buffered glycerol-complex medium (BMGY), buffered methanol-complex medium (BMMY), and YPD-Zeocin [22] were prepared with the *Pichia* Multi-Copy Expression Kit (version A, Invitrogen B.V., Breda, The Netherlands). Plasmids used in this study were listed in Table S3.

2.2. Construction of CRL1 and molecular chaperone expression vectors

The *LIP1* gene was optimized using OptimumGene® technology and the optimized gene encoding CRL1 was synthesized in vitro and cloned into pUC57 by GenScript Biotech Co. (Nanjing, China). The *LIP1* gene was amplified by PCR using the primer pair CRL1-F1/R1 (see Table 1, the XhoI and NotI sites are underlined). The gene encoding mature CRL1 with an α-mating factor signal peptide and a Kozak translational initiation sequence was amplified by PCR using the primer pair pAO815-CRL1-F1/R1 (see Table 1, the EcoRI sites are underlined). The plasmid pAO815-α-mCRL1 was obtained by MEGAWHOP PCR [27] using the primer pair pAO815-M-F1/R1, which eliminated the BglII and BamHI sites in *LIP1*. The first PCR was conducted using PrimeSTAR™ HS DNA polymerase with the following program: pre-denaturation at 98 °C for 5 min, 30 cycles at 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Using the purified PCR product as a primer and pAO815-α-mCRL1 as template, a second PCR was conducted with PrimeSTAR™ HS DNA polymerase with the following program: pre-denaturation at 68 °C for 5 min, and pre-denaturation at 98 °C for 3 min, followed by 24 cycles at 98 °C for 10 s, and 68 °C for 5 min, followed by a final extension at 68 °C for 10 min.

To study the effect of gene dosage on the expression of CRL1, *LIP1* was ligated into the vector pPICZαA at the EcoRI and NotI sites, resulting in pPICZαA-CRL1, with the *S. cerevisiae* α-mating factor secretion signal under the control of the AOX1 promoter.

Several kind of molecular chaperone expression vectors were constructed, including two kinds of single molecular chaperone expression vectors and one vector that expressed two molecular chaperones. The *ERO1* gene (GenBank accession number FN392319) was amplified from the chromosomal DNA of *P. pastoris* GS115 using the primer pair ERO-F/R (see Table 1, the XhoI and NotI sites are underlined). The PCR product was cloned into XhoI/NotI-digested pPICZA generating pPICZA-ERO1 under the control of the AOX1 promoter without the *S. cerevisiae* α-mating factor secretion signal. The plasmids pPICZA-BiP and pPICZAERO1-pGAPZABiP were constructed beforehand in our laboratory as the starting vectors for this work.

2.3. First transformation of P. pastoris

P. pastoris GS115 was electrotransformed with linearized pAO815-α-mCRL1 and pAO815 using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA). According to the manufacturer's protocol, the parameters were 1500 V, 200 Ω, and 25 μF using a 0.2-cm cuvette (Invitrogen). Transformants were selected on MD plates after incubation for 2–3 days at 28 °C.

2.4. Quantitative lipase assay screening and shake flask cultures

The transformants were plated onto BMMY-rhodamine B-olive oil (BRBO) medium plates containing 0.008% (w/v) rhodamine B and 1% (v/v) emulsified olive oil, and about two days later, white colonies grew on the BRBO plates. Subsequently, 200 μL of methanol was added to the culture dish every day to induce the production of CRL1, and 30 colonies with large, clear, transparent circles were picked and inoculated into a 500 mL Erlenmeyer flask containing 50 mL of BMGY. These selected recombinant cells were harvested by centrifugation and transferred into 50 mL of BMMY after incubation at 28 °C for 24 h. In addition, 1% (v/v) methanol was added to the culture every 24 h to induce lipase expression. Aliquots of the cultures (0.5 mL) were harvested every 24 h. The biomass concentration and enzyme assay were determined immediately. Then, quantitative PCR was performed to determine the