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# Functional expression of lipase A from *Candida antarctica* in *Escherichia coli*—A prerequisite for high-throughput screening and directed evolution

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**Abstract**

We report for the first time the functional and heterologous expression of lipase A from *Candida antarctica* (CalA) in the cytoplasm of *Escherichia coli* Origami™ B cells. Expression under control of the *lac* promoter in the pUC18 vector yielded 0.7 U $mg^{-1}$ lipase activity, whereas expression of a thioredoxin–CalA fusion protein using the pET-32b(+) vector yielded 1.7 U $mg^{-1}$. The native enzyme was most efficiently expressed under control of the *cspA* promoter (9.63 U $mg^{-1}$) using the pColdIII vector. Co-expression of various chaperones led to a significant increase in formation of active protein (up to 13.1 U $mg^{-1}$). This expression strategy was validated in microtitre plates and therefore is suitable for high-throughput screening of large gene libraries and applications including directed evolution of CalA.
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*Keywords:* Lipase; *Candida antarctica*; Functional expression; *Escherichia coli*; Chaperone co-expression

## 1. Introduction

Lipases [triacylglycerol-hydrolases (EC 3.1.1.3)] play an important role in several industrial applications. They catalyse the hydrolysis of triacylglycerols at the interface between water and the hydrophobic substrate. Besides the hydrolysis of triacylglycerols, lipases also catalyse the enantio- and regioselective hydrolysis or synthesis of a wide range of natural and unnatural esters [1–3]. Especially lipases from microorganisms received a lot of interest because they are useful catalysts for many industrial applications such as ester synthesis [4,5], optical resolution [6,7], transesterification [8] or washing processes [9].

From *Candida antarctica* two lipases were isolated: lipases A and B [10]. Lipase B shows high enantioselectivity against secondary alcohols and due to its high stability in organic solvents and at high temperature it is one of the most frequently used enzymes for industrial applications nowadays [11].

Lipase A is reported to be a thermostable lipase [12] and thus could be interesting for industrial use. Moreover, CalA is described to be the only lipase displaying a preference for position *sn*-2 of triglycerides [13] opening a wide range of possible applications in synthesis of structured triglycerides. Recently CalA was expressed in high yields in the methylotrophic yeast *Pichia pastoris*, purified and generally characterised [14].

Furthermore, the enzyme was found to exhibit high activity towards sterically hindered alcohols, including both secondary and tertiary alcohols [15] and hydrolyses a range of *tertiary*-butyl esters of protected amino acids [16]. These results indicate that CalA could be applied for the conversion of highly branched and bulky substrates where most other lipases fail to display any activity. In the patent literature lipase A was claimed to be able to esterify tertiary alcohols. Unfortunately the protein structure of CalA is not solved yet and thus rational protein design is impossible. Consequently the only way to tailor the enzyme is "directed evolution" [17] which requires functional expression in a fast-growing and easy-to-handle organism.

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Table 1
Primers used for the amplification of the calA gene

| Primer | Sequence (5′–3′) | Function |
|---|---|---|
| CalA_EcoRI-fw | ccggaattcggcggcgctgcccaacccc | Cloning into pUC18 and pET-32b(+) |
| CalA-NotI-r | ttttccttttgcggccgcctaaggtggtgtgatggggc | Cloning into pET-32b(+) |
| CalA_Nde1-r | gggaattccatatgctaaggtggtgtgatggg | Cloning into pUC18 |
| CalA_pColdIII_Nde1-fw | gggaattccatatggcggcgctgcccaacccc | Cloning into pColdIII |
| CalA_pColdIII_EcoRI-r | ccggaattcctaaggtggtgtgatggg | Cloning into pColdIII |

Up to now CalA has not been functionally expressed in *Escherichia coli*, the standard expression system for high-throughput screening of large mutant libraries.

Expression of eukaryotic proteins in *E. coli* is challenging as the cellular environment, folding machinery and conformational quality-control checkpoints of prokaryotes are different from those of eukaryotes [18]. Generally expression problems can occur during transcription of the encoding gene, translation of the mRNA, protein folding and posttranslational modification, e.g. formation of disulfide bonds. Misfolding often leads to proteins displaying hydrophobic patches on the surface, thus favoring aggregation and insolubility of proteins. There are several methods to improve the solubility of proteins expressed in *E. coli*: domain optimisation [19], optimisation of translation initiation regions [20], introduction of solubilising amino acids [21] or co-expression of chaperones as reported in this work.

A recently published review on CalA [22] documented the increasing interest this unique biocatalyst attracts and the multitude of applications the enzyme can be used for. Our work represents the basis for optimisation of CalA by directed evolution aiming to improve regioselectivity and to enhance the substrate spectra or other properties of the enzyme. In this context we summarised the use of different expression strategies and the impact of co-expressed chaperones.

## 2. Material and methods

### 2.1. Cloning of the lipase gene

The yeast *C. antarctica* (DSM 70725) was cultivated in Universal Medium For Yeasts (DSMZ) (yeast extract 3 g $l^{-1}$, malt extract 3 g $l^{-1}$, peptone from soybeans 5 g $l^{-1}$, glucose 10 g $l^{-1}$) at 30 °C and 180 rpm. After cultivation for 3 days the cells were harvested and disrupted with glass-beads (0.75–1.00 mm) using a Retsche-mill for 30 min. Cellular proteins were precipitated with SDS (2 vol.%) at 65 °C. After centrifugation the genomic DNA was precipitated with 3 M Na-acetate and 2-propanol and finally resuspended in 100 μl TE (Tris 100 mM, EDTA 10 mM) supplemented with RNase (0.1 vol.%).

The lipase gene was amplified by PCR using DyNAzyme™ EXT DNA Polymerase (Finnzymes) and primers containing the restriction sites for *Eco*RI and *Not*I for cloning into pUC18 (MBI Fermentas, St. Leon-Rot, Germany), *Eco*RI and *Not*I for cloning into pET-32b(+) (Novagen, Darmstadt, Germany) and *Nde*I and *Eco*RI for cloning into pColdIII (Takara, Otsu, Japan). The PCR-products were cloned into the corresponding vector using standard procedures and *E. coli* Origami™ B or Origami™ 2(DE3) (Novagen, Darmstadt, Germany) cells were transformed with the construct. Tables 1–3 give an overview of the strains, plasmids and primers used.

### 2.2. CalA expression and co-expression of chaperones

#### 2.2.1. pUC18 expression

Origami™ B cells were transformed with CalA-pUC18 constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 μg $ml^{-1}$) up to an optical density $OD_{600}$ of 0.4–0.6 at 180 rpm and 37 °C. The expression of the lipase was induced by adding IPTG (final concentration 1 mM). Cells were grown for additional 24 h at 180 rpm and 30 °C and were harvested by centrifugation.

#### 2.2.2. pET-32b(+) expression

Origami™ 2(DE3) cells were transformed with CalA-pET-32b(+) constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 μg $ml^{-1}$) up to an optical density $OD_{600}$ of 0.4–0.6 at 180 rpm and 37 °C. The

Table 2
Plasmids used for the expression and co-expression

| Plasmid | Gene of interest | Promoter | Inducer | Ori | Resistance marker | Supplier |
|---|---|---|---|---|---|---|
| pUC18/calA | calA | Lac | IPTG | pBR322 | Ampicillin | MBI Fermentas |
| pColdIII/calA | calA | cspA | Cold shock + IPTG | ColE1 | Ampicillin | TaKaRa |
| pET32-b(+)/calA | Trx-calA | T7 | IPTG | pBR322 | Ampicillin | Novagen |
| pKJE7 | dnaK-dnaJ-grpE | araB | L-Arabinose | pACYC | Chloramphenicol | TaKaRa |
| pG-KJE8 | dnaK-dnaJ-grpE | araB | L-Arabinose | pACYC | Chloramphenicol | TaKaRa |
| | GroES-GroEL | Pzt1 | Tetracycline | | | |
| pGro7 | GroES-GroEL | araB | L-Arabinose | pACYC | Chloramphenicol | TaKaRa |
| pG-Tf2 | GroES-GroEL-tig | Pzt1 | Tetracycline | pACYC | Chloramphenicol | TaKaRa |
| pTf16 | tig | araB | L-Arabinose | pACYC | Chloramphenicol | TaKaRa |

Table 3
Strains used for the expression experiments

| Strains | Genotype | Reference |
|---|---|---|
| DH5α | supE44 ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 end A1 gyrA96 thi1relA1 | Clontech (Heidelberg, Germany) |
| Origami™ B | Δara-leu7697 ΔlacX74 ΔphoAPvull phoR araD139 ahpC galE galK rpsL F′[lac$^+$ (lacI$^q$)pro] gor522::Tn10 (Tc$^R$) trxB::kan | Novagen 2004 |
| Origami™ 2(DE3) | Δ(ara-leu)7697 ΔlacX74 ΔphoA Pvull phoR araD139 ahpC gale galK rpsL F′[lac$^+$ (lacI$^q$)pro] (DE3) gor522::Tn10 trxB (StrR, TetR) | Novagen 2004 |

expression at 30 and 15 °C, induction and harvest were done as described before.

*2.2.3. pColdIII expression*

Origami™ B cells were transformed with CalA-pColdIII constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 μg ml$^{-1}$) up to an optical density $OD_{600}$ of 0.4–0.6 at 180 rpm and 37 °C. The expression at 15 °C, induction and harvest were done as described before.

*2.2.4. Co-expression of chaperones using pET-32b(+) and pColdIII constructs*

CalA-pET-32b(+) and CalA-pColdIII clones were co-transformed with chaperone plasmids from the Takara Kit and selected on LB agar containing ampicillin (final concentration 100 μg ml$^{-1}$) and chloramphenicol (final concentration 34 μg ml$^{-1}$).

For expression, cells were grown in LB media containing the corresponding antibiotics and 1 mg ml$^{-1}$ L-arabinose (in case of pGro7, pKJE7 and pTf16), 5 ng ml$^{-1}$ tetracycline (in case of pG-Tf2) or L-arabinose and tetracycline (in case of pG-KJE8) in concentrations given above at 180 rpm and 37 °C up to an optical density $OD_{600}$ of 0.4–0.6. Lipase expression was induced by adding IPTG (final concentration 1 mM). Cells were grown for additional 24 h at 180 rpm and 37 °C (pUC18), 30 °C (pUC18, pET-32b(+)) or 15 °C (pET-32b(+), pColdIII). Afterwards they were harvested by centrifugation.

All expression experiments were done three- to four-fold; therefore all published data are mean values.

*2.3. Cell disruption, pH-stat assay, SDS-PAGE and densitometric analysis*

Cell disruption was performed using three times 30 s sonification in 50 mM sodium phosphate buffer pH 7.5. The cell debris (insoluble fraction) was removed by centrifugation at 20,000 × *g* for 1 h.

Lipase activity was determined using a pH-stat application with tributyrine as substrate at 50 °C (pH 7.5). Tributyrine (5% (v/v)) was emulsified in distilled water containing 5% (w/v) gum Arabic as stabiliser using a homogeniser for 7 min at maximum speed. Twenty mililiters of the substrate emulsion were heated to the reaction temperature and the pH was adjusted using 0.01 M NaOH. Liberated fatty acids were titrated automatically with 0.01 M NaOH to maintain a constant pH. One unit (U) of lipase activity is defined as the amount of lipase that liberates 1 μmol fatty acids per minute.

For protein-concentration determination, the Bradford-assay (Bio-Rad, Muenchen Germany) according to the instructors' manual was used [23].

For determination of the amount of solubly and insolubly expressed lipase, the supernatant and the pellet fraction after cell disruption were analysed using SDS-PAGE on a 12.5% separation gel under the conditions developed by Laemmli [24]. A Minigel-Twin cell (Whatman, Biometra, Goettingen, Germany) was used for electrophoresis. Molecular weight marker was purchased from Amersham Biosciences. After electrophoresis the gels were stained using Coomassie Brilliant blue.

The percentage of soluble and insoluble CalA on the total cell protein was measured densitometrically using the software "Scion Image".

*2.4. Tributyrin agar plate assay*

For this assay agar plates containing 1% emulsified tributyrin and the appropriate antibiotics were used. After cell growth for 24 h at 37 °C, the agar plates were covered with soft agar (0.6% agar in water) containing 1 mM IPTG and incubated at 30 °C for expression. Expression of functional lipase was indicated by formation of halos around the colonies.

## 3. Results and discussion

In this publication we compare the activity and solubility of lipase CalA heterologously expressed in *E. coli* using different expression strategies.

*3.1. Cloning of calA gene*

The *calA* gene dispensed from the N-terminal pre-pro-peptide sequence [14] was amplified from genomic *C. antarctica* DNA using the appropriate primers (Table 1) and afterwards cloned into *E. coli* expression vectors (Table 2). Successful cloning was confirmed by sequencing [25] of the isolated plasmids.

*3.2. Lipase expression using three different vector systems*

CalA has not been functionally expressed in *E. coli* so far. Two *E. coli* strains (Table 3) were used for expression experiments: *E. coli* Origami™ B and Origami™ 2(DE3). Common to both strains is the deficiency of their thioredoxin and glutathione reductase genes. These deficiencies are reported to enhance disulfide bond formation in the *E. coli* cytoplasm [26].

DH5α cells transformed with *pUC18-calA* did not show any halo formation on tributyrin agar plates, but Origami™ B cells transformed with the same plasmid showed halo formation. The

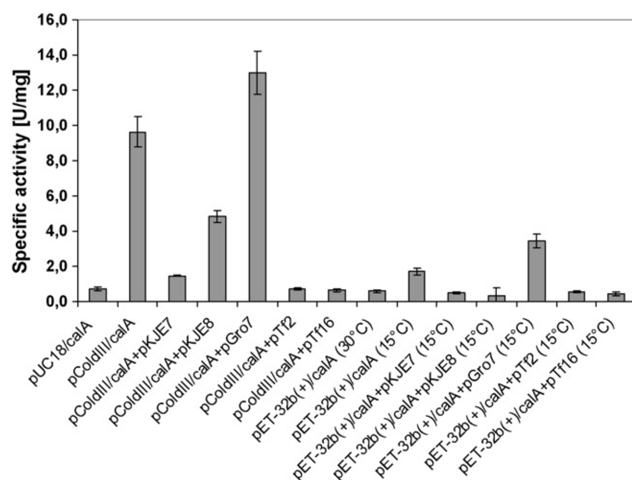


Fig. 1. Specific lipase activity yielded in the various expression experiments. The hydrolytic activity was determined using the pH-stat assay with the cleared cell lysates from *E. coli* Origami$^{TM}$ B cells (pUC18 and pColdIII constructs) and Origami$^{TM}$ 2(DE3) (pET32-b(+) constructs). The error bars show the standard deviation of three independent experiments.

CalA activity in the Origami$^{TM}$ B lysate was very weak; the hydrolysis activity against tributyrin was about 0.7 U mg$^{-1}$ of total soluble protein (Fig. 1). The fact that *pUC18-calA* showed halo formation in Origami$^{TM}$ B cells, but not in DH5α cells indicates that the formation of correct disulfide bonds is a major bottleneck in functional expression of CalA in the *E. coli* cytoplasm.

In SDS-PAGE analysis, the corresponding band of CalA (43 kDa) was not detectable in the soluble fraction, while the CalA content in the insoluble fraction was 13% of whole insoluble cell proteins (data not shown).

To enhance the yield of active enzyme, the *calA* gene was fused to a thioredoxin tag (Trx·Tag$^{TM}$) using the vector pET-32b(+) and expressed in *E. coli* Origami$^{TM}$ 2(DE3). Cultivation at 37 °C did not result in any detectable lipase activity using the pH-stat assay (data not shown). Expression of Trx-CalA at 30 °C yielded 0.6 U mg$^{-1}$ lipase activity in the cleared cell lysate (Fig. 1). This expression protocol resulted in a strong overexpression of CalA. The insoluble fraction consisted of 25% CalA. This fits with the general observation, that high expression levels can lead to an increase in the proportion of incorrectly folded protein. Finally expression of the *Trx-calA* construct at 15 °C yielded 1.7 U mg$^{-1}$ (Fig. 1) and 22% CalA content in the insoluble fraction (Table 4). A high yield expression protocol for functional lipases in *E. coli* cells using the T7 *lac* promoter has recently been reported [27,28]. However, in these expression experiments, most of the target proteins were prokaryotic enzymes with no need for folding assistance and posttranslational modifications as required by most eukaryotic enzymes. By fusion to the extremely soluble TrxA protein several eukaryotic enzymes, e.g. mammalian cytokines and growth factors were functionally expressed in the *E. coli* cytoplasm [29]. Using both, the T7 *lac* promoter and the *trxA* fusion tag provided in the vector system pET-32b(+), the murine interleukin-2 (IL-2) was expressed highly soluble in *E. coli* [30].

Table 4
Densitometric analysis of CalA contents in the soluble and insoluble fractions of cell extracts

| Clone | Soluble (%) | Insoluble (%) |
| --- | --- | --- |
| pColdIII/calA | 0.22 | 8.5 |
| pColdIII/calA + pKJE7 | 19.2 | 22.8 |
| pColdIII/calA + pKJE8 | 14.4 | 17.8 |
| pColdIII/calA + pGro7 | 22.4 | 18.5 |
| pColdIII/calA + pG-Tf2 | 17.2 | 17.8 |
| pColdIII/calA + pTf16 | 14.8 | 22.7 |
| pET32-b(+)/calA | 0.35 | 22.3 |
| pET32-b(+)/calA + pKJE7 | 12.1 | 9.4 |
| pET32-b(+)/calA + pKJE8 | 17.8 | 12.9 |
| pET32-b(+)/calA + pGro7 | 18.9 | 19.7 |
| pET32-b(+)/calA + pG-Tf2 | 11.7 | 11.3 |
| pET32-b(+)/calA + pTf16 | 10.3 | 9.3 |

A further increase of functional CalA expression level to 9.6 U mg$^{-1}$ was achieved using the temperature-inducible pColdIII vector system (Fig. 1). Nevertheless, analysis by SDS-PAGE and densitometry still showed a high amount of insoluble CalA (18.5%) (Table 4).

Decreasing the expression temperature increased the yield of functional enzyme slightly in the case of pET32-b(+) and strongly in the pColdIII system. The positive influence of decreased expression temperature on the yield of functionally expressed enzyme was reported previously [31,32]. This dependency of the yield of correctly folded enzyme on the expression temperature can be theoretically explained: whereas folding rate is only slightly decreased by a temperature switch from e.g. 37 to 15 °C, the rate of transcription and translation in *E. coli* is significantly reduced. These facts provide sufficient time for protein refolding, yielding active enzyme and avoiding the formation of insoluble aggregates of misfolded protein [33].

The fusion of an enzyme to a tag, like TrxA can lead to changes in catalytic properties [34]. This may explain the fact that compared to the pColdIII results, the activity of the enzymes expressed in the pET-32b(+) clones are low. Therefore screening of mutants carrying a TrxA tag would not be suitable for the detection of improved variants of the native CalA. From this point of view the use of the pColdIII system seems to be more favorable than utilising pET-32b(+). Moreover, expression using pColdIII led to higher amounts of soluble CalA indicating a positive effect of the cold-responsive promoter *cspA* on efficient gene expression at reduced temperatures as already reported [35].

### 3.3. Functional lipase expression using co-expression of molecular chaperones

A strong increase in soluble heterologous protein concentration in the *E. coli* cytoplasm by co-expression of chaperones has already been reported [36–38]. The pColdIII and the pET-32b(+) constructs both were co-expressed with several combinations of chaperones from the TaKaRa Chaperone Plasmid Set (Table 2) [39]. The specific activity (Fig. 1) and the amount of soluble protein (Table 4, Figs. 2 and 3) expressed by the various constructs differed significantly. Increased yield of active enzyme

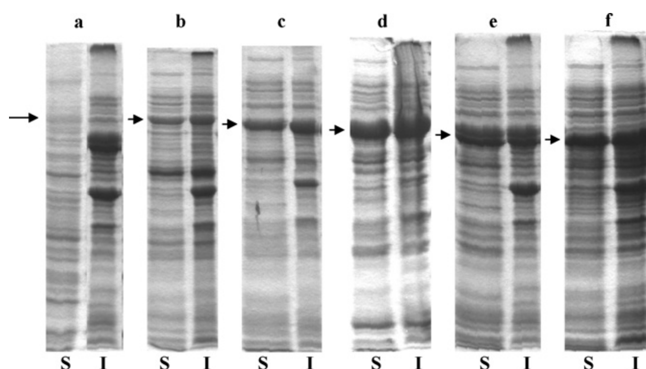


Fig. 2. SDS PAGE analysis of soluble (S) and insoluble (I) fractions obtained from various CalA expression experiments at 15 °C using pColdIII constructs in Origami™ B cells. The CalA bands (43 kDa) are marked with arrows. Abbreviations for constructs: (a) pColdIII/calA (without chaperones), (b) pColdIII/calA + pKJE7, (c) pColdIII/calA + pKJE8, (d) pColdIII/calA + pGro7, (e) pColdIII/calA + pG-Tf2, (f) pColdIII/calA + pG-Tf16.

by co-expression of different chaperones was reported recently [36–38].

CalA was most efficiently expressed in the pColdIII vector with co-expression of pGro7 resulting in 13.1 U $mg^{-1}$. Generally expression from the pColdIII constructs resulted in higher specific activity than expression from the pET-32b(+) constructs. The specific activity of the pET-32b(+) constructs expressed at 30 °C was lower than 1 U $mg^{-1}$. Enzyme expression at 15 °C increased the specific activity significantly, still activity was lower than with the pColdIII constructs. Also among the pET-32b(+) constructs, the best activity value was reached co-expressing pGro7 (3.4 U $mg^{-1}$) (Fig. 1).

Co-expression using the pGro7 construct surpassed the specific activity of the expression of CalA-pColdIII without chaperone. All other expression experiments using co-expression of chaperones yielded lower activity (Fig. 1).

Generally co-expression of chaperones increased the amount of soluble CalA (10–22% of total soluble protein) compared to expression without chaperones (0.22–0.35% of total soluble protein) (Table 4, Figs. 2 and 3).

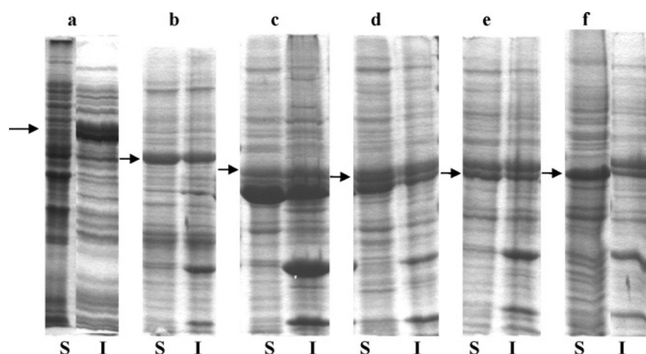


Fig. 3. SDS PAGE analysis of soluble (S) and insoluble (I) fractions obtained from various CalA expression experiments at 15 °C using pET-32b(+) constructs in Origami™ 2(DE3) cells. The Trx-CalA fusion protein bands (55 kDa) are marked with arrows. Abbreviations for constructs: (a) pET-32b(+)/calA (without chaperones), (b) pET-32b(+)/calA + pKJE7, (c) pET-32b(+)/calA + pKJE8, (d) pET-32b(+)/calA + pGro7, (e) pET-32b(+)/calA + pG-Tf2, (f) pET-32b(+)/calA + pG-Tf16. The first SDS PAGE was performed under different conditions than the five others; therefore the Trx-CalA band is shifted to the top.

With both expression systems CalA was most efficiently expressed when co-expressing Gro7, indicating strong impact of the GroES/GroEL chaperone system on the correct folding of CalA. It is known that these molecular chaperones play an important role in folding α/β structures enriched in hydrophobic and basic residues. This is underlined by the fact, that the molecular mass of CalA (43 kDa) fits with the size of GroES/GroEL most preferred substrates (proteins < 60 kDa) [18]. Recently a lipase with the molecular weight of 33 kDa was also functionally expressed in *E. coli* using co-expression of Gro7 [40]. In combination with *trxB gor* suppressor cells (e.g. Origami™) the yield of properly disulfide-bonded proteins was increased by the co-expression of these folding modulators [41–43]. Co-expression of the trigger factor (encoded by pG-Tf2 and pTf16) did not show any positive effect in functional enzyme expression and co-expression of DnaK-DnaJ-GrpE showed only a slight increase of functional CalA expression. These results fit to the observation that a positive influence of the DnaK-DnaJ system is mostly restricted to target proteins with higher molecular weight than 60 kDa [44].

We reported the first expression system for soluble and active CalA variants in a prokaryotic host. This is a prerequisite for a high-throughput-screening system for CalA. The *E. coli* system has some major advantages compared to other high-throughput systems like for example *S. cerevisiae*. Besides lower costs and easier handling, the total amount of clones that can be screened daily is much higher in *E. coli*. The total cultivation period in microtiter plates is 48 h, while the *S. cerevisiae* system needs a 9-day period. Therefore the described *E. coli* expression system is a very important achievement for directed evolution of CalA. The method described here is a useful approach for the expression of proteins which up-to-now failed to be functionally expressed in *E. coli*.

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