Process Biochemistry 48 (2013) 1317–1323

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Combined strategies for improving the heterologous expression of an alkaline lipase from *Acinetobacter radioresistens* CMC-1 in *Pichia pastoris*

Xiaolan Zhao, Wanyong Xie, Ying Lin, Xiaoqiong Lin, Suiping Zheng, Shuangyan Han *

Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, PR China

ARTICLE INFO

Article history:
Received 2 February 2013
Received in revised form 10 June 2013
Accepted 16 June 2013
Available online 28 June 2013

Keywords:
Acinetobacter radioresistens CMC-1
ARL
Lipase
Secretion expression
Pichia pastoris

ABSTRACT

In this study, a series of strategies was developed to enhance the expression of an alkaline lipase from *Acinetobacter radioresistens* (ARL) in *Pichia pastoris*. Activity of the lipase from recombinant strain carrying a single copy of codon-optimized ARL gene was 65 U/mL in shake flask culture with p-nitrophenyl caprylate as the substrate. The lipase yield was increased to 104 U/mL by introducing a short N-extension spacer peptide coding for the 10 amino acids (EEAEAEAEPK) between α-factor signal peptide and ARL. The N-terminal extension spacer did not affect the pH or temperature properties of the recombinant ARL. After the multi-copy constructs were identified by Q-PCR assay, a higher lipase activity of 180 U/mL was obtained. Further introduction of the spliced HAC1 gene into multi-copy integrants (>6 copies) extensively enhanced the ARL yield by 30–40%. As a result, the ARL yield reached 1.06×10^4 U/mL in a 10-L scaled-up fed-batch fermenter as well as the lipase showed some better properties compared to that wild one from *A. radioresistens*.

1. Introduction

Of the numerous lipases, those with a high activity at an alkaline pH and a middle temperature are desired for use as additives in various detergents and this is one of the largest areas for the application of industrial lipases [1]. The alkaline lipase A1 from *Acinetobacter radioresistens* CMC-1 (ARL, GenBank Accession No. AF073953) has received considerable attention because of its high activity at an alkaline pH and in a thermal environment as well as a broad range of specificity toward long chain triacylglycerols or *p*-nitrophenyl esters [2,3]. Initially, the maximum ARL yield was only 54 U/mL when the wild type host, *A. radioresistens* was cultivated during repeated batch culture [4]. Li et al. used a fed-batch culture with a pH-stat feeding method and the maximum lipase activity was increased to 120 U/mL [2]. In 2005, the lipase productivity was further improved as high as 3.3 times that in the pH-stat fed-batch culture method mentioned above after introducing an adequate feeding method to control the cell growth rate [5]. This lipase yield was still too limited for bulk production so a gene engineering approach to enhanced productivity is necessary.

The alpha mating factor signal sequence of *Saccharomyces cerevisiae* is the most common choice for driving recombinant protein secretion in *Pichia pastoris*, a popular productive expression system [6,7]. The encountered problem is that the signal peptide did not ensure efficient translocation and/or cleavage after the signal peptide, which resulted in lower secretion efficiency [8]. A synthetic N-terminal extension sequence was confirmed to improve the heterologous protein secretion and yield significantly in patent No. US 6500645. The researchers developed a new theory that signal peptide-leader peptide(-X1-X2-X3-X4-X5-X6-X7)-heterologous protein not only increased the fermentation yield but protected the interest protein against proteolytic cleavage during fermentation [9].

Another point valuable to consider is that the specific gene copy number of the heterologous protein might affect the outcome of heterologous protein expression in *P. pastoris.* In most cases, increasing gene dosage may greatly enhance recombinant protein expression of in *P. pastoris* although this improvement can reach a plateau, even sometimes, high copy number can be detrimental for productivity [10–12]. Besides, a non-conventional spliced HAC1 mRNA is supposed to encode an active transcription factor binding to UPR-responsive elements in the promoter of unfolded protein response (UPR) target genes. It has been known the expression of HAC1 will be unregulated when the UPR is activated in yeast, which helps to eliminate bottlenecks during secretion [13].

In this study, we used a series of combined genetic modification strategies to enhance ARL production in *P. pastoris*, which involved the introduction of an extension spacer in front of the lipase gene's

* Corresponding author. Tel.: +86 20 3938 0618; fax: +86 20 3938 0618.
E-mail address: syhan@scut.edu.cn (S. Han).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.06.021

Table 1
Primers used for plasmid construction and quantitative PCR analysis.

Name	Sequence (5′–3′)	Annotation
P_{ARL1}	CGGAATTCTGTAATGACGACCACGACGA	*Eco*RI site (underlined)
P_{ARL2}	ATTAAATAGCGGCCGCCTGAATTGGCATAAGACT	*Not*I site (underlined)
P_{eARL1}	TGAATTCGAAGAAGCTGAAGCTGAAGCTGAACCAAAGTGTAATGACGACCACGACGAC	*Eco*RI site (underlined)
P_{eARL1}	AAGCTGGCGGCCGCCTGAATTGGCATAAGACTCTG	*Not*I site (underlined)
P_{G1}	GTATGCGGCCGCTGTGAGGCTGAAATGTG	*Not*I site (underlined)
P_{G2}	CTTCTAGACACATGCCAACTCAATCC	*Xba*I site (underlines)
P_{rtA1}	GAGAAATACCGCTCCTAC	qPCR for ARL
P_{rtA2}	GCAACATACTTCCACCAA	qPCR for ARL
P_{rtG1}	GTCGGGACACGCCTGAAACT	qPCR for G
P_{rtG2}	CCACCTTTTGGACCCTATTGAC	qPCR for G

N-terminus, the augmentation of the gene dose of AOX1-driven ARL by head-to-tail cassette multimerization, and overexpression of the spliced HAC1 gene.

2. Materials and methods

2.1. Strains and medium

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the host for DNA manipulation. The recombinants were screened at 37 °C using low Luria-Bertani medium plates (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, and 2% agar), containing 25 μg/mL Zeocin (Invitrogen, Carlsbad, CA, USA) or kanamycin (Invitrogen, Carlsbad, CA, USA), as necessary. *P. pastoris* GS115 (Invitrogen, USA) was used as the host. The yeast grows in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or BMGY/BMMY medium (pH 6.0) (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base [YNB], 4×10^{-5}% biotin, and 1% glycerol or 0.5% methanol). The yeast recombinants were screened using minimal dextrose (MD) medium plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, and 2% agar) or YPDS (YPD containing 100 μg/mL zeocin), followed by inoculated onto tributyrin emulsion plates (1% tributyrin, 1% yeast extract, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 2% agar). Race metal solution [PTM1] ($CuSO_4 \cdot 5H_2O$ 0.6%, KI 0.009%, $MnSO_4 \cdot H_2O$ 0.3%, H_3BO_3 0.002%, $MoNa_2O_4 \cdot 2H_2O$ 0.024%, $CoCl_2$ 0.05%, $ZnCl_2$ 2%, $FeSO_4 \cdot H_2O$ 6.5%, biotin 0.02%, and H_2SO_4 0.5%) and basal salt medium [BSM] (glycerol 4%, K_2SO_4 1.82%, $MgSO_4 \cdot 7H_2O$ 1.49%, KOH 0.413%, H_3PO_4 2.67%, and $CaSO_4$ 0.093%, PTM1 0.435%) were used in fed-batch cultivation. A 50% (w/v) glycerol solution containing 1.2% (v/v) PTM1, and a 100% methanol solution containing 1.2% (v/v) PTM1 are used as feed solutions according to the protocol of '*Pichia* fermentation process guideline' (Invitrogen, San Diego, CA, USA).

The vector pPICZaA was purchased from Invitrogen Co. We substituted HIS4 and kanamycin genes for a zeocin expression cassette in the vector. The resultant was named as pPICHKA and used as a cloning and expression vector. The antibodies used for the Western blot analysis were obtained from Abgent (mouse anti-6 × His monoclonal antibody; San Diego, CA, USA). The secondary antibody was purchased from Invitrogen (Alexa Fluor-488-conjugated goat anti-mouse IgG; San Diego, CA, USA). Restriction endonuclease, PrimeSTAR® HS DNA polymerase, and T4 DNA ligase were bought from TaKaRa Biotechnology Co. (Dalian, China). The DNA molecular weight marker was obtained from Fermentas (Burlington, Canada). A QIAquick Gel Purification Kit was purchased from Qiagen (Qiagen Co., Hilden, Germany). Other chemicals were of analytical grade and commercially available.

2.2. Vector construction and yeast transformation

To express the mature *A. radioresistens* lipase (ARL) gene in *P. pastoris* at a high level, the mature ARL gene was optimized using OptimumGene® technology and the optimized gene encoding the mature *A. radioresistens* lipase lacking a stop codon was synthesized and cloned into pUC57 by GenScript Biotech Co. (Nanjing, China). The ARL gene was amplified by PCR using the primer pair P_{ARL1} and P_{ARL2} (Table 1, the *Eco*RI and *Not*I sites are underlined). The gene encoding mature ARL with a short extending peptide (EEAEAEAEPK) was amplified by PCR using the primer pair P_{eARL1} and P_{eARL2} (Table 1, the *Eco*RI and *Not*I sites are underlined). PCR was carried out using PrimeSTAR® HS DNA polymerase in the following program: pre-denaturation at 94 °C for 2 min, 30 cycles at 98 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 7 min. The purified amplicon and the pPICHKA plasmid were digested by *Eco*RI and *Not*I before ligation using T4 ligase and then transformed into *E. coli* TOP10. The constructed recombinant plasmids were confirmed by sequencing (Majorbio, China) and designated as pPICHKA/ARL and pPICHKA/epARL.

Digestion of pPICHKA/epARL with *Bgl*II and *Bam*HI releases the expression cassette (PAOX1 plus ARL gene). After digestion of the *Bgl*II-*Bam*HI expression cassette and linearization of pPICHKA/epARL using the same *Bam*HI, the insert and the plasmid were ligated using T4 ligase to obtain the two-copy plasmid pPICHKA/$(epARL)_2$. This process was repeated until the desired multimer was constructed. The Kpn21-linearized pPICHKA/ARL, pPICHKA/epARL and pPICHKA/$(epARL)_n$ were transformed into *P. pastoris* GS115 using the lithium chloride (LiCl) method, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The spliced HAC1 gene from *P. pastoris* was cloned into pPICZA to construct pPICZA-HAC1, then transformed into a multi-copy recombinant *P. pastoris* that harbored >6 copies of the ARL gene. The restriction enzyme digestion and DNA sequencing assured that all of the plasmids matched their designs (data not shown).

P. pastoris transformants were screened at 28 °C on MD plates and selected using tributyrin emulsion plates (GS115/pPICHKA as control). The integration of the recombinant plasmid into the GS115 genome was verified by colony PCR using 5′AOX1 and 3′AOX1 as the sequencing primers (date not shown).

2.3. Determination of the ARL copy number by quantitative PCR

The quantitative PCR (qPCR) assay protocol was based on the Pfaffl method [14]. A standard plasmid pPICHKA-AG containing the ARL gene and a 600-bp G fragment was constructed. The single 600-bp non-coding region G (part of GAPDH, the interval partial sequence between *P. pastoris* GS115 genes 8198905 and 8198906) was used as the standard DNA template to calculate the amplification efficiency of ARL (E_{ARL}) and G (E_G). pPICHKA-AG was transformed into the *E. coli* TOP10F′ strain for propagation. To prepare the standard plasmid working solution for qPCR assay, pPICHKA-AG was extracted from a positive recombinant and diluted to produce a 100 pg/μL solution using ultrapure water. The qPCR assay was performed using a gradient dilution pPICHKA-AG working solution series (1×100^{-1} to 10^{-6}) as templates and the primers P_{rtA1}/P_{rtA2} and P_{rtG1}/P_{rtG2}. For each gradient series sample, the crossing points of the amplification curve and the threshold line (CT) versus the pPICHKA-AG concentration input were plotted to calculate the slope. The amplification efficiencies of ARL and the G fragment (E_{ARL}, E_G) were calculated during the exponential phase according to the following equation: $E = 10^{(-1/\text{slope})}$. Genomic DNA was prepared from the yeast recombinants using a yeast DNA extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and adjusted to produce an approximately 1000 pg/μL solution for the qPCR assay. The yeast recombinant DNA and the standard plasmid were analyzed simultaneously using an Applied Biosystems®7500 fast real-time PCR instrument (Applied Biosystems Inc., Foster City, CA, USA). The quantitative PCR (qPCR) assay was repeated 3 times for one sample and the mean CT values of ARL and G from the standard plasmid (CT-ARL control, CT-G control) and the samples (CT-ARL sample, CT-G sample) were entered into Eq. (1) to calculate the ARL copy number (ratio).

$$\text{ratio} = \frac{(E_{CALB})^{\Delta C_{T\text{-}CALB}(\text{control-sample})}}{(E_G)^{\Delta C_{T\text{-}G}(\text{control-sample})}} \quad (1)$$

2.4. Shake flask cultures

Single colonies with bigger transparent zone on tributyrin emulsion plates were transferred into a 250 mL flask containing 25 mL of BMGY medium and incubated at 28 °C for 24 h. The cells were collected by centrifugation at 6000 rpm for 10 min and transferred into 25 mL BMMY medium to produce a final OD_{600} = 1.0. 1% (v/v) methanol were added into the culture every 24 h to induce lipase expression. Aliquots of the cultures (250 μL) were harvested every 24 h. The biomass concentration and an enzyme assay were determined immediately. Cell concentration was indirectly measured via absorbance at 600 nm. The lipase activity was measured spectrophotometrically using an assay based on the hydrolysis of 0.05 M p-nitrophenyl caprylate (pNPC), with the GS115/pPICHKA supernatant as the negative control. The enzyme reaction mixture was incubated at 55 °C at pH 9.0 for 5 min. The p-nitrophenol (p-NP) produced in the reaction mixture was quantified at 405 nm [15]. One unit of activity was defined as the amount of enzyme that released 1 μmol of p-NP per minute under the assay conditions. When the clone with the highest lipase activity was determined, the time course of its fermentation and enzyme activity was determined by triplicate shake flask culture at 28 °C and 250 rpm for 144 h.

2.5. Fermentation scale-up

The fed-batch cultivations were performed in a 10-L standard mechanical agitated fermenter with an initial medium volume of 5 L (FUS10-A, Shanghai Guoqiang