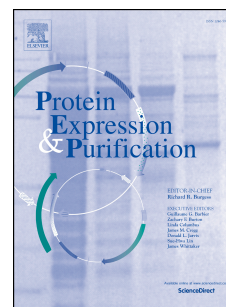


# Journal Pre-proof

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PII: S1046-5928(19)30307-9

DOI: <https://doi.org/10.1016/j.pep.2019.105498>

Reference: YPREP 105498

To appear in: *Protein Expression and Purification*

Received Date: 12 June 2019

Revised Date: 4 September 2019

Accepted Date: 11 September 2019

Please cite this article as: L. Van Tassel, A. Moilanen, L.W. Ruddock, Efficient production of wild-type lipase B from *Candida antarctica* in the cytoplasm of *Escherichia coli*, *Protein Expression and Purification* (2019), doi: <https://doi.org/10.1016/j.pep.2019.105498>.

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**Efficient production of wild-type lipase B from *Candida antarctica* in the cytoplasm of *Escherichia coli*.**

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Highlights (3-5 bullet points)

- Wild-type lipase B from *Candida antarctica* (CalB) was produced solubly in high yields in the cytoplasm of *E.coli* using a catalyzed system for cytoplasmic disulfide bond formation
- Scale up to fermentation in chemically defined media was possible
- The CalB produced had stereospecific activity
- The CalB produced had higher activity than commercial enzyme.

## Abstract

*Candida antarctica* lipase B (CalB) is a very efficient catalyst and is used in a wide range of industries from food flavour to pharmaceutical, and biodiesel manufacturing. It has a high degree of enantioselective and regioselective substrate specificity and is stable over a wide range of biophysical conditions including pH, temperature and solvent conditions. High-level expression of biologically active wild-type CalB has been problematic, partly due to folding events. Consequently, focus has been on modified CalB, which has allowed orders of magnitude increase in yields of protein. However, these modifications alter the quaternary structure of the protein. Here we produce soluble wild-type CalB in high yields in the cytoplasm of *E.coli* using a catalyzed system for cytoplasmic disulfide bond formation both in shake flasks and in fermentation in chemically defined media. The CalB produced had the expected stereospecific activity and had a higher activity than CalB from a commercial source.

Keywords (max 6) *Escherichia coli*, CalB, lipase, disulfide bond formation, cytoplasmic oxidative folding

## Introduction

Lipases are a family of enzymes that naturally hydrolyze triglycerides to fatty acids, diacylglycerol, monoacylglycerol, and glycerol, by acting on ester bonds of carboxylic esters. Their mechanism of activation at the lipid-water interface differentiates lipases from other esterases that mainly hydrolyze water-soluble esters. Lipases are compelling catalysts in a multitude of industries (including food, flavour, pharmaceutical, and biodiesel manufacturing) as they can also catalyze stereospecific esterification and other reactions.

The yeast species *C. antarctica* produces two lipases; A and B (CalA and CalB). CalB is the more extensively studied enzyme due to its strong selectivity and catalytic functionality (for example [1]). Uppenberg and co-workers first described CalB in 1994 as a 33 kDa secreted protein [2]. The active site of CalB consists of a nucleophilic serine (Ser105) which forms a catalytic triad with histidine (His224) and aspartate (Asp187). CalB shows a high degree of substrate specificity, both enantioselective and regioselective [3]. It remains stable from pH 3.5 – 9.5 (optimal catalysis at pH 7) and has been observed to function at 150°C [4], in polar organic solvents [5], within ionic liquids and supercritical carbon dioxide [4]. The enzyme is an efficient biocatalyst and can be utilized in a broad range of processes, from biodiesel production (reviewed in [6-12]), glycerol carbonate [13], diacylglycerol [14-15], benzyl acetate [16], olvanil [17] and drug synthesis, [18] racemate resolutions [19-23], lignin valorization [24] and aminolysis [25].

Sixteen crystal structures exist for CalB in the protein data bank [2, 26-31] with the protein either purified from the natural source or recombinant expression in *P. pastoris*, *A. oryzae*, *A. niger* or *E. coli*. However, high-level expression of biologically active wild-type CalB has been problematic. Low

secretion efficiency was observed in *S. cerevisiae* [32] and the majority of the enzyme is expressed as inclusion bodies in *E. coli* [33,34]. Part of the problem of efficient production of CalB is the formation of native post-translational modifications. CalB has one non-essential N-glycosylation site and three sequential structural disulfide bonds (C47-C89, C241-C283, C318-C336). In addition, while the natively folded protein is thermally stable there appears to be an issue with folding events, including the solubility of folding intermediates, such that soluble protein expression is usually obtained at lower temperatures, e.g. 20°C.

To date, expression in *E.coli* has focused primarily on targeting the protein to the periplasm (for example [33,35]) to allow native disulfide bond formation, though a fusion protein approach expressed in a  $\Delta trxB / \Delta gor$  strain has also been used [36-37]. The highest yield we are aware of reported for the wild-type enzyme expressed in *E.coli* is 5.2mg/L [33] from shake flasks or 113mg/L after optimization in fermentation [38].

To address this problem, multiple approaches have been adopted, most of which offer only modest increases in yield. The most successful approach has been the addition of polyanionic tags to the N- or C-termini of the protein [39]. These appear to increase the solubility of the folding intermediates and allow production levels of 1.9 g/L from fed-batch cultivation [39]. However, these additions change the quaternary structure of the protein [39].

Here we express wild-type CalB in the cytoplasm of *E.coli* with the assistance of the CyDisCo folding apparatus. CyDisCo comprises two catalysts of disulfide bond formation, a sulfhydryl oxidase (usually Erv1p) and a disulfide isomerase (usually PDI). CyDisCo can efficiently produce some disulfide bond containing proteins, both prokaryotic and eukaryotic [40-41]. CyDisCo works in all *E.coli* strains tested

to date and works in fed batch fermentation in chemically defined media [42]. Expression of wild-type CalB with CyDisCo resulted in purified yields up to 0.5 g/L from non-optimized fed-batch fermentation. The purified CalB shows the same stereospecificity as commercial enzyme produced in *Aspergillus oryzae*, but higher specific activity.

## Materials and Methods

### *Cloning*

A gene encoding *Candida antarctica* CalB residues A19-P342 (lacking the N-terminal signal peptide) was purchased from GenScript with the sequence:

```
ATGGCAACCCCGCTGGTGAAACGCCTGCCGTCTGGTAGCGACCCGGCTTTTCCCAACCGA
AATCAGTGCTGGATGCTGGCCTGACCTGTCAGGGCGCGTCGCCGAGCTCTGTTAGCAAAC
CGATTCTGCTGGTCCCGGGTACCGGTACCACGGGTCCGCAGAGCTTTGATTCTAACTGGAT
TCCGCTGTCTACCCAACCTGGGTTATACGCCGTGCTGGATCAGCCCGCCGCCGTTTCATGCTG
AACGACACCCAGGTCAATACGGAATATATGGTGAATGCAATTACCGCTCTGTACGCGGGC
TCCGGTAACAATAAACTGCCGGTGCTGACCTGGAGTCAGGGCGGTCTGGTTGCGCAATGG
GGCCTGACCTTTTCCCGAGTATCCGTTCCAAAGTTGATCGCCTGATGGCGTTTGCCCCGG
ACTACAAAGGCACCGTCCTGGCAGGTCCGCTGGATGCACTGGCTGTTTCAGCTCCGTCGGT
CTGGCAGCAAACCACGGGTAGCGCTCTGACCACGGCACTGCGTAACGCCGGCGGTCTGAC
CCAGATTGTGCCGACCACGAATCTGTATAGCGCCACGGATGAAATCGTCCAGCCGCAAGT
GTCAAACCTCGCCGCTGGACAGTTCTTACCTGTTCAACGGCAAAAATGTGCAGGCACAAGC
AGTTTGCGGTCCGCTGTTTGTTATTGATCATGCCGGTAGCCTGACCTCTCAGTTCAGTTATG
TGGTTGGTCGTTCCGCACTGCGCTCAACCACGGGTCAAGCACGTTCTGCAGATTACGGTAT
CACCGACTGTAACCCGCTGCCGGCGAATGATCTGACGCCGGAACAGAAAGTGGCAGCAGC
AGCTCTGCTGGCACCGGCAGCAGCAGCTATTGTTGCAGGTCCGAAACAGAATTGCGAACC
GGACCTGATGCCGTATGCACGTCCGTTTGCTGTGCGCAAACGCACCTGTAGCGGTATCGTG
ACGCCGTAA
```

The gene was inserted into a modified pET23-based expression vector using NdeI/BamHI restriction sites. The resulting plasmid was named pHIA633. The polycistronic vector pMJS205 encoding codon

optimized *S.cerevisiae* Erv1p and mature human PDI (D18-L508) cloned into a modified version of pLysS was constructed previously [43]. A negative control, pAG82, for pMJS205 which lacked folding factors was also constructed previously [43]. All plasmids were sequenced to confirm the correct insertion of genes.

### *Expression*

pHIA633 and pMJS205 or pAG82 were transformed into the *E.coli* K12 strain BW25113 using calcium chloride. In brief, 1  $\mu$ L of each plasmid was added to 50  $\mu$ L of competent cells, incubated on ice for 30 minutes and then heat-shocked at 42°C for 45 seconds, and incubated again on ice for 2 minutes. 200  $\mu$ L super optimal broth (SOB) media was added and the tubes were placed in a shaking incubator at 37°C for 1 hour. Transformations were plated onto chloramphenicol (35 mg/L) and ampicillin (100 mg/L) plates and incubated at 37°C overnight. Glycerol stocks were made from the strains after two rounds of replating single colonies. Stocks were stored at -70°C.

### *Small scale expression tests*

The expression strain was streaked onto selective plates and incubated at 37°C overnight. The following morning, a single colony was picked and a starter culture was prepared. For small scale expression, starter cultures comprising of 2 mL of Luria-Bertani (LB) media supplemented by 2 g/L glucose and appropriate antibiotics were incubated at 30°C / 250 rpm for 5 hours. A 1:100 dilution of this pre-culture was used to inoculate the primary culture comprised of 50 mL autoinduction media [44] with appropriate antibiotics in a 500 mL conical flask sealed with an oxygen permeable membrane. Cultures were grown for 17 hours at 30°C / 250 rpm. The temperature was then dropped to 20°C and 0.5 mM of IPTG added (BW25113 cells cannot autoinduce). After 24 hours growth, the cells were harvested. Cells were collected by centrifugation (3220 g, 20 minutes, +4°C). The supernatant



was removed and the cells were resuspended in 50 ml of 50 mM phosphate buffer pH 7.4, supplemented with 20 mg/L DNase and 100 mg/L lysozyme. The cultures were incubated at room temperature for 10 minutes before freezing to allow action of the lysozyme. Cells were lysed by repeating a freeze-thaw cycle twice. Total and soluble lysate fractions were taken for SDS-PAGE analysis.

#### *Fed-batch fermentation*

For fermentation scale production, the expression strain was similarly streaked onto selective plates and incubated at 37°C overnight. A single colony was used to inoculate 50 mL of vegitone LB (Fluka Analytical) with appropriate antibiotics in a 500 mL flask and incubated at 30°C / 250 rpm for 18 hours.

Fermentation was carried out using a 1 L Biostat B system with a 0.5 L working volume, using standard lab protocols, i.e. no protein specific optimization. The initial fermentation medium contained per liter 8.92 g  $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$ , 3.0 g  $\text{KH}_2\text{PO}_4$ , 1.0 g  $\text{NH}_4\text{Cl}$ , and 0.5 g  $\text{NaCl}$  which was autoclaved in the vessel. The sterile medium at growth temperature (30°C) was supplemented with 5 g/L glucose, 2 mM  $\text{MgSO}_4$ , 10 mg/L Fe(III) citrate hydrate, 100 mg/L ampicillin, 35 mg/L chloramphenicol, 0.3 mM  $\text{CaCl}_2$ , 0.3 mM  $\text{NaSO}_4$ , 1 mg/L biotin and trace elements containing per liter 0.25 mg of  $\text{CoCl}_2 \times 6 \text{H}_2\text{O}$ , 1.5 mg  $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$ , 0.15 mg  $\text{CuCl}_2 \times 2 \text{H}_2\text{O}$ , 0.3 mg  $\text{H}_3\text{BO}_3$ , 3.4 mg  $\text{Zn}(\text{CH}_3\text{COO})_2 \times 2 \text{H}_2\text{O}$ , 0.23 mg  $\text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O}$ , and 1.25 mg of EDTA (Titriplex II).

Freshly supplemented 0.5 L medium at 30°C was inoculated with 2 mL of the preculture (1:250 volume ratio). Oxygen concentration was kept constant at 30% (relative to maximum dissolved oxygen from pressurized air at 30°C) controlled by a cascade of first adjusting air flow (0.075 – 1.5 Lplm) and

then stirring (200 rpm – 1200 rpm). The pH was kept at 7.0 by 10% ammonium hydroxide and foaming was controlled with Antifoam 204 (Sigma-Aldrich). The fed-batch phase started 15 hours from inoculation by feeding 50% (v/v) glycerol solution containing the chemically defined medium components minus glucose and magnesium. Glycerol was fed at a rate of 6.3 g/hr in three one hour pulses after 15, 18, and 21 hours from inoculation and then constantly with the same feed rate from hour 23 onwards until the end of the fermentation. Additional magnesium (2 mM  $\text{MgSO}_4$ ) was supplied to the culture at hours 22.5, 26, and 29 from inoculation. The temperature was lowered from 30°C to either 20°C or 23°C one hour before induction. Protein production was induced by the addition of 0.5 mM IPTG 27 hours after inoculation. Cultures were harvested 19 hours after induction by centrifugation (6000 x g, 20 min, +4°C) and resuspending to culture volume of 20 mM sodium phosphate, pH 7.3, 100 mg/L lysozyme, and 20 mg/L DNase. Cells were lysed by two cycles of freeze-thaw.

#### *Protein purification*

His-tagged CalB produced in shake flask cultivation was purified by immobilized metal affinity chromatography (IMAC) using HisPur Cobalt resin (Thermo Scientific) as previously described [43]. The protein produced in fermentation was purified by (IMAC) using a 5ml NTA column (Amersham Pharmacia) charged with nickel as described [45], except elution using an imidazole gradient (0.01-0.5M) in 20 mM sodium phosphate, pH 7.3. Fractions containing purified CalB as determined by SDS-PAGE were pooled. Purified proteins were stored either at 4°C or at -20°C.

Protein concentration for purified CalB or commercial CalB was determined by using absorbance at 280nm using a calculated molar extinction coefficient of 41,285  $\text{M}^{-1}\text{cm}^{-1}$ .

#### *Activity Assay:*

Activity of CalB was measured using an assay developed by Magnusson and co-workers [46] in which hydrolysis of the substrate is linked to a pH change due to the formation of an acidic product. The pH change is monitored using the chromophore para-nitrophenol which loses its absorbance at 400 nm upon protonation. This method allowed us to examine the reactivity of CalB to four different substrates, to examine substrate specificity and stereoselectivity. The activity of CyDisCo produced CalB was compared with that of commercial CalB produced in the fungus *Aspergillus niger* (Sigma).

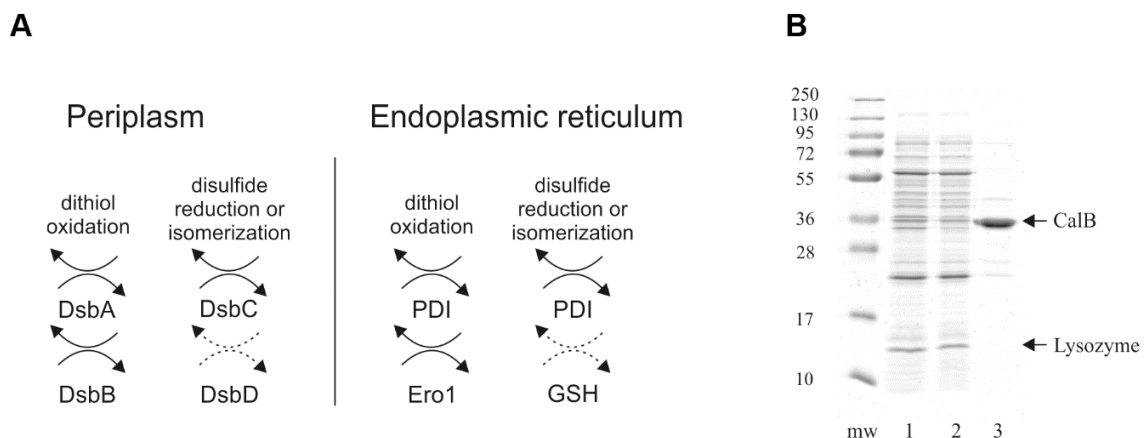
The assay was performed in triplicate in 200  $\mu$ l total volume, 2.5 mM MOPS buffer, 0.125 mM para-nitrophenol, 0.5  $\mu$ M CALB in 96 well plates. Time-dependent changes in absorbance were monitored on a TECAN M-1000 pro plate reader at 400nm. After thermal equilibration, 5 mM of substrate was added and the change in absorbance monitored every 30 or 60 seconds for 30 minutes. A linear change in absorbance was observed up to  $\Delta\text{abs} = 0.3$  for three of the substrates, equivalent to consumption of circa 13% of the substrate, while for ethyl propanoate linearity was only observed up to  $\Delta\text{abs} = 0.2$ .

To convert changes in absorbance into substrate turnover, standard curves were prepared for each substrate with increasing amounts of acetic acid added as a mimic of substrate hydrolysis to release the acidic product. Standard curves were prepared in duplicate with 200  $\mu$ l total volume containing 2.5 mM MOPS buffer, 0.125 mM para-nitrophenol, and 5 mM substrate. Absorbance at 400 nm was monitored on a TECAN M-1000 pro plate reader.

## Results and Discussion

### *Production of soluble wild-type CalB in the cytoplasm of E.coli*

The production of wild-type CalB in *E.coli* is problematic. The highest yield we are aware of being reported from production in shake flasks is 5.2 mg/L, where CalB was targeted to the periplasm [33]. CalB contains three structural disulfide bonds and these will be formed in the periplasm by the Dsb-system which is comprised of the transmembrane enzymes DsbB and DsbD and the soluble proteins DsbA and DsbC (reviewed in [47]). The Dsb system is strikingly different from the systems used to make disulfide bonds in the endoplasmic reticulum (ER) of eukaryotes (reviewed in [48]). In the human ER, the primary route for disulfide bond formation is thought to be by the action of the sulfhydryl oxidase on PDI which in turn oxidizes folding proteins. PDI also plays a role in the isomerization of non-native bonds aided by reduced glutathione (reviewed in [49]). Hence PDI efficiently plays the role of both DsbA and DsbC (Fig 1A).



**Figure 1. CalB production in shake flask scale**

**Panel A)** Schematic of the primary route for disulfide bond formation in the periplasm (left side) vs disulfide bond formation in the endoplasmic reticulum (right side). Note that PDI plays the role of both DsbA and DsbC. In the CyDisCo system, the sulfhydryl oxidase Erv1p replaces the sulfhydryl oxidase Ero1. **Panel B)** Coomassie stained SDS-PAGE analysis of CalB production in the cytoplasm of *E.coli* with the CyDisCo system for disulfide bond formation. Lane 1 total lysate, lane 2 soluble lysate, lane 3 IMAC purified. The position of added lysozyme used for densitometric analysis of total yield is indicated.

We hypothesised that the use of eukaryotic folding factors may aid the more efficient folding of the eukaryotic protein CalB and that this in turn may increase the yield of soluble protein obtained. For this we turned to the CyDisCo system, which uses a yeast sulfhydryl oxidase and human PDI to catalyse disulfide bond formation in the cytoplasm of *E.coli* [40-42].

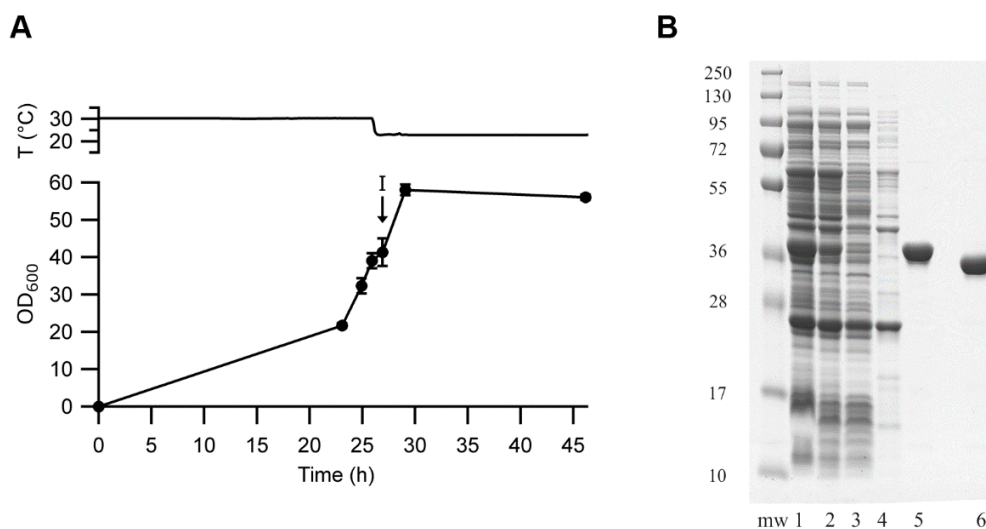
Expression of CalB at 37°C with CyDisCo resulted in no soluble CalB being produced (data not shown). This is not surprising as the yeast sulfhydryl oxidase Erv1p folds poorly at temperatures above 30°C and CALB is from a psychrophilic organism and hence is usually expressed at 20°C. Dropping the expression temperature to 20°C resulted in high level soluble expression of wild-type CalB in chemically defined autoinduction media (Fig 1B). Densitometric analysis suggested yields of circa 95 mg/L of soluble protein, equivalent to 5.8 mg/L/OD. Purified yields were 40 mg/L (2.4 mg/L/OD).

### *Scale up production*

While small scale production of CalB can be useful for high-throughput screening, e.g. enhancing stability [28] or altering specificity [50], large scale production is required for most industrial use. It

has been reported that CyDisCo can be used in fed-batch cultures in chemically defined media [42]. However, relatively few proteins have been produced in scale up using CyDisCo and it is unclear what limitations, if any, there may be on the range of proteins of interest that can be produced using it.

As a proof of concept of scale-up production of CalB, we undertook fed-batch fermentation in chemically defined media using glycerol as a carbon source. No optimization of the process was undertaken, rather our standard starting point fermentation conditions were used, with the exception that the temperature of the culture was reduced to either 20°C or 23°C one hour prior to induction (Fig 2A). Culture growth was inhibited more at the lower temperature, but both cultivation conditions resulted in CalB production similar to that in DWP and shake flasks, i.e. high yields of protein, with circa 45% being solubly expressed (Fig 2B). Purification of CalB resulted in yields of  $8.5 \pm 0.2$  mg/L/OD (equivalent to 0.48g/L from the 23°C fermentation; Final OD<sub>600</sub> = 56.0), with differences in yield between the fermentation runs coming from differences in cell density. The higher yield from fermentation compared with shake flasks arises both from higher cell culture density and circa 3.5x higher yield per cell. This higher yield per cell may arise from better oxygenation of the culture as Ery1p uses molecular oxygen to make disulfide bonds in the CyDisCo system.



**Figure 2. CalB production in fermentation**

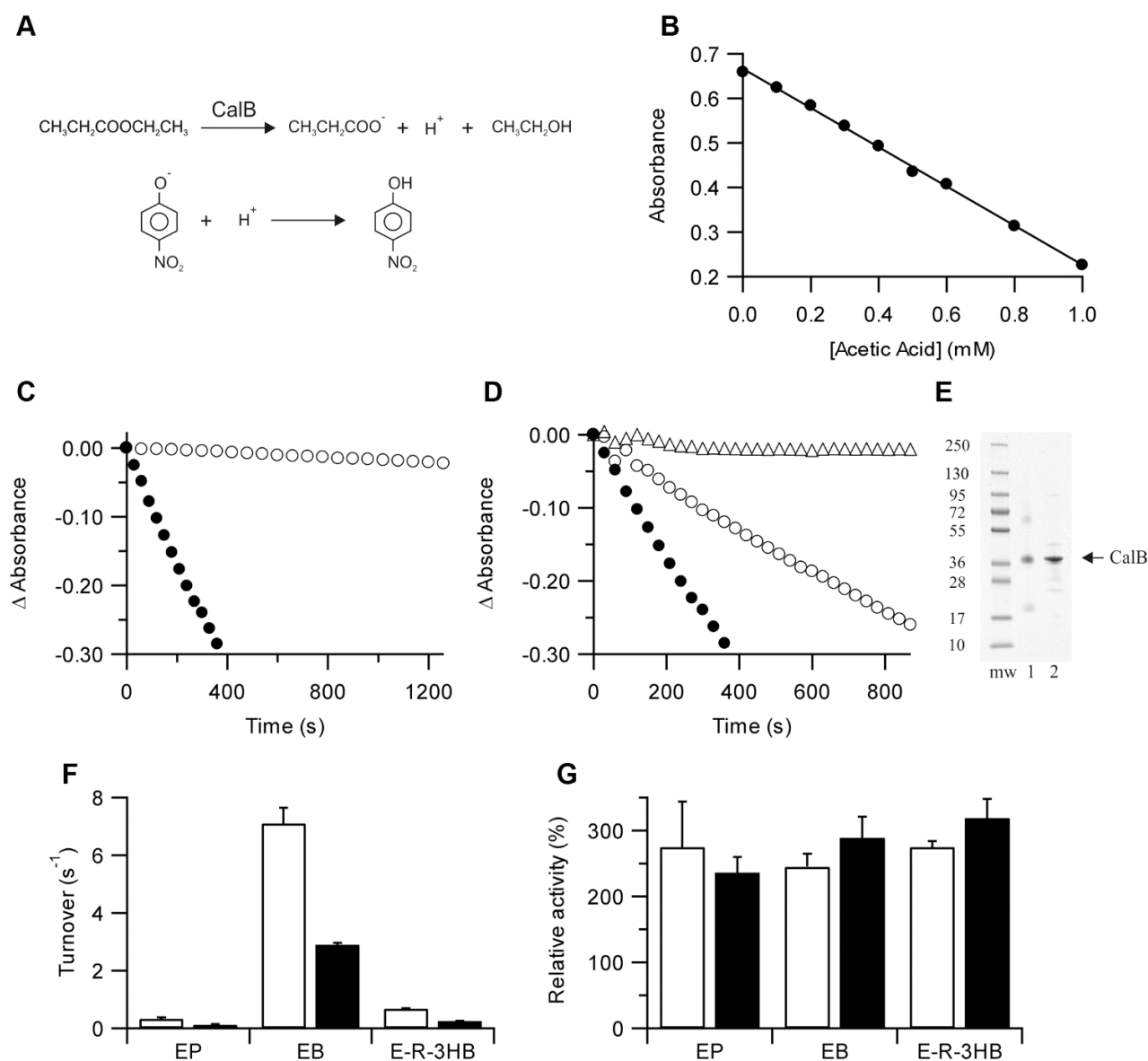
**Panel A)** Growth (lower panel) and temperature (upper panel) profile for CalB production in fermentation. The induction point (I) is marked. **Panel B)** Coomassie stained SDS-PAGE analysis of CalB production in the cytoplasm of *E.coli* in fermentation with the CyDisCo system for disulfide bond formation. The samples are molecular weight markers (mw), total lysate (lane 1), soluble lysate (lane 2), flow through from IMAC (lane 3), wash from IMAC (lane 4), purified protein (lanes 5 and 6). Lanes 1-5 are reducing SDS-PAGE, lane 6 is non-reducing SDS-PAGE.

The CalB produced showed a mobility shift in reducing vs non-reducing SDS-PAGE (Fig 2B, lane 5 vs 6) implying the formation of structural disulfide bonds. On non-reducing SDS-PAGE only a single band was observed, suggesting that only a single redox state was formed.

*Recombinant CalB is active and specific*

While the production levels of soluble wild-type CalB were significantly higher than previously reported in *E.coli*, soluble expression alone does not demonstrate that the protein is biologically active. The activity of recombinant CalB is often tested using p-nitrophenyl butyrate (p-NPB) as a substrate. While this allows direct spectrophotometric determination of the rate of hydrolysis, by itself it would not demonstrate whether our CalB showed the same substrate range and stereospecificity as commercial enzyme produced in a eukaryotic expression system. Instead, we adopted the assay format from Magnusson et al [46], where hydrolysis is linked to a pH change due to the formation of an acid product (Fig 3A). This pH change is monitored using a pH-sensitive dye. This method allowed us to examine the reactivity of CalB to four different substrates, including stereoselectivity.





**Figure 3 CalB produced using CyDisCo is biologically active.**

**Panel A)** Schematic of the assay used to monitor CalB activity using ethyl butyrate. The assay works in the same manner for other substrates. **Panel B)** Absorbance change for the pH-sensitive dye para-nitrophenol in the presence of the CalB substrate ethyl-(S)-3-hydroxybutyrate upon addition of acetic acid. A linear decrease in absorbance was observed with increasing acetic acid ( $R^2 = 0.998$ ). Similar linear dependencies were observed in the presence of the other substrates tested. **Panel C)** Stereospecificity of CalB activity produced towards ethyl-(R)-3-hydroxybutyrate (●) versus ethyl-(S)-

3-hydroxybutyrate (○). **Panel D)** The relative activity of CalB produced using CyDisCo (●) vs commercial CalB (○) and the uncatalysed reaction (Δ) towards ethyl-R-hydroxybutyrate. **Panel E)** Coomassie stained reducing SDS-PAGE analysis of commercial CalB (lane 1) vs IMAC purified from shake flask production (lane 2). **Panel F)** Turnover of CalB produced using CyDisCo in shake flasks (white bars) vs commercial CalB (black bars) for three substrates (EP = ethyl propionate; EB = ethyl butyrate; E-R-3HB = ethyl-(R)-3-hydroxybutyrate). **Panel G)** Relative activity of CalB produced using CyDisCo in shake flasks (white bars) and fermentation (black bars) vs commercial CalB for three substrates (EP = ethyl propionate; EB = ethyl butyrate; E-R-3HB = ethyl-(R)-3-hydroxybutyrate)

To confirm that the change in absorbance observed was proportional to the activity of the enzyme, i.e. the amount of acid derivative produced, we titrated acetic acid into the reaction mix and followed absorbance as a function of [acid]. The results showed that for the addition of up to 1mM acid, a linear decrease in absorbance was observed with increasing acetic acid added (Fig 3B).

The activity of our recombinant CalB was then examined. The CalB we produced showed activity towards all four substrates tested (Fig 3), with the expected stereospecificity towards the ethyl-(R)-3-hydroxybutyrate vs the ethyl-(S)-3-hydroxybutyrate (Fig 3C). The relative activities towards the four substrates is consistent with previously published results [46].

We then compared the activity of the CalB we produced with that of commercially available material. Our enzyme was more active towards all substrates (Fig 3), with an average of 273% activity compared with the commercially available material and no significant differences between CalB produced in shake flasks or fermentation. SDS-PAGE analysis revealed that the commercially available material contained multiple bands (Fig 3D), suggesting that the difference in activity may arise from being

unable to determine the enzyme concentration of the commercial CalB accurately. Mass spectrometric analysis of the commercial CalB revealed that a major contaminant was alpha-amylase from *A. oryzae*, i.e. a recombinant host protein.

## Conclusions

The results presented here show that it is possible to generate significant yields (circa 0.5g/L of purified protein) of wild-type CalB from non-optimized fed-batch fermentation through the use of the co-expression of eukaryotic folding factors in the cytoplasm of *E.coli*. The CalB produced was biologically active and showed the expected specificity indicating that it was correctly folded. It may be possible to increase the yields of protein produced either via optimization of the fermentation process, e.g. increasing final cell density or via the addition of a polyanionic tag – as previously proved so successful in periplasmic expression.

## Acknowledgements

The use of the facilities and expertise of the Biocenter Oulu core facilities, a member of Biocenter Finland, is gratefully acknowledged.

## Funding

This work was supported by the Academy of Finland [grant number 266457] and Biocenter Oulu.

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