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Molecular Cloning and Expression of *Candida antarctica* lipase B in *Corynebacterium* genus

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This study, for the first time, reports the functional expression of lipase B derived from the yeast *Candida antarctica* (CALB) in *Corynebacterium* strain using the *Escherichia coli* plasmid PK18. The CALB gene fragment encoding a 317-amino-acid protein was successfully obtained from the total RNA of *C. antarctica*. CALB was readily produced in the *Corynebacterium* strain without the use of induction methods described in previous studies. This demonstrated the extracellular production of CALB in the *Corynebacterium* strain. CALB produced in the *Corynebacterium* MB001 strain transformed with pEC-CALB recombinant plasmid exhibited maximum extracellular enzymatic activity and high substrate affinity. The optimal pH and temperature for the hydrolysis of 4-nitrophenyl laurate by CALB were 9.0 and 40℃, respectively. The enzyme was stable at pH 10.7 in the glycine-KOH buffer and functioned as an alkaline lipase. The CALB activity was inhibited in the presence of high concentration of Mg^{2+}, which indicated that CALB is not a metalloenzyme. These properties are key for the industrial application of the enzyme.

Keywords: *Candida antarctica* lipase B, *Corynebacterium* genus, protein engineering, extracellular enzymatic activity, biochemical characterization

Introduction

For the production of heterologous proteins, several cell factories have been developed and applied for different purposes. Each system has its own advantages, but also possible drawbacks. No doubt, a major advantage of using Gram-positive bacteria is that target proteins, when equipped with the necessary secretion system, can be secreted outside of the cell enabling correct folding and accompanying functionality as such facilitating downstream processing. As secretion yield is very important to make the process economically viable, several attempts to improve the secretion process have been reported [1].

With this purpose, the engineering of the Lipase B from *Candida antarctica* (CALB) is an urgent issue, necessary to obtain better properties by protein engineering approaches. It is therefore an attractive and challenging goal for many researchers. CALB is a macromolecule with a molecular mass of about 33 kDa and 317 amino acid residues in its polypeptide chain [2]. This extracellular enzyme is one of the most widely used biocatalysts [3].

CALB has been cloned and expressed in different hosts, such as *Aspergillus oryzae*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica* [4]. However, to date there are no reports describing the gene cloning and level expression of CALB in *Corynebacterium* genus. Several problems, including generation of insoluble proteins in *E. coli*, low secretion efficiency in *S. cerevisiae*, and poor transformation efficiency in *P. pastoris*, have limited the develop-

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ment of efficient screening protocols and thus the identification of CALB variants with interesting properties [3]. On the other hand, gram-positive bacteria are considered to be more suitable for whole-cell catalysts and whole-cell adsorbents because of the robust structure of their cell walls.

Studies in *E. coli* have shown that the ability to control the activity of the promoter is an absolute prerequisite for maintaining a stable expression plasmid in the host [5]. These studies were based on the *Escherichia coli* plasmids pK18 and pK19 by combining the useful properties of the pK plasmids (e.g., multiple cloning site, lacZα fragment, sequencing with M13 primers) with the broad-host-range transfer machinery of plasmid RP4. New pK derivatives can be transferred by RP4-mediated conjugation into a wide range of Gram negative and positive bacteria and should facilitate gene disruption and allelic exchange by homologous recombination [6] .

Corynebacterium genus is asporogenous Gram-positive bacteria with moderately high G+C content. A wild type of these bacteria is widely used as producers of amino acids. Recently, *Corynebacterium glutamicum* has attracted attention as a potential cell factory for the production of recombinant proteins since it exhibits numerous ideal features for protein secretion [7, 8]. First, as a Gram-positive bacterium, it has a single cellular membrane, which allows proteins to be secreted into the extracellular medium by simply crossing a single membrane barrier. Second, *C. glutamicum* secretes only a few endogenous proteins into the culture medium, which allows for the simple purification of target proteins in downstream process compared to the protein production and purification processes of other bacterial hosts such as *Escherichia coli*. Third, secreted proteins from *C. glutamicum* can be kept stable because extracellular protease activity is rarely detectable [9]. Fourth, it is capable of much more protein secretion than the dozen or so documented to date [10]. All these characteristics make *C. glutamicum* a very favorable and versatile host for heterologous protein productions.

There had been only few reports concerning heterologous protein secretion in *C. glutamicum* [10–12], but recent studies indicated that *C. glutamicum* indeed possesses a variety of secretory proteins [8, 13, 14]. Based on secretome analysis of *C. glutamicum*, Yim *et al.* [9] identified one major protein encoded by cg1514 and

demonstrated that a secretome analysis can be a useful method for discovering overexpressed proteins in extracellular medium.

Taking into account all of the above mentioned ideas, in the present investigation we report the successful cloning, expression and partial biochemical characterization of CALB produced in culture supernatant from *C. antarctica* in Coryneform strain expression system.

Materials and Methods

Bacterial strains, plasmids, chemicals, media and growth conditions

Candida antarctica strain, *C. glutamicum* S9114, *C. glutamicum* MB001, *C. ammoniagenes* ATCC 6872, *Escherichia coli* DH5α cells and plasmid pUC18 used in this study were purchased from TSINGKE Company. All CALB expression systems were designed and constructed on the basis of the wild type of *Corynebacteria* strains that encoded one major protein from efficient protein secretion.

Candida antarctica was routinely cultivated at 200 rpm and 30℃ in Luria-Bartani (LB) medium (per 1 L distilled sterile water): 10 g tryptone, 5 g yeast extract, 10 g NaCl and 50 mg kanamycin. Epo medium was used for Corynebacterium strains growth and electroporation of competent cells [6] (per 100 ml LB medium): 1 g tryptone, 0.5 g yeast extract, 1 g NaCl, 400 mg isonicotinic acid hydrazide (isoniazid), 2.5 g glycine and 0.1 ml Tween 80.

BHIS (Brain Heart Infusion, Sorbitol) medium (per 1 L distilled sterile water): 5 g tryptone, 5 g NaCl, 2.5 g yeast extract, 18.5 g Brain Heart Infusion powder (Difco), all autoclaved together, and separately autoclaved 91 g sorbitol [16]. LBHIS plates for growth of transformants were composed of BHIS medium, adding (per 1 L) 15 g agar and 25 mg kanamycin.

CGXII medium [9] for the expression of CALB in shake flasks was composed of (per 1 L distilled sterile water): 40 g glucose, 20 g $(NH4)_2SO_4$, 5 g urea, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 42 g 3-morpholino-1-propanesulfonic acid (MOPS), 10 mg $CaCl_2$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 310 μg $CuSO_4 \cdot 5H_2O$, 20 μg $NiCl_2 \cdot 6H_2O$, 200 μg Biotin, 30 mg Protocatechuic acid (PCA) and 25 mg Kanamycin. 2 M KOH was used to adjust pH 7.0 [17].

Cloning and sequencing of lipase gene

Candida antarctica cells were harvested after overnight culture in LB medium. Chromosomal DNA was obtained using TIANamp Genomic DNA Kit (DP304, TIANGEN China) according to manufacturer protocol and amplified by PCR. All PCR experiments were performed using Thermal Cycler S1000™ (Biorad, USA). Briefly, 50 µl PCR reaction mixture included 25 µl I-5™ 2X High Fidelity Master Mix, 2 µl plasmid template, 2 µl forward primer and 2 µl reverse primer (primer sequences are available in Table 1). For the initial denaturation, the mixture was first heated to 98℃ for 2 min. This was followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 60℃ for 15 s, elongation at 72℃ for 1 min per kb of template length with the final elongation step at 72℃ for 5 min.

DNA fragments were ran in 1% agarose gel and the band with 6–7 kb range identified by molecular weight marker was carefully cut from the gel. It was purified by GeneJET Gel Extraction Kit (Thermo Scientific, USA) followed by Gibson Assembly Master Mix (New England Biolabs, China) for successful assembly of DNA fragments.

For secretory production, two plasmids were constructed for CALB expression: pEC-CALB and pEC-H36-CALB. For pEC-CALB, M-13F and M-13R primers containing SacI and XbaI restriction sites were used. Plasmid pEC-H36-CALB was made based on pEC-CALB and pK18-lysC vector using V-F and V-R. PH36 fragment was extended in front of the cg1514 signal sequence by PCR with primers H36-F and H36-R. Plasmids design was conducted using the sequence analysis software SnapGene Viewer (Version 3.1.2.0, GSL Biotech LLC, USA) as shown in Figure A in supplementary material. All sequences used in this study are listed in Table 1. Recombinant plasmids were transformed by heat shock with competent *E. coli* DH5α cells. Colonies containing clones with insert were selected to check for the correct insert size and analyzed by agarose gel electrophoresis. New plasmids were extracted by Mini Plasmid Extraction Kit and samples were sent to a sequencing laboratory. DNA concentration and purity were determined by measuring absorbance at 260 nm and 280 nm using a Multiskan GO Thermo Scientific, version 1.00.40, USA.

Preparation of bacterial competent cells and transformation

Transformation of Corynebacterium competent cells was carried out using the procedure of M. E. van der

Table 1. Bacterial strains and plasmids used in this study.

Material used	Relevant characteristic	Reference or source
Strain		
C. glutamicum	Wild type	S9114
C. glutamicum	Wild type	MB001
C. ammoniagenes	Wild type	ATCC6872
E Coli DH5α	F- φ80lacZ ΔM15 Δ *(lac ZYA-argF)endA1, recA1hsdR17 (r_k- m_k+) supE44 λ, thi-1 gyrA96 rel A1, phoA*	TIANGEN BIOTECH CE1200227
Plasmids		
pEC-CALB	7023bp Linearized vector plasmid containing an *E. coli* origin of replication, kan and PCR fragment of cg1514 (5782–5877)	This work
pEC-H36-CALB	7112bp containing plasmid Pec-CALB and H36 promoter	Yim *et al.* [2016]
Primers		
M13F	5'- ATGTTAAACAGAGTCAGTCGTATTGCAGG -3'	This work
M13R	5'- CTCCTGATCATGTAGGTGTCCTGAAA -3'	This work
H36-F	5'- GACACCTACATGATCAGGAGCAAAAGCTGGGTACCTCTATCTG -3'	This work
H36-R	5'- CGACTGACTCTGTTTAACATGGATCCCATGCTACTCCTACC -3'	This work
V-F	5'- CTCCTGATCATGTAGGTGTCCTGAAA -3'	
V-R	5'- ATGTTAAACAGAGTCAGTCGTATTGCAGG -3'	

Rest *et al.* [16]. Briefly, *Corynebacterium* genus was inoculated in LB medium with 2% (w/v) glucose, cultivated overnight and used for the inoculation of Epo medium. The latter was centrifuged and *Corynebacteria* cells were stored in 10% (v/v) ice-cold glycerol at -70℃. Thawed cells were mixed with DNA and electroporated using a Gene Pulser Xcell electroporation system (Bio-Rad, USA). After that, optimal heat transfer was conducted at 46℃ in BHIS medium. Recovery and expression of the antibiotic resistance marker was assured by incubation at 30℃, 300 rpm for 3 h in a dry block (Thermomixer F1.5) and *Corynebacteria* cells possessing the plasmid were selected on solid LBHIS plates.

Expression of CALB in shake flasks

Different recombinant *Corynebacteria* colonies from LBHIS plates were used to inoculate CGXII medium. 40 ml cells was cultivated in a shaker incubator (SUKUN SKY-211B, China) at 30℃ and 200 rpm for 72 h. Controls from different *Corynebacteria* were run to enable comparison of results.

Protein quantification was conducted using the BCA (bicinchoninic acid) protein assay kit (LEAGENE, China). A 96 well plate procedure was adopted according to the manufacturer's instructions and Bovin Serum Albumin (BSA) was used as standard. Absorbance was read at 562 nm in Multiskan.

SDS-PAGE analysis

For quantification of the expressed lipase, the culture supernatant was analyzed using SDS-PAGE on a 5% acrylamide stacking gel and 10% separating gel using a Mini-gel system (Bio-Rad). Gels were cast with 0.75 mm spacers, samples were mixed with 2X reducing loading buffer and heated at 100℃ for 5 min before electrophoresis. About 2 µg/µl were deposited in each well. Proteins in the gel were stained with Coomassie brilliant blue G-250 and distained by heating in water in a microwave oven [18].

Fed-batch fermentation conditions

Bioreactor cultivation. Fed-batch cultivations were carried out using the procedure of Yim *et al.* [9]. For secretory analysis of CALB, culture medium was centrifuged at 5000 g, 4℃ for 10 min, supernatant concentrated 19 times using 10K Centricon filters and CALB

production was followed up in time by SDS-PAGE. Protein quantification was done using a Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad). For glucose quantification, supernatant was 0.2 µm filtered and injected in HPLC-RI system (SHIMADZU Instruments, CTO-20A model, Japan). Aminex HPX-87H column (Bio-Rad, Laboratories, Inc., USA) was used at 65℃ and 5 mM H_2SO_4 mobile phase at 0.8 ml/min.

Lipase activity determination with titrimetric method. This method was used to determine the enzymatic activity over time in bioreactor cultures. It is based on the titrimetric determination of free fatty acids released from triacylglycerols by lipase-catalyzed hydrolysis. Reaction mixture consisted of 1 ml tributyrin (TCI, Japan) as enzyme substrate, 3 ml of 0.05 M glycine-NaOH buffer (pH 9.0) and 0.1 ml enzyme supernatant. Flasks were incubated at 45℃, 175 rpm for 10 min and 50 ml ethanol was added to terminate the reaction. The released fatty acids were estimated by titration with 0.05 M NaOH in the presence of Phenolphthalein. Negative (without enzyme) and positive (Novozym 435, China) controls were considered. One unit activity was defined as the amount of enzyme capable of releasing 1 µmol fatty acid, per minute and under the test conditions. Lipase activity was calculated using the following formula:

$$\text{Lipase activity} = \frac{\text{Vol. of NaOH consumed (ml)} \times \text{Molarity of NaOH}}{\text{Vol. of Lipase (ml)} \times \text{Reaction Time (min)}}$$

Lipase activity assay with 4-nitrophenyl laurate. This assay was used to determinate biochemical characteristics of Lipase B from bioreactor cultivation of *C. glutamicum* MB001/pEC-CALB. The spectrophotometric method make use of synthetic substrates, which are transformed upon enzyme-catalyzed hydrolysis into spectrophotometrically detectable products. Because lipase B has maximal activity on laurate [21], 4-nitrophenyl laurate (4-NPL) was selected as substrate and the activity was assayed by a modified method [20] in 96 well plate with Multiskan, (Thermo Scientific version 1.00.40, USA). The reaction mixture consisted of 0.65 ml of 0.05 M phosphate buffer (pH 7.2), 0.1 ml enzyme supernatant and 0.1 ml of 0.025 M 4-NPL prepared in

absolute ethanol. The hydrolytic reaction was carried out at 65℃ for 30 min and then terminated adding 0.25 ml of 0.1 M Na₂CO₃. After centrifugation, absorbance was read at 410 nm and lipase activity was calculated using the following formula:

$$\text{Enzymatic activity (µmoles/min)} = \frac{V\,(\mu l)\times OD410\;nm\;(cm^{-1})}{\varepsilon\times\text{incubation time (min)}}$$

Under the conditions used, the extinction coefficient of 4-nitrophenol was $\varepsilon = 7.15\;mM^{-1}\times cm^{-1}$. One unit activity was defined as the amount of enzyme capable of releasing 1 µmol 4-nitrophenol, per minute under the assay conditions.

Effect of temperature on activity. Effect of temperature on lipase B activity produced by *C. glutamicum* MB001/pEC-CALB was studied at temperatures ranging from 30 to 80℃ in 0.05 M phosphate buffer (pH 7.2) using 4-NPL assay. Enzyme was incubated in the presence of substrate for 30 min and the activity was measured. Optimum temperature for CALB activity was determined.

Effect of pH on enzyme activity. The pH effect on CALB activity from *C. glutamicum* MB001/pEC-CALB was studied in a pH range of 3.0–9.0 using different buffer systems (50 mM): citric acid-sodium citrate buffer (pH 3.0–5.0), Phosphate Buffer Saline (PBS, pH 4.6–7.5) and Glycine-KOH buffer (pH 9.0). Activity was measured using 4-NPL assay at optimum temperature for CALB activity (40℃).

Effect of metal ions. Certain enzymes require a metal ion for their optimal activity. As reported from studies on this isolate, some metal ions could affect the enzyme activity at determinate concentration [25].

The effect of two concentrations (1 and 10 mM) of various metal ions on CALB activity of *C. glutamicum* MB001/pEC-CALB was investigated using Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+}. The enzyme was treated with the latter at 40℃ for 30 min in 0.05 M phosphate buffer (pH 7.2). The relative activity was measured under the 4-NPL standard assay conditions. Sample without the addition of metal ions and surfactants was taken as control (100%) and all samples were analyzed in duplicates.

Results

Cloning, expression and Sequence Analysis of CALB gene

The new obtained fragment encoded a putative protein of 317 amino acids with a molecular mass of 33 kDa and fragment size of 7023 bp (Fig. B, Supplementary material). Successful cloning was confirmed by sequencing of the isolated plasmids and analysis in 1% agarose gel. A multisequence alignment using BLAST showed that pEC-CALB had 80% amino acid sequence identity with the CALB from *C. antarctica* (LF 058) gene for lipase B, accession No Z30645.1.

Analysis of recombinant lipase from Corynebacteria genus. The absorbance values from S9114/pEC-CALB, MB001/pEC-CALB, ATCC6872/pEC-CALB, S9114/pEC-H36-CALB, MB001/pEC-H36-CALB and ATCC6872/pEC-H36-CALB were 25.58, 20.54, 13.44, 17.66, 14.12
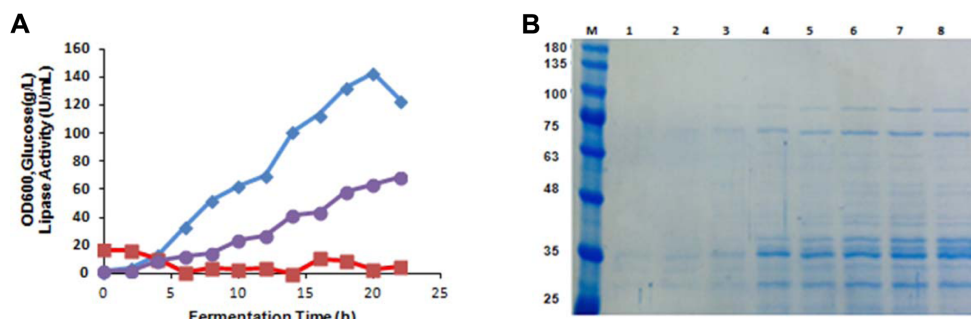

Fig. 1. Fed-batch cultivation of *C. glutamicum* MB001 producing CALB. (A) Time profiles of cell growth (◆), glucose concentration (■), and Enzymatic Activity (●) in the culture supernatant. (B) SDS–PAGE analysis of culture supernatant during fed-batch cultivation. M: molecular mass marker proteins. Lanes 1 to 8, samples at 2, 4, 6, 10, 16, 18, 20 and 22 h, respectively. At each time point, 12 µl of samples (without concentration) was loaded onto each lane. The arrowhead indicates CALB (33 kDa).
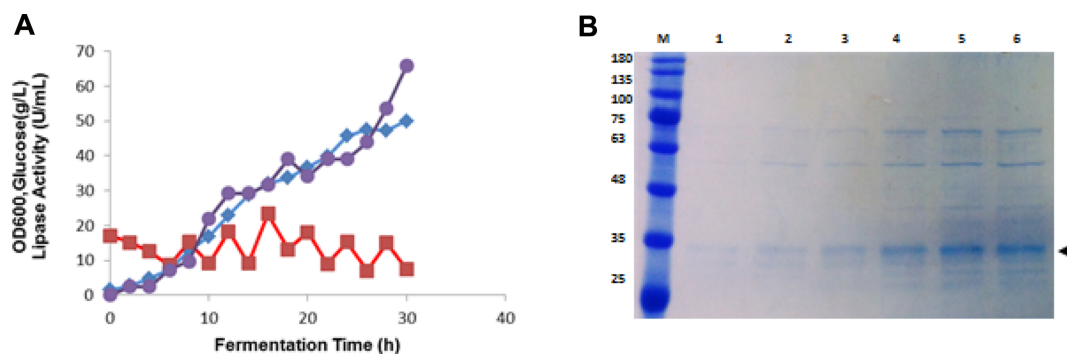
Fig. 2. Fed-batch cultivation of *C. glutamicum* S9114 producing CALB. (A) Time profiles of cell growth (◆), glucose concentration (■), and Enzymatic Activity (●) in the culture supernatant. (B) SDS–PAGE analysis of culture supernatant during fed-batch cultivation. M: molecular mass marker proteins. Lanes 1 to 6, samples at 4, 6, 10, 16, 24 and 30 h, respectively. At each time point, 12 µl of samples (without concentration) was loaded onto each lane. The arrowhead indicates CALB (33 kDa).

and 24.58 UFC/ml, respectively. Mutant lipase samples showed several bands like the controls, but also a distinctive band estimated to be approximately 35 kDa corresponding to the secretory productions of CALB. This is consistent with the predicted molecular weight of lipase (33 kDa). Comparable molecular weights have also been reported from other studies [4–6, 9, 15, 22–24]. Strain S9114 has another strong band about 50 kDa position but the corresponding sequence was not identified in the database**.** By measuring the intensities of the bands obtained in the SDS-PAGE, it was possible to estimate the CALB lipase content in supernatant (Fig. C, Supplementary material). Results for S9114/pEC-CALB, MB001/pEC-CALB and ATCC6872/pEC-CALB showed in figure C (1) were about 27%, 22% and 16% of the total protein, respectively. Of the three pEC-H36-CALB transformants that we constructed only one secreted in *C. glutamicum* MB001, as shown in Fig. C(2).

According to absorbance values and SDS-PAGE results, fed-batch cultivations were carried out for S9114/pEC-CALB and MB001/pEC-CALB. The secretory production yields were analyzed. *C. glutamicum* MB001 harboring pEC-CALB entered exponential growth at 6 h. The cells significantly increased to OD_{600} 142.6 UFC/ml for 20 h with specific growth rate of 0.4906 h^{-1} in the exponential growth phase and then decreased (Fig. 1A). In the case of fed-batch cultivation of *C. glutamicum* S9114, it continued to grow up to OD_{600} of 50 UFC/ml for 30 h with specific growth rate of 0.2661 h^{-1} in the exponential growth phase (Fig. 2A). The maximum CALB content was approximately 17 %

and 43.5% of the total extracellular proteins in MB001 (22 h) and S9114 (32 h), respectively, at the end of fermentation (Figs. 1B and 2B). The maximum production yield was about 5953.8 mg/l and 3191.8 mg/l, which means a productivity of 297.7 mg/l and 106.4 mg/l in 1h for MB001/pEC-CALB and S9114/pEC-CALB, respectively. The isolates MB001/CALB and S9114/CALB were grown in optimum culture conditions and lipase activity was observed to start soon after beginning of inoculation where the concentration gradually increased reaching a maximum activity of 68.38 U/ml and 63.44 U/ml, respectively. Taking into consideration the higher productivity and activity results of CALB from *C. glutamicum* MB001/pEC-CALB, the latter was selected for biochemical characterization of Lipase B enzyme.

Characterization of the recombinant lipase
Effect of temperature on activity. As shown in Fig. 3, the optimal temperature range was found to be from 40
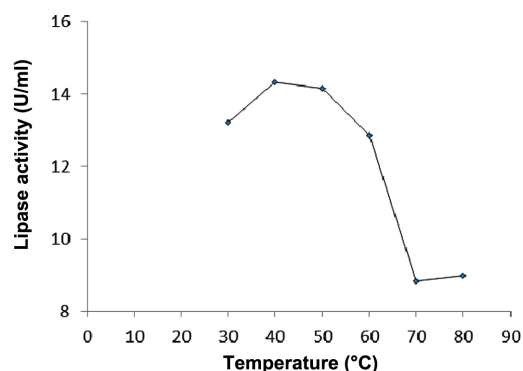

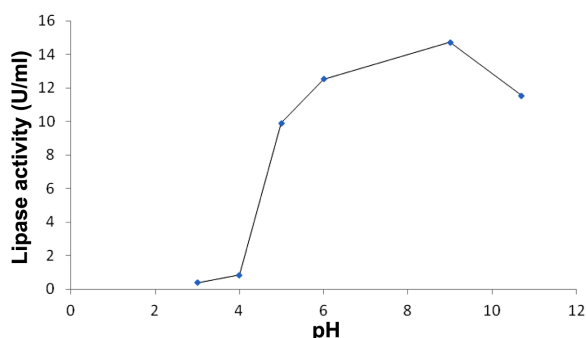**Fig. 3. Effect of temperature on the recombinant lipase.**

Fig. 4. Effect of pH on the activity of the recombinant lipase. Buffer systems: Citric acid-sodium citrate buffer (pH 3.0–5.0), PBS buffer (pH 6), and Glycine-KOH buffer (pH 9.0–10.7).

to 50℃, and the optimal activity was attained at 40℃. More than 60% of the activity was lost at temperatures above 60℃. This showed that the recombinant lipase obtained in this study was a heat-sensitive enzyme.

Optimal pH for recombinant CALB activity. The recombinant CALB was found to be most active at pH 9.0 in Glycine-KOH buffer and exhibited very low activity at pH 3–4 (Fig. 4). The activity of CALB decreased after incubation at buffer with the pH below 5, while it was relatively stable in an alkaline environment.

Effect of metal ions on the lipase activity. The effect of various chemicals on CALB enzymatic activity is shown in Table 2 with 0.05 standard deviation of duplicated samples. It was not observed a significant effect using different concentrations (1 mM and 10 mM) of Fe^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+}. CALB activity was only inhibited in the presence of high concentration of Mg^{2+}, indicating that CALB is not a metalloenzyme.

Table 2. Effect of metal ions on the activity of recombinant lipase.

Metal ions	% Relative activity (1 mM)	% Relative activity (10 mM)
Control	100	100
MgSO$_4$	92	69
FeSO$_4$	91	97
MnSO$_4$	96	91
ZnSO$_4$	101	96
CaCl$_2$	102	101

Discussion

This study demonstrates the ability of extracellular production of CALB in Coryneforms strain like others [4, 28].

The best results were obtained from the transformants in *C. glutamicum* S9114 and MB001. This is a favorable host for protein production and secretion because it lacks extracellular proteolytic activities [15]. The sequence analysis and the molecular weight measurement of recombinant CALB confirmed the integrity of the structure.

CALB was readily produced without the use of induction methods described in previous papers [4, 6]. Our expression system was based in three important elements: Conjugation mediated by the broad-host-range (bhr), the most efficient system to introduce foreign DNA [17], the Cg1514 signal sequence directly fused to the mature portion of CALB, and the good culture medium. Their combination resulted in a successful CALB production.

A recent study compared the secretion capacity of different extracellular proteins in five types of yeasts and the results showed that *Saccharomyces cerevisiae* had lower secretion capacity than other yeasts such as *Pichia pastoris*, *Kluyveromyces lactis* and *Yarrowia lipolytica*. Longer incubation time was consequently needed before first detection of lipase activity in *Saccharomyces cerevisiae* (7 days compared to 2 days for *Pichia pastoris*). This has been attributed to the culture conditions, where the protein secretion requirements were minimal and several process parameters affected the production of recombinant proteins in yeasts. Good optimization of growth conditions should have considered how the recombinant strain was constructed. These factors combined, we can note that secretion system of *C. glutamicum* MB001 mutant grown in properly-optimized culture conditions remains efficient and advantageous for use in high-scale CALB production [34].

The use of the Cg1514 signal sequence for secretory expression is one major reason for the successful production. In his article [9], Yim demonstrated the feasibility of the Cg1514 based system for the secretion of proteins with a broader range of molecular weights examining different proteins.

Cells were grown and studied in CGXII and BHI, where CGXII was the best medium because the cells grew more than BHI and the bands were clearly observed. *C. glutamicum*, when cultured on defined CGXII minimal medium with glucose as single carbon source, has revealed more than 800 genes that exhibited full-length or almost full-length antisense transcripts [11]. It became the standard for Metabolic Engineering and Systems Biology with *C. glutamicum* and has been cited over 250 times [26].

In fermenter process, CALB was successfully secreted in the culture medium with high efficiency. The maximum production yield was about 6 g/l and 3.2 g/l from MB001/pEC-CALB and S9114/pEC-CALB, respectively. These results were higher compared to values reported in *C. glutamicum* as a host [9, 27]. The enzymatic activity was higher too, reaching values of 68.38 and 63.44 U/ml in short time. Most studies in the literature reported lower lipase activity values in fed-batch fermentation after 100 h [4, 28]. Very few represented higher values and were obtained with the assistance of additional factors.

The characterization of lipase B from *C. antarctica*, tested in the presence of 4-NPL substrate, was conducted in different conditions and was compared with various expression strategies. CALB was stable at pH 10.7 compared with *Acinetobacter* species SY-01 lipase (pH 9–11) [29]. Optimum value was at pH 9 similar to *Bacillus* sp LBN2 [30]. Besides, the optimum temperature (40℃) is similar to *Bacillus amyloliquefaciens* PS35 lipase [31]. When the temperature was higher than 60℃, the thermostability of the lipase enzyme was reduced more than 60%, indicating that the recombinant lipase is moderately thermostable enzyme. These results showed that lipase production is species specific and has its own temperature optima which favored maximum enzyme production [32].

An overview of current literature demonstrates that it is difficult to predict a priori how one or another metal will influence the enzyme. This depends not only on the nature of the metal and the enzyme, but also on other specific conditions. The most important of those are various factors like the concentration of the metal [33]. During this study, it was not observed a significant effect in the concentration of metals indicating that CALB is not a metalloenzyme.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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