Increase of *Candida antarctica* lipase B production under PGK promoter in *Pichia pastoris*: effect of multicopies

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Abstract

The effect of gene dosage on the production of *Candida antarctica* lipase B (CalB) in the methylotrophic yeast *Komagataella phaffii*, at high densities in a simple medium containing crude glycerin as the sole carbon source, is described. The use of crude glycerin, the main by-product of biodiesel production from vegetable oils, will reduce the production cost of the bioprocess. Two *K. phaffii* strains were constructed with one or three copies of LipB, an optimized version of the gene encoding CalB under the control of the constitutive P_{PGK1} promoter. These two constructs were tested and compared on batches using minimal-salts medium with crude glycerin. The strain with three copies achieved a higher enzyme yield (48,760 U/L, 2.3-fold higher than the one-copy strain), with 42 g/L biomass, with no effects on growth.

Keywords *Candida antarctica*, lipase B · *Komagataella phaffii* · Constitutive expression · Multicopy vector integration · Gene dosage

Introduction

The yeast *Candida antarctica* is capable of producing two lipases, lipase A (CalA) and lipase B (CalB). The latter is one of the lipases most used for biocatalysis thanks to its high enantioselectivity with a broad range of substrates and good thermal stability [1–3]. From an industrial angle, CalB tested has high efficiency in transesterification [4] and also catalyze alcoholysis to produce biodiesel [5, 6]. CalB is

produced commercially by Novozymes as a recombinant enzyme from *Aspergillus niger*, which is widely used in organic synthesis for resolution of racemic mixtures of alcohols, amines, and acids or in the preparation of optically active compounds [2, 7]. The 33 kDa CalB has the following characteristics: isoelectric point of 6.0, 15% retained activity after 20 min of incubation at 70 °C pH 7, stable in basic pH [7–10] and regiospecificity of hydrolysis of triacylglycerol Sn-3 [7].

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The methylotrophic yeast *Komagataella phaffii* (formerly known as *Pichia pastoris*) has several advantages over other eukaryotic or prokaryotic systems as an expression platform for CalB production [1]: high productivity in simple medium containing glycerol as the sole carbon source [2]; growth at high cell densities [3]; well-established fermentation protocols [4]; GRAS status [5]; ability to perform various post-translational modifications, including glycosylation, methylation, acylation, and proteolysis; and [6] extracellular soluble-protein purification [8, 9].

Most vectors developed for *K. phaffii* are based on the methanol-induced P_{AOXI} promoter [10]. However, the use of methanol may be undesirable in large-scale fermentation processes because of its flammability and toxicity. Variants of P_{AOXI} are being developed, in order to regulate the production without the need for methanol, but the production rates are not as well studied as the classic P_{AOXI} [11]. Constitutive expression systems represent an attractive alternative, with the advantage that different carbon sources can be employed, including crude glycerol, a by-product of the biodiesel industry [12]. The most commonly used promoter is P_{GAP} , which is strongly regulated and capable of hyperexpression [13]. Other promoters of the glycolytic pathway can be explored, such as a reduced-version P_{PGKI} used in this study, which has potential for use in the development of new engineered pathways [14].

In order to improve the protein production of *K. phaffii*, several studies have focused on increasing the copy number of the desired gene, using different approaches such as in vitro multimerization, PVTA (post-transformational vector amplification), integration targeted to the rDNA locus, and the use of defective markers [15]. The drug-resistance genes *Sh ble* and *kan* (zeocin and G418 resistance, respectively) are typically used to select yeast multicopy clones. Although the use of these markers is laborious and requires expensive antibiotics, they can be used in wild-type yeast strains and provide the opportunity to obtain a multicopy strain. In most cases, a higher gene dosage may greatly enhance the expression of recombinant protein. However, an excessively high gene dosage will lead to a plateau in expression, and may even be detrimental [16, 17]. A high level of overexpression of heterologous proteins in *K. phaffii* saturates or overloads the secretory pathway, or even triggers the protein to unfold [18].

Defective markers are typically auxotrophic genes, which are transcriptionally impaired. In this case, prototrophy is normally obtained when several copies of the transforming vector are present to compensate for the weak transcription from the defective marker [19]. This approach has been used successfully to obtain multicopy transformants in many types of yeast, such as *Saccharomyces cerevisiae* [20–22], *Yarrowia lipolytica* [23, 24], and *Hansenula polymorpha* [25]. Recently, Betancur et al. [26] developed a transformation system for *K. phaffii* based on the leucine-defective marker *leu2-*

d, which proved to be an easy and efficient way to obtain multicopy integration of the desired gene after a single transformation event.

In this study, we sought the development of *K. phaffii* strains that express a synthetic gene coding for CalB under the control of a constitutive promoter, P_{PGKI} , and evaluated the effect of gene dosage in order to improve lipase production. In addition, we evaluated several fermentation batches based on crude glycerol as the sole carbon source, in order to compare the productivity of each strain in a controlled environment.

Material and methods

Material

The crude glycerol used in this study was derived from biodiesel synthesis from soybeans and was kindly provided by Petrobras (Rio de Janeiro, Brazil). The main features of this crude glycerol were: density 1.25 g/L, NaCl content about 7 g/L, and less than 1000 ppm methanol. The purity of the glycerol was 84%.

Strains and media

Chemically competent *Escherichia coli* Stellar™ (Clontech, USA) and XL10-Gold® (Stratagene, USA) were used for the DNA cloning procedures. Bacterial cells were cultured in LB modified medium (in w/v, 1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.2), with 100 µg/mL ampicillin or 50 µg/mL kanamycin added when necessary, at 37 °C and 200 rpm. Low-salt LB (0.5% w/v NaCl) was used for selection with zeocin (25 µg/mL). Solid medium was prepared by adding 1.5% agar.

K. phaffii X-33 (Invitrogen) and M12 (*leu2*) [26] were used for lipase expression. *K. phaffii* was routinely cultured in YPD medium (in w/v, 1% yeast extract, 2% peptone, 2% glucose) at 28 °C and 200 rpm. For transformation, *K. phaffii* X-33 cells were plated on YPDS (YPD with 1 M sorbitol) with 100 µg/mL zeocin added, while M12 cells were selected on MD medium (1.34% yeast nitrogen base without amino acids—YNB, 2% glucose, $4 \times 10^{-5}\%$ biotin). Lipase activity was detected on MDT (MD plus 100 mM phosphate buffer and 1% tributyrin) or YPDT (YPD plus 1% tributyrin) medium plates. For solid medium, 2% agar was added.

Plasmid construction

A 954-bp version of the *C. antarctica* CalB gene corresponding to the mature protein was codon-optimized for expression in *K. phaffii* and synthesized de novo by Epoch Biosciences (USA). The synthetic gene, LipB, contained in its 5'-end a

XhoI site followed by 18 nt required to reestablish the Kex2 and Ste13 proteolytic sites present in the *S. cerevisiae* α -factor signal sequence; while the 3'-end had a NotI site after the stop codon. LipB was cloned into pBluescript II SK, resulting in pBSK_LIPB. LipB was removed after double digestion of this vector with XhoI/NotI following subcloning in pPGK Δ 3AMY [14] digested with the same enzymes, resulting in pPGK Δ 3LIPB. To further improve CalB production, the original α -factor secretion sequences were replaced with codon-optimized sequences, resulting in pPGK Δ 3PRO_LIPB. This vector has the *Sh ble* dominant marker, and LipB is under the control of the constitutive *pPGK1* promoter.

For multicopy integration of LipB mediated by a defective marker, the pK-ld vector [26] was used. This vector contains *pPGK1* and the *leu2-d* selection marker. A fragment corresponding to LipB and the optimized α -factor sequences was amplified by PCR from pPGK Δ 3PRO_LIPB with the primers EGFif-F (GATTACGAAAGGATCACGATGAGATTCCCATCTATCTTCACTG) and Liprec-R (ATGGTCCGACGCGGCCGCTTATGGAGTAACGATACCAGAG) using CloneAmp HiFi PCR Premix (Clontech). The ~1.2 kb amplicon was cloned into pK-ld previously double-digested with BamHI/NotI by in vitro recombination (In-Fusion® HD Cloning Kit, Clontech). The resulting vectors pPGK Δ 3PRO_LIPB and pKLip-ld were linearized with SacI and PvuI, respectively, for direct integration into the *K. phaffii* *PGK1* locus. Schemes of the constructed plasmids are shown as supplemental figures.

Yeast transformation

K. phaffii cells were transformed by electroporation following the protocol described in the *Pichia* Expression Kit (Invitrogen). Transformed cells were plated on YPDS containing zeocin (pPGK Δ 3PRO_LIPB) or MD (pKLip-ld). Transformants were screened for lipase activity on YPDT (pPGK Δ 3PRO_LIPB) or MDT (pKLip-ld) plates.

DNA extraction

Genomic DNA (gDNA) was isolated using a DNA purification kit (Wizard® Genomic DNA Purification Kit, Promega). DNA concentration and quality were verified with Nanodrop™ (Thermo Scientific™).

Primer design

For DNA amplification, a set of primers for the gene of interest (LipB) was used, and the reference β -actin gene (*ACT1*) was used as an endogenous control to normalize the data. Primers for ddPCR were designed according to the Bio-Rad guidelines. Primer sequences are listed in Table 1.

ddPCR methodology

To ensure accurate quantification and avoid a negative impact on the droplet volume, genomic DNA was digested with EcoRI (Thermo Scientific). As a result, different gDNA fragments were obtained, containing individual expression cassettes. EcoRI was selected on the following basis: (i) the LipB expression cassette was not cut by this enzyme and (ii) LipB was cloned into a standard plasmid backbone such as pPICZ α , i.e., containing a single restriction site for EcoRI in its multicloning site. ddPCR reactions contained 10 μ L of QX200™ ddPCR™ EvaGreen® Supermix, 200 nM of forward primer, 100 nM of reverse primer, 0.4 ng of digested gDNA, and the required amount of DNase/RNase-free water up to 20 μ L of final volume. Reactions were incubated at 95 °C for 10 min, followed by a denaturation (94 °C, 30 s), and an annealing/extension step (58 °C, 1 min for LipB primers; 56.5 °C, 1 min for ACT1 primers) during 40 cycles. Droplets were detected by using the QX100™ Droplet Digital™ PCR System and the software QuantaSoft™ v. 1.7.4.0917. All the reagents used in the ddPCR were purchased from Bio-Rad. This methodology was adapted based on [16].

Storage and inoculum

Transformed *K. phaffii* cells were stored in 20% glycerol and maintained at –80 °C for storage. A Petri dish with YPD (in w/v, 1% yeast extract, 2% peptone, and 2% glucose) and 2% w/v agar-agar was maintained in a refrigerator to be used as the inoculum. A single colony from the Petri dish was propagated in shaker flasks at 200 rpm and 30 °C in YPD medium.

Culture medium

The culture medium used for fermentation in bioreactors was adapted from [27] containing (per liter): 2 g citric acid, 12.4 g (NH₄)₂HPO₄, 0.022 g CaCl₂·2H₂O, 0.9 g KCl, 0.5 g MgSO₄·7H₂O, 100 g crude glycerol, and 4.6 mL of PTM₁ trace salts. The pH was maintained at 7.0 using 5% H₂SO₄ and 20% NH₄OH.

The composition of PTM₁ trace salts used contains (per liter): 2 g CuSO₄·5H₂O, 0.08 g KI, 3 g MnSO₄·H₂O, 0.2 g MnSO₄·H₂O, 0.02 g H₃BO₃, 0.5 g CoCl₂·6H₂O, 6.7 g ZnCl₂, 21.7 g FeSO₄·7H₂O, 0.2 g biotin, and 1.7 mL H₂SO₄.

Fermentation process

The bioreactor used was a bench-top Multifors from Infors with three vessels of 500 mL working volume. The bioreactor was maintained at 30 °C. Aeration was controlled in order to try to maintain the dissolved-oxygen saturation at 30%. This control was effected through a cascade with stirring between

Table 1 Construction design of primers for LipB and *ACT1*

Oligonucleotide	Sequence	Tm	%GC	Amplicon size (nt)
<i>LipB</i> Fw	5'-TGGCTTTCGCTCCAGACTAC-3'	63	55	137
<i>LipB</i> Rev	5'-AGTCAAACCACCAGCGTTTC-3'	63	50	
<i>ACT1</i> Fw	5'-CACCACACCTTCTACAAC-3'	56	50	139
<i>ACT1</i> Rev	5'-AGAAGGCTGGAACGTTG-3'	58	52	

250 and 700 rpm and gas flow between 0 and 1 vvm. The inoculum was produced as mentioned in the “[Storage and inoculum](#)” section and used to inoculate the bioreactor at the desired concentration, typically between 0.7 and 1 g/L. The fermentations were performed in duplicate.

Titrimetric activity

Titrimetric activity was performed with 56 mM tributyrin for hydrolysis at 40 °C and pH 7.0, based on the method described by [28] using a pHstat and 0.06 M NaOH as titrating reagent. One unit of lipase activity (U) is defined as the quantity of enzyme needed to catalyze the production of 1 μmol butyric acid (volumetric analysis) per minute under the assay conditions. This analysis was conducted at the peak of production activity, to obtain the parameters associated with the fermentation. The results were determined as the mean of triplicates.

Biomass measurement

Cell growth was quantified based on a standard curve of dry weight, related to the measurement of optical density at 600 nm in a spectrophotometer (Hach Lange DR 3900™): DCW (g/L) = 0.4382 abs.

Glycerol concentration

Glycerol was determined using HPLC (HPLC Agilent Technologies) equipped with an HPX-87H column (Bio-Rad 300 mm × 7.8 mm). The temperature was maintained at 65 °C, using as the mobile phase a 0.005 M sulfuric acid solution at a flow rate of 0.6 mL/min. The sample volume injected was 20 μL.

N-NH₄⁺ concentration

This was measured using the method adapted from [29, 30]. Ammonium sulfate was used as a standard. The nitrogen concentration was determined as the mean of triplicates.

Results

Copy number determination

In order to produce CalB in *K. phaffii*, a synthetic gene (*LipB*) was designed with codon optimization for this yeast. *LipB* was cloned under the transcriptional control of the *K. phaffii* constitutive promoter *P_{PGK1}*, resulting in two vectors: pPGKΔ3PRO_LIPB and pKLip-ld. *K. phaffii* was transformed with linearized pPGKΔ3PRO_LIPB and pKLip-ld in order to target integration to the *PGK1* locus. Over 85% of transformants from both systems showed lipolytic activity on plates containing tributyrin (data not shown). A clone showing the largest halo from each system was selected for further study. Clones derived from pPGKΔ3PRO_LIPB and pKLip-ld were denominated 04-MR and 34-MR, respectively.

DNA quality and concentration

Because of the low quality and quantity of DNA samples, genomic extractions were repeated. The results of the Nanodrop quantification are shown in Table 2.

Gene dosage quantification

For each sample, five replicates were performed. The number of copies of *LipB* was determined by calculating the ratio between positive droplets of *LipB* and *ACT1* amplification (Fig. 1).

These results established that strain 04-MR has one gene copy and strain 34-MR has three copies.

Table 2 Results obtained from Nanodrop quantification for both strains tested

Strain	Concentration (ng/μg)	260/280 ratio	260/230 ratio
04-MR	1018	2.09	1.56
34-MR	523	1.95	1.2

Fig. 1 In blue, positive droplets (PCR amplification detected). Pink line indicates threshold between positive and negative droplets. **a** Amplification with CalB primers. **b** Amplification with Act1 primers

Comparison of CalB production between *K. phaffii* strains

In a previous study, the best conditions for Clone 04-MR in terms of production were evaluated, and the use of 100 g/L of crude glycerin as initial in a batch culture was the best condition obtained [3]. In order to conduct a comparative analysis of the new strain, the same conditions were used in this study. Figure 2 shows the culture progress of CalB production using Clone 04-MR.

The fermentation attained a biomass/substrate yield ($Y_{x/s}$) of 0.47 gX/gS. The specific growth rate was 0.10 h^{-1} and the volumetric productivity was $453 \text{ U/L}\cdot\text{h}$, with the crude glycerin being completely consumed after 46 h of growth. No nitrogen limitation occurred; as can be seen, the NH_4OH added to maintain the pH assured a nearly constant nitrogen concentration in the culture.

Fig. 2 Kinetics of cell growth, enzymatic activity, and consumption of glycerol and nitrogen with Clone 04-MR on minimal medium with 100 g/L crude glycerin, pH 7, with a cascade control to maintain oxygen saturation at 30%

Figure 3 shows the fermentation process using Clone 34-MR. Biomass/substrate yield ($Y_{x/s}$) was 0.44 gX/gS , the specific growth rate was 0.11 h^{-1} and the volumetric productivity and activity were 2.3-fold higher than in the previous case. Figure 4 shows the comparison of the main parameters obtained with the two strains.

Discussion

Two integrative vectors for lipase production were constructed in this work, pPGK Δ 3PRO_LIPB and pKLip-Id. The main difference between these vectors is that the latter allows the direct isolation of transformants with multiple copies of the expression cassettes [26]. This vector contains the defective auxotrophic marker *leu2-d*, which is an allele bearing only 29 bp of the 5' flanking region of the *S. cerevisiae* LEU2 gene

Fig. 3 Kinetics of cell growth, enzymatic activity, and consumption of glycerol and nitrogen of Clone 34-MR on minimal medium with 100 g/L crude glycerin, pH 7, with a cascade control to maintain oxygen saturation at 30%

[31]. In *S. cerevisiae*, this marker is typically used to maintain multiple copies of episomal plasmids [31] but since this class of vectors is not common in *K. phaffii*, prototrophy is necessarily achieved after multiple plasmid integration events in the host's chromosome as previously shown [21].

Over 85% of transformants from both systems showed lipolytic activity on plates containing tributyrin (data not shown), thus showing that CalB was efficiently secreted by the *S. cerevisiae* α -factor signal sequence.

The recommended 260/230 ratio (related to some of the extraction contaminants, e.g., phenol and guanidine) to the ddPCR methodology is around 2 [32, 33], but the samples were of sufficient quality to continue with the procedure, although it achieved only 1.6 and 1.2, respectively.

For both cultivations, the growths were coupled to the production of CalB as expected, since a constitutive promoter was used. In general, both clones had the same behavior of production. Nevertheless, the biomass/substrate yield ($Y_{x/s}$) for strain 34-MR was slightly smaller (0.44 gX/gS); this is explainable since the strain used for the modification is

auxotrophic. Volumetric productivity and activity were 2.3-fold higher in this case, which shows that this multicopy construction generates higher lipase production, as has previously been reported for other multicopy integration processes. The production of tumor necrosis factor (TNF) increased 200-fold with more than 20 integrated copies [34]; hepatitis B surface antigen showed a linear correlation between copy number, mRNA and protein [35]; and proinsulin secretion increased 13-fold with 11 copies of the expression cassette [36]. Marx et al. (2009) [37] found a linear correlation between copy number and hSOD production. Specifically, for *K. phaffii*, some studies with lipase expression found an increase in protein production with multicopy integration [38–41].

For all parameters, the 34-MR strain showed better performance, around 2.4-fold. This behavior was expected because of the larger number of copies integrated; the increase was nearly linearly related to the number of integrations, as there are 3 times more copies of the lipase B gene from *C. antarctica*. It is also possible that this number of copies is close to saturation, due to limits on secretion or transcription,

Fig. 4 Comparison of biomass/product yield, enzymatic activity, and volumetric and product productivity for both strains on minimal medium with 100 g/L crude glycerin, pH 7, with a cascade control to maintain oxygen saturation at 30%

and the integration of more copies would reduce the correlation until the productivity becomes stable, independently of the number of copies, as for the HSA (human serum albumin) productivity reported by Marx et al. [37]. Notably, the maximum specific growth rate achieved was half the rate normally obtained with glycerol (0.2 h^{-1}), with both copy numbers, also a reflection of the auxotrophic characteristic.

The effect of gene dosage on the production of different microbial recombinant lipases in *K. phaffii* has also been reported. Under P_{AOXI} , the maximum gene dosage was around five copies for *C. rugosa* Lip1 [18] and *Rhizopus chinensis* CCTCC M201021 lipase [39]. For the constitutive promoter P_{GAP} the gene dosage was lower, three copies; for *C. rugosa* Lip2 [42], the same optimal copy number found in this study.

Although this strain produced much less than the highest cell density (which would be about 120 g/L as previously achieved in *K. phaffii* culturing [43]), because of the decrease in $Y_{x/s}$, the fed-batch strategy can circumvent this problem and further increase the parameters obtained. Attempts to find a strong constitutive promoter and construction to match or even exceed the activities obtained with the P_{AOXI} promoter are important, in order to eliminate the use of methanol. This system produces the highest standards reported in the literature, as obtained by Vadhana et al. [44]: 300 U mL^{-1} at 6 days of induction. In some cases, such as the hepatitis B surface antigen, constitutive expression is better than induction because no induction is necessary and the specific growth rate is generally higher using glucose or glycerol than using methanol, which leads to faster processes [45]. Obviously, one must be careful when comparing activity numbers, because different substrates and methods used generate divergent results. Vadhana and colleagues used the same method to measure the activity as in this study, and they achieved a production, per hour, very near that obtained with methanol induction ($1.87 \text{ U/mL}\cdot\text{h}$ with methanol induction, while the present study achieved $1.5 \text{ U/mL}\cdot\text{h}$). These results demonstrate the excellent potential of *K. phaffii* for CalB production, and different culture strategies should be studied in order to explore its full potential. The enzyme produced on this work was already used to produce myo-inositol derivatives that are intermediates from pharmacological pathways involved in the synthesis of building blocks for drugs that inhibit the etiological agent of Chagas disease, *Trypanosoma cruzi* [46].

Conclusion

Evaluating the newly constructed *K. phaffii* strain with a multicopy gene of *C. antarctica* lipase B, the increase in the number of gene copies directly impacted the lipase productivity. Clone 34-MR, with three copies, showed around 2.4-fold better performance in terms of volumetric and product productivity, although the final biomass decreased by 10%.

The optimal gene dosage to maximize CalB production under control of the P_{PGKI} promoter was three copies, similar to the number obtained with the well-known constitutive promoter P_{GAP} .

Therefore, the novel constitutive promoter P_{PGKI} showed high potential for production of this lipase when coupled with multicopy integration, close to the productivities achieved when using methanol induction, but without the downsides of toxicity and hazards involved in using methanol. The process described here can easily be scaled up (pilot and industrial), as it is simple and low in cost.

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