



# Streamlined Preparation of Immobilized *Candida antarctica* Lipase B

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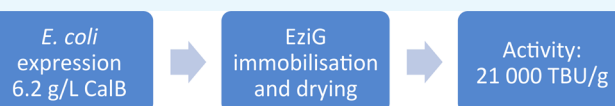
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**ABSTRACT:** *Candida antarctica* lipase B (CalB) was efficiently expressed (6.2 g L<sup>-1</sup>) in *Escherichia coli* by utilizing an N-terminal tag cassette and the XylS/Pm expression system in a fed-batch bioreactor; subsequent direct binding to EziG from crude extracts resulted in an immobilized catalyst with superior activity to Novozym 435.



## INTRODUCTION

In pharmaceutical and fine chemical industries, there is a drive toward greener and more economic alternatives to current production methods, partly because of pressures on cost and demands from environmental legislation.<sup>1</sup> Biological systems and, in particular, isolated or immobilized enzymes offer a sustainable and greener alternative to traditional catalysis for advanced chemical manufacture.<sup>2</sup> It is of great importance that streamlined production systems are used to produce enzymes. This allows the enzyme to compete head to head with traditional catalysts in terms of cost, performance, and waste generation of the process. Consequently, enzyme production should be reviewed in relation to culturing time, fermentation productivity, expression level, and the necessity for immobilization to minimize the overall cost for the target chemical process.

CalB is among the most widely used enzymes for biocatalytic applications,<sup>3–6</sup> possessing many of the desirable characteristics of an efficient and industrially viable biocatalyst, that is, excellent stereoselectivity, chemical compatibility, and stability.<sup>4</sup> The enzyme is monomeric, does not have a cofactor requirement, and has high substrate specificity, stereoselectivity, and regioselectivity. Furthermore, it is easy to immobilize on hydrophobic resins because of its specific surface properties and is active and stable in organic solvents at elevated temperatures.<sup>7</sup> Various methods have been developed for immobilization of CalB,<sup>8,9</sup> which in practical terms is a prerequisite for employment in organic solvents.

Even with its high substrate specificity, CalB is known to be promiscuous in terms of substrate acceptance, reaction condition, and reaction mechanism.<sup>10,11</sup> Therefore, the enzyme

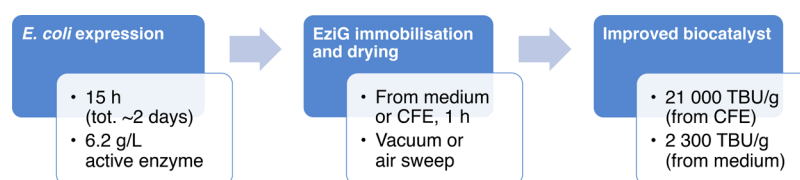
is used in a broad range of applications, including polymerizations, resolutions of alcohols and amines, sugar modifications, desymmetrization reactions, and ring opening of  $\beta$ -lactams.<sup>4,12–15</sup> Today, the most commercially applied enzyme preparation of CalB is Novozym 435,<sup>6</sup> where the enzyme is immobilized on an acrylic resin. This biocatalyst is used in numerous industrial reactions,<sup>16</sup> including the production of biodiesel,<sup>17</sup> enantiomerically enriched ketoprofen,<sup>18</sup> and glycerol carbonate.<sup>19</sup>

For recombinant expression of CalB, various host organisms have been applied, namely *Aspergillus oryzae*,<sup>20,21</sup> *Escherichia coli*,<sup>22–30</sup> *Hansenula polymorpha*,<sup>31</sup> *Pichia pastoris*,<sup>13,25,32–34</sup> *Saccharomyces cerevisiae*,<sup>35,36</sup> and *Yarrowia lipolytica*.<sup>37</sup> *E. coli* generally provides a high transformation efficiency, high plasmid copy number, and high protein yield, making it the first choice for routine cloning, protein engineering or sequencing work, and recombinant protein production. However, if the native target protein undergoes posttranslational modifications or complex domain organizations, other host systems are often needed. Eukaryotic host systems for recombinant expression provide glycosylation, albeit in patterns that differ from those of the native host system.<sup>38</sup> By contrast, prokaryotic host systems, such as *E. coli*, are known to provide nonglycosylated proteins.<sup>39</sup> CalB is a monomeric enzyme containing three disulphide bonds and one N-glycosylation sequon,<sup>7,40</sup> and it is therefore not an ideal candidate for *E. coli* expression. However, mutagenesis has been used to explore the

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**Figure 1.** Streamlined process for preparing immobilized CalB, giving a drastically improved biocatalyst (compared to Novozym 435) in terms of standard lipase units (TBU) when prepared from the cell free extract (CFE).

lack of glycosylation on CalB. An exchange of the glycosylated asparagine residue for a serine (N74S) did not affect the processing or folding of the enzyme, although the activity level of the variant enzyme was reduced.<sup>25</sup> Because glycosylation of N74 is not required, both prokaryotic and eukaryotic expression systems may be considered for CalB, as long as the disulphide bonds can be formed.

CalB has been expressed using *E. coli* in the cytosol,<sup>22,27,28,41,42</sup> periplasmic space,<sup>23–25,43</sup> and culture medium.<sup>29,30</sup> Extracellular expression (to the culture medium) of CalB from *E. coli* has resulted in reasonably high expression levels of CalB; 0.55 g/L (0.5 L shake flask culture)<sup>30</sup> and 1.9 g/L (1 L bioreactor fed-batch culture),<sup>29</sup> wherein the lion's share of examples is in the single digit milligram per liter range with intracellular or periplasmic expression. Extracellular expression of recombinant protein can reduce the cost and makes downstream processing easier.<sup>44</sup> Because this organism does not usually secrete recombinant proteins to any significant amount, examples of this process with *E. coli* are valuable.

## RESULTS AND DISCUSSION

This research aimed to streamline the preparation of immobilized CalB by further improving the expression in *E. coli* and the immobilization method to develop a reusable and effective biocatalyst that can be produced in a fast and simple manner. We achieved an improved expression level compared to previously published results and could utilize the EziG enzyme carrier for fast and efficient immobilization,<sup>45</sup> as described in Figure 1.

First, we reproduced the recombinant expression system applied for extracellular expression of CalB in *E. coli* BL21 by Kim et al.<sup>29</sup> The applied expression cassette template was designed in the common pET26b(+) plasmid containing a pelB leader sequence (P), which translocates the expressed protein to the periplasmic space by the SecB secretory system. As the protein is transported over the inner membrane from the cytosol into the periplasmic space, the P sequence is cleaved off.<sup>44</sup> In the periplasmic space, disulphide bonds and proper tertiary structure can be formed. After the P sequence, an anionic tag consisting of five aspartic acid residues (D5-tag) was inserted before the *E. coli* codon optimized mature CalB gene sequence. This expression cassette system, called P-D5-CalB, has given protein yields of 0.031 g/L D5-CalB in a shake flask culture and 1.9 g/L in a bioreactor culture.<sup>29</sup> In our hands, the P-D5-CalB expression system provided 0.040 g/L active CalB in shake flask cultures.

To further improve the production yield of CalB in *E. coli*, the proprietary expression system by Vectron Biosolutions AS (Norway) was employed: the plasmid pVB-1A1B1 contains a backbone based on the previously published pJB658 and pIFN30SpelB carrying the wild type XylS/*Pm* regulatory promoter system<sup>46</sup> and *trfA* with cop271 mutation,<sup>47,48</sup> which results in an elevated plasmid copy number compared to the

wild type. The codon optimized P-D5-CalB sequence was cloned into this vector with the addition of a C-terminal His-tag (H6), resulting in the pVB-1A1B1-PelB-D5-CalB-H6 expression plasmid. The His-tag enables immobilization on EziG, with concurrent purification directly from crude extracts or culture media.

EziG, provided by EnginZyme AB (Sweden), is a material based on controlled pore glass, which is coated with organic polymer and chelated Fe(III) for His-tag binding. Because of efficient mass transfer through interconnecting pores and selective and nondestructive binding through His-tags, a high enzyme mass loading can be reached without the loss of activity caused by a high degree of diffusion limitation and deactivation. His-tag binding to Fe(III), rather than the commonly used Ni(II) or Co(II), gives a stronger (albeit less specific) bond resulting in less or no leaching. Iron is also nontoxic; thus, the immobilized preparation is suitable for a wide range of applications including processing for pharmaceutical intermediates and active pharmaceutical ingredients. EziG is available in three different versions with varied surface hydrophobicities. In this work, the semihydrophobic EziG Amber was used, because it was previously showed to be the most efficient for CalB immobilization.<sup>45</sup>

In comparison to the P-D5-CalB gene construct, the new expression system produced a greater than tenfold higher yield of enzyme in shake flask cultures; that is, 0.46 g/L (expression in the Luria broth medium, 20 °C, 72 h), where the active enzyme was found in the medium (22%) as well as the cell extract (78%). The active enzyme content was calculated by the tributyrin hydrolysis activity (TBU) assay using a pH-stat and enzyme active-site titration.<sup>49,50</sup> No significant difference in the activity (440 TBU/mg) was observed compared to CalB expressed in yeast.<sup>51</sup>

Following the procedure by Kim et al.<sup>29</sup> (except that induction was performed with 2.0 mM *m*-toluic acid, instead of isopropyl  $\beta$ -D-1-thiogalactopyranoside), the combined N-terminal cassette with the XylS/*Pm* expression system was used for a proof-of-concept fed-batch bioreactor production (*E. coli* BL21). An increased extracellular expression was observed, and the yield was significantly improved: 6.2 g/L of enzyme was achieved after 15 h of expression, of which 4.2 g/L was found in the cell free medium (extracellular) and 2.0 g/L was found intracellular (presumably periplasmic). This yield is roughly 3 times higher than that achieved with the previous expression system omitting optimizations.<sup>29</sup> Inactive enzymes, misfolded or otherwise, were not quantified.

The new CalB-variant was immobilized on EziG, both from the culture medium and the cell extract after osmotic shock. Prior to immobilization, the cell extract was buffered (20 mM 3-(*N*-morpholino)propanesulfonic acid, 250 mM NaCl, pH 7.5), EziG was then added, and immobilization was performed with end-over-end mixing for 1 h before rinsing with the same buffer and vacuum drying for 16 h (air sweeping is also

suitable). The culture medium was filtered and the pH was set to 7.5 following the same immobilization procedure. No optimizations were completed as part of this study, as the purpose was to test the result directly to a standardized procedure. When tested with the TBU assay, more than eightfold higher activity was observed for the EziG preparation made from the *E. coli* cell extract compared to Novozym 435 (Table 1), whereas the EziG preparation made from the medium displayed comparable activity.

**Table 1. Enzyme Activities of the Tagged CalB-Variant Immobilized on EziG Amber, Compared to Novozym 435**

| immobilized enzyme preparation | standard assay (TBU/g) <sup>a</sup> |
|--------------------------------|-------------------------------------|
| EziG—D5-CalB-H6 (from the CFE) | 21 000                              |
| EziG—D5-CalB-H6 (from medium)  | 2300                                |
| Novozym 435                    | 2500                                |

<sup>a</sup>Measured by pH-stat,<sup>48</sup> 10 v/v % tributyrin emulsion, pH 7.5, 40 °C.

It is conceivable that the difference of the activity between the preparations made from the cell extract and culture medium can be attributed to binding of contaminants occupying space on the EziG enzyme carrier, thus giving a lower enzyme loading on the support. This aspect, as well as optimization of the fed-batch cultivation to further increase the titer and minimize the putative presence of inactive enzymes, remains to be explored. Further work also includes testing of the EziG-CalB for biocatalysis in organic solvents and for the plethora of reactions for which CalB is used.

## CONCLUSIONS

An improved yield of active CalB expressed in *E. coli* BL21 was obtained (6.2 g/L) by combining a previously published N-terminal expression cassette with the XylS/*P<sub>m</sub>* expression system. When the enzyme was directly immobilized on EziG Amber from crude extracts without prior purification steps, the resulting preparation showed roughly an eightfold increase in the hydrolytic activity compared to the commercially available CalB immobilized on acrylic beads (Novozym 435). Thus, in terms of production and immobilization, this simplified preparation of immobilized CalB does not compromise biocatalyst performance. On the contrary, the achieved and still unoptimized procedure gives an improved biocatalyst. CalB can be efficiently immobilized by hydrophobic adsorption on an acrylic resin, in contrast to most enzymes. Because an improved biocatalyst was achieved with the standardized binding method used in the EziG technology and compared with the excellent case of hydrophobic CalB immobilization, these results indicate that the simple method used (Figure 1) can be applied to give efficient immobilized enzyme preparations for a wide variety of enzymes.

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All authors have given approval to the final version of the manuscript.

## Notes

The authors declare no competing financial interest.

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## REFERENCES

- (1) Ferreira-Leitão, V.; Cammarota, M.; Aguiéras, E. G.; de Sá, L. V.; Fernandez-Lafuente, R.; Freire, D. *Catalyst* **2017**, *7*, 9.
- (2) Lancaster, M. *Green Chemistry: An Introductory Text*, 2nd ed.; The Royal Society of Chemistry: Cambridge, 2010.
- (3) Patkar, S. A.; Björklund, F.; Zundel, M.; Schulein, M.; Svendsen, A.; Heldt-Hansen, H. P.; Gormsen, E. *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **1993**, *32*, 76–80.
- (4) Anderson, E. M.; Larsson, K. M.; Kirk, O. *Biocatal. Biotransform.* **1998**, *16*, 181–204.
- (5) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 1999.
- (6) Kirk, O.; Christensen, M. W. *Org. Process Res. Dev.* **2002**, *6*, 446–451.
- (7) Uppenberg, J.; Hansen, M. T.; Patkar, S.; Jones, T. A. *Structure* **1994**, *2*, 293–308.
- (8) Adlercreutz, P. *Chem. Soc. Rev.* **2013**, *42*, 6406–6436.
- (9) Petkar, M.; Lali, A.; Caimi, P.; Daminati, M. *J. Mol. Catal. B Enzym.* **2006**, *39*, 83–90.
- (10) Hult, K.; Berglund, P. *Trends Biotechnol.* **2007**, *25*, 231–238.
- (11) Humble, M. S.; Berglund, P. *Eur. J. Org. Chem.* **2011**, 3391–3401.
- (12) Homann, M. J.; Vail, R.; Morgan, B.; Sabesan, V.; Levy, C.; Dodds, D. R.; Zaks, A. *Adv. Synth. Catal.* **2001**, *343*, 744–749.
- (13) Rotticci-Mulder, J. C.; Gustavsson, M.; Holmquist, M.; Hult, K.; Martinelle, M. *Protein Expr. Purif.* **2001**, *21*, 386–392.
- (14) Jaeger, K.-E.; Eggert, T. *Curr. Opin. Biotechnol.* **2002**, *13*, 390–397.
- (15) Park, S.; Forró, E.; Grewal, H.; Fülöp, F.; Kazlauskas, R. J. *Adv. Synth. Catal.* **2003**, *345*, 986–995.
- (16) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*, 2nd ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2006.
- (17) Shimada, Y.; Watanabe, Y.; Samukawa, T.; Sugihara, A.; Noda, H.; Fukuda, H.; Tominaga, Y. *J. Am. Oil Chem. Soc.* **1999**, *76*, 789–793.
- (18) Ong, A. L.; Kamaruddin, A. H.; Bhatia, S. *Process Biochem.* **2005**, *40*, 3526–3535.
- (19) Kim, S. C.; Kim, Y. H.; Lee, H.; Yoon, D. Y.; Song, B. K. *J. Mol. Catal. B Enzym.* **2007**, *49*, 75–78.
- (20) Höegh, I.; Patkar, S.; Halkier, T.; Hansen, M. T. *Can. J. Bot.* **1995**, *73*, 869–875.
- (21) Tamalampudi, S.; Talukder, M. M. R.; Hama, S.; Tanino, T.; Suzuki, Y.; Kondo, A.; Fukuda, H. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 387–395.
- (22) Liu, D.; Schmid, R. D.; Rusnak, M. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1024–1032.
- (23) Blank, K.; Morfill, J.; Gump, H.; Gaub, H. E. *J. Biotechnol.* **2006**, *125*, 474–483.
- (24) Jung, S.; Park, S. *Biotechnol. Lett.* **2008**, *30*, 717–722.
- (25) Larsen, M. W.; Bornscheuer, U. T.; Hult, K. *Protein Expr. Purif.* **2008**, *62*, 90–97.
- (26) Narayanan, N.; Chou, C. P. *Appl. Environ. Microbiol.* **2009**, *75*, 5424–5427.
- (27) Seo, H.-S.; Kim, S.-E.; Han, K.-Y.; Park, J.-S.; Kim, Y.-H.; Sim, S. J.; Lee, J. *Biochim. Biophys. Acta, Proteins Proteomics* **2009**, *1794*, 519–525.

- (28) Hong, S. Y.; Jung, E. J.; Joo, J. C.; Yoo, Y. J. *Biotechnol. Bioprocess Eng.* **2012**, *17*, 687–692.
- (29) Kim, S.-K.; Park, Y.-C.; Lee, H. H.; Jeon, S. T.; Min, W.-K.; Seo, J.-H. *Biotechnol. Bioeng.* **2015**, *112*, 346–355.
- (30) Ujiie, A.; Nakano, H.; Iwasaki, Y. J. *Biosci. Bioeng.* **2016**, *121*, 303–309.
- (31) Kim, S. Y.; Sohn, J. H.; Pyun, Y. R.; Yang, I. S.; Kim, K. H.; Choi, E. S. *J. Microbiol. Biotechnol.* **2007**, *17*, 1308–1315.
- (32) Vadhana, A. K. P.; Samuel, P.; Berin, R. M.; Krishna, J.; Kamatchi, K.; Meenakshisundaram, S. *Enzyme Microb. Technol.* **2013**, *52*, 177–183.
- (33) Eom, G. T.; Lee, S. H.; Song, B. K.; Chung, K.-W.; Kim, Y.-W.; Song, J. K. *J. Biosci. Bioeng.* **2013**, *116*, 165–170.
- (34) Yang, J.-K.; Liu, L.-Y.; Dai, J.-H.; Li, Q. *PLoS One* **2013**, *8*, No. e53939.
- (35) Zhang, N.; Suen, W.-C.; Windsor, W.; Xiao, L.; Madison, V.; Zaks, A. *Protein Eng., Des. Sel.* **2003**, *16*, 599–605.
- (36) Suen, W.-C.; Zhang, N.; Xiao, L.; Madison, V.; Zaks, A. *Protein Eng. Des. Sel.* **2004**, *17*, 133–140.
- (37) Emond, S.; Montanier, C.; Nicaud, J. M.; Marty, A.; Monsan, P.; André, I.; Remaud-Siméon, M. *Appl. Environ. Microbiol.* **2010**, *76*, 2684–2687.
- (38) Cereghino, J. L.; Cregg, J. M. *FEMS Microbiol. Rev.* **2000**, *24*, 45–66.
- (39) Andryushkova, A.; Glieder, A. Expression hosts for protein discovery and production. In *Biocatalysis for the Pharmaceutical Industry: Discovery, Development and Manufacturing*; Tao, J. A., Lin, G.-Q., Liese, A., Eds.; Wiley-WCH Verlag GmbH & Co. KGaA: Singapore, 2009; pp 21–44.
- (40) Seo, H.-S.; Kim, S.-E.; Han, K.-Y.; Park, J.-S.; Kim, Y.-H.; Sim, S. J.; Lee, J. *Biochim. Biophys. Acta, Proteins Proteomics* **2009**, *1794*, 519–525.
- (41) Xu, Y.; Lewis, D.; Chou, C. P. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 1035–1044.
- (42) Jung, S.; Park, S. *Biotechnol. Lett.* **2008**, *30*, 717–722.
- (43) Xu, Y.; Yasin, A.; Wucherpennig, T.; Chou, C. P. *World J. Microbiol. Biotechnol.* **2008**, *24*, 2827–2835.
- (44) Mergulhão, F. J. M.; Summers, D. K.; Monteiro, G. A. *Biotechnol. Adv.* **2005**, *23*, 177–202.
- (45) Engemark Cassimjee, K.; Kadow, M.; Wikmark, Y.; Svedendahl Humble, M.; Rothstein, M. L.; Rothstein, D. M.; Bäckvall, J.-E. *Chem. Commun.* **2014**, *50*, 9134–9137.
- (46) Gawin, A.; Valla, S.; Brautaset, T. *Microb. Biotechnol.* **2017**, *10*, 702–718.
- (47) Blatny, J. M.; Brautaset, T.; Winther-Larsen, H. C.; Karunakaran, P.; Valla, S. *Plasmid* **1997**, *38*, 35–51.
- (48) Sletta, H.; Tondervik, A.; Hakvag, S.; Aune, T. E. V.; Nedal, A.; Aune, R.; Evensen, G.; Valla, S.; Ellingsen, T. E.; Brautaset, T. *Appl. Environ. Microbiol.* **2007**, *73*, 906–912.
- (49) Holmquist, M.; Martinelle, M.; Clausen, I. G.; Patkar, S.; Svendsen, A.; Hult, K. *Lipids* **1994**, *29*, 599–603.
- (50) Rotticci, D.; Norin, T.; Hult, K.; Martinelle, M. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2000**, *1483*, 132–140.
- (51) Martinelle, M.; Holmquist, M.; Hult, K. *Biochim. Biophys. Acta Lipids Lipid. Metabol.* **1995**, *1258*, 272–276.