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# High-level production of *Bacillus cereus* phospholipase C in *Corynebacterium glutamicum*

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ABSTRACT

Enzymatic oil degumming (removal of phospholipids) using phospholipase C (PLC) is a well-established and environmentally friendly process for vegetable oil refining. In this work, we report the production of recombinant *Bacillus cereus* PLC in *Corynebacterium glutamicum* ATCC 13869 in a high cell density fermentation process and its performance in soybean oil degumming. A final concentration of 5.5 g/L of the recombinant enzyme was achieved when the respective gene was expressed from the *tac* promoter in a semi-defined medium. After treatment with trypsin to cleave the propeptide, the mature enzyme completely hydrolyzed phosphatidylcholine and phosphatidylethanolamine, which represent 70% of the phospholipids present in soybean oil. The results presented here show the feasibility of using *B. cereus* PLC for oil degumming and provide a manufacturing process for the cost effective production of this enzyme.



## 1. Introduction

Phospholipases C (PLC) are hydrolytic enzymes that cleave phospholipids into 1,2-diacylglycerol (1,2-DAG) and phosphate monoesters, and have been isolated from a variety of microorganisms including *Streptomyces*, *Clostridium* and *Bacillus* (Bai et al., 2010; Nygren, 1962; Ottolenghi, 1965). The PLC from *B. cereus* (BC-PLC) is a secreted monomeric protein of 28.5 KDa. Mature BC-PLC is the result of two processing events, the cleavage of the 24-amino acid presequence by the type I signal peptidases during export and the removal of the 14 amino acid prosequence, made by unidentified extracellular proteases to give the functional enzyme with 245 AA (Johansen et al., 1988).

The BC-PLC has a broad substrate specificity (Martin et al., 2000) and it has received considerable attention due to its capacity to efficiently hydrolyze phosphatidylcholine (PC) and phosphatidylethanolamine (PE), two of the most abundant phospholipids present in vegetable oils (Szuhaj, 1989). This specificity makes BC-PLC an interesting candidate to be used for the enzymatic degumming of edible oils at industrial scale, an environmentally friendly process that removes phospholipids and increases the recovery of oil during the refining stage. In this process, an extra-yield is obtained as a consequence of (i) the generated 1,2-DAGs that are miscible with triacylglycerols (TAGs) and (ii) the lower amount of TAGs trapped by the reduced volume of gums (Dijkstra, 2010; Dijkstra and Van Opstal, 1989; Yang et al., 2008).

*B. cereus* is a pathogenic microorganism and therefore heterologous expression of the *plc* gene is desirable to establish a safe industrial production process. Production of recombinant BC-PLC in *Pichia pastoris* and *Bacillus subtillis* has been reported under laboratory conditions (Durban et al., 2007; Seo and Rhee, 2004), although the obtained yields were far below the required to establish a cost-effective process. Expression experiments were also made in *Escherichia coli* (Tan et al., 1997), where the recombinant protein formed inclusion bodies, making its recovery in an active form problematic since it requires multiple steps, including a large volume refolding process. Thus, a host capable of providing high quantities of BC-PLC from a cost-effective robust fermentation process is desirable.

*Corynebacterium glutamicum* is a gram-positive, nonpathogenic and nonsporulating bacterium that has been used for decades in the industrial production of amino acids such as glutamate and lysine for human food, animal feed and pharmaceutical industry

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(Hermann, 2003). Because of its robust growth in fermenters using inexpensive substrates and the lack of production of hazardous toxins and extracellular proteases, it is considered an attractive host for the production of recombinant proteins for both pharmaceutical and industrial use (Eggeling and Bott, 2005; Kumagai, 2000; Tateno et al., 2007; Yukawa and Inui, 2007). Like other bacteria, *C. glutamicum* possesses two-protein secretory pathways. The Sec-system catalyzes the transmembrane translocation of proteins in their unfolded conformation while the Tat-pathway promotes the translocation of folded secretory proteins. In both cases, N-terminal sequences direct the proteins to these pathways (Natale et al., 2008). *C. glutamicum* has been used to produce several proteins at high yields, including transglutaminases (Date et al., 2004), amylases (Watanabe et al., 2009), proteases (Billman-Jacobe et al., 1995), xylanases (Adham et al., 2001), glucanases (Paradis et al., 1987) and recombinant proteins for therapeutic use (Salim et al., 1997; Yim et al., 2014). Thus we decided to investigate this microorganism to be used as a host for the production of BC-PLC.

Recently, we created a plasmid-based platform to examine and classify synthetic DNA parts comprising of promoters, ribosome binding sites (RBS) and secretion sequences that provide high level expression and secretion of recombinant proteins in *C. glutamicum* (Ravasi et al., 2012). Here, we used selected synthetic elements to engineer this host for the efficient production of BC-PLC, and we developed a high cell density fed batch fermentation for the industrial production of this enzyme to be used in oil degumming processes.

## 2. Materials and methods

### 2.1. Strains, plasmids, and growth conditions

The bacterial strains used were *E. coli* DH5α (Hanahan, 1985) and *C. glutamicum* ATCC 13869. *E. coli* was grown in LB (Maniatis et al., 1982) at 200 rpm and 37 °C and *C. glutamicum* was grown in BHI-Sorbitol (brain–heart infusion from Difco Laboratories, supplemented with 0.5 M sorbitol) at 200 rpm and 30 °C. The concentration of kanamycin used in the study was 50 mg/L for *E. coli* and 25 mg/L for *C. glutamicum*. Plasmids used in this work are listed in Table 1.

### 2.2. DNA preparation and PCR techniques

Phusion High-Fidelity DNA Polymerase, restrictions enzymes, T4 ligase and DNA ladders were purchased from New England Biolabs. Plasmid DNA was prepared by using the Axygen Biosciences Axy-Prep™ Plasmid Minipreps Kit. DNA sequencing was performed by Macrogen (Korea).

In order to obtain the pTGR100 construction, *B. cereus* PLC was amplified by PCR using the primers PLCBCF (5′-GAGCATAT GAAAAAGAAAGTACTTGC-3′) and PLCBCR3 (5′-GAGCCTAGGTTAACGATCTC CGTACG-3′) and *B. cereus* genomic ATCC 14579 DNA as template. pTGR101 and pTGR102 constructions were obtained by crossover PCR. For the pTGR101 a first PCR was made in order to amplify the signal sequence using the primers CSPBF (5′-TTTTTCATATGTTTAACAA CCGTATC-3′) and CSPBZOEPLCPROR (5′-GTCCCCCATCATTTTCATGAGCG AATGCTGGGATAGC-3′) with *C. glutamicum* genomic DNA as template. A second PCR to amplify the PLC gene was performed with the primes PLCPROF (5′-CATGAAAATGATGGGGGACAGAG-3′) and PLCBCR3 (5′-GAGCCTAGGTTA ACGATCTCCGTACG-3′) and *B. cereus* genomic ATCC 14579 DNA as template. Then a final PCR, using the primers CSPBF and PLCBCR3 and the former PCR product as template, was done to obtain the final construction.

For the pTGR102 construction, a first PCR was made in order to amplify the signal sequence using the primers PHODBSF (5′-GAGCATATGGCATAC GACAGTCG-3′) and PHODZOEPLCPROR (5′-GTCCCCCATCATTTTCA TGAGCATTTACTTCAAAGGCC-3′) with *Bacillus subtilis* JH642 genomic DNA as template. A second PCR was performed to amplify the PLC gene with the primers PLCPROF and PLCBCR3 and *B. cereus* genomic ATCC 14579 DNA as template. Then a final PCR, using the primers PHODBSF and PLCBCR3 and the former PCR product as template, was done to obtain the final construction. The pTGR103 plasmid was synthesized by Genescript (NJ, USA).

### 2.3. Preparation of competent cells and transformation

Preparation of competent cells and transformation of *E. coli* and *C. glutamicum* were performed according to the protocols described (Eggeling and Bott, 2005; Sambrook et al., 1982).

### 2.4. SDS PAGE PLC quantification

Culture supernatants were separated by SDS-PAGE on 12% gels, stained with Coomassie Brilliant blue and quantified by densitometry using a scanner and Bovine serum albumin (BSA, Sigma) as a standard. GelPro Analyzer 4.0 software was used to perform the quantitation of the scanned images.

### 2.5. Enzyme purification and activity assays

BC-PLC was recovered by precipitation using $(NH_4)_2SO_4$. 100 mL of batch culture supernatant were supplemented with 37 g $(NH_4)_2SO_4$ for 1 h at 4 °C, centrifuged and the obtained pellet was dissolved and dialyzed extensively in 20 mM sodium acetate pH 6, 10% glycerol, 1 mM $ZnSO_4$. Protein concentration was determined by bicinchoninic acid method using BSA as standard (Smith et al., 1985). To recover the protein from the fermentation supernatant the same procedure was performed using 20 mL of supernatant.

PLC activity was measured in 96 well microplates using 1 mM O-(4-Nitrophenylphosphoryl) choline (ONPC) as a substrate and 2.2 μM PLC in buffer 250 mM HEPES pH 7, 60% sorbitol, 0.1 mM $ZnSO_4$. Absorbance at 405 nm was monitored for 1 h at 50 °C. The obtained data were fitted to the integrated Michaelis–Menten rate equation to obtain the corresponding curves to determine $V_{max}/K_{m.}$ 1 PLC unit corresponds to the amount of enzyme releasing 1 nmol of *p*-nitrophenol per minute. *Bacillus cereus* PLC purchased from Sigma was used as standard.

### 2.6. Enzymatic oil degumming

Oil degumming experiments were performed using 1 kg of crude soybean oil. Crude oil was preincubated in a beaker immersed in a thermostatic bath equipped with an agitation unit which consisted of a 3-bladed propeller-type impeller (70 mm diameter) to maintain continuous stirring during the reaction. BC-PLC enzyme was pre-incubated in the presence of trypsin 2 μg/mL during 1 h at 37 °C in order to produce the PLC activation by cleaving the propeptide. 5 mg of this enzyme in 30 mL of 50 mM citrate buffer pH 6 were added to 1 kg of crude soybean oil. The mixture was emulsified using a Ultra-Turrax T50Homogenizer and continuous stirring (500 rpm) was maintained at 50 °C for 3 h. Finally, heat inactivation of the enzyme was done by incubating 10 min at 85 °C to end the reaction.

### 2.7. 1,2-DAG determinations

1,2-DAGs were determined following the AOCS recommended method (CD 11-d-96). This method quantifies DAG by HPLC

**Table 1**
List of plasmids used in this work.

| Plasmid | Characteristics | References |
|---|---|---|
| pTGR5 | Synthetic *C. glutamicum–E.coli* shuttle vector. eGFP expression controlled by *tac* promoter and *sod* RBS. $Km^R$ | Ravasi et al. (2012) |
| pTGR100 | pTGR5 carrying *B. cereus* PLC with its own signal sequence | This work |
| pTGR101 | pTGR5 carrying *B. cereus* PLC with *C. glutamicum* CspB signal sequence | This work |
| pTGR102 | pTGR5 carrying *B. cereus* PLC with *B. subtilis* PhoD signal sequence | This work |

Ravasi, P., Peiru, S., Gramajo, H., Menzella, H.G., 2012. Design and testing of a synthetic biology framework for genetic engineering of *Corynebacterium glutamicum*. Microb. Cell Fact. 11, 147.

(Agillent LC1260) coupled with evaporative ligth scattering detector (ELSD) (SEDERE model Sedex 75) using 1,2-dipalmitin (Sigma) as a standard. 20 μl of sample/standard are separated in a silica column (Chromegasphere Silica, Adsorption Phase HPLC Columns, 60 Å, ES Industries) incubated at 40 °C in the column oven and DAG's present are determined with ELSD set at gain 5, 40 °C and 3 bar for nitrogen pressure. The solvent gradient used hexane (channel A) and a hexane based solvent (channel B: hexane/isopropanol/ethyl acetate/10% formic acid, 80:10:10:1 v/v/v/v) all HPLC grade.

### 2.8. NMR analysis of crude and PLC treated oil

Oil degumming experiments were performed as described above using BC-PLC treatment for 2 h at 50 °C. Treated oil was emulsified using an Ultra-Turrax T50Homogenizer (IKA) for 1 min before taking 300 mg samples for further analysis. Oil samples were extracted with 900 μl of NMR solution (100 mM Tris–HCl pH 10.5, 50 mM EDTA, 2.5% sodium deoxycholate) during 1 h at 37 °C with constant agitation and the resulting aqueous phase was extracted with 600 μl hexane. Finally, 50 μl of $D_2O$ was added to the aqueous phase. $^{31}P$ NMR phospholipids profile was acquired using a Bruker 300 Ultrashield equipment. Samples of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol were used as standards.

### 2.9. N-terminal sequencing

Protein samples (secreted protein before or after trypsin treatment) were run on SDS PAGE, transferred to PVDF membrane (Amersham Hybond P) and stained with 0.1% Ponceau red (Sigma). Protein bands were cut for N-terminal sequencing by Edman degradation (Laboratorio Nacional de Investigación y Servicios en péptidos y proteínas, LANAIS PROEM, UBA).

### 2.10. Sonication cell lysis

Cells and culture supernatant were separated by centrifugation. Cells were resuspended in 1 mL buffer 10 mM Tris/HCl pH 8.0, 25 mM $MgCl_2$, and 200 mM NaCl and disrupted on ice in a GEX 600 Ultrasonic Processor (Sonics & Materials Inc.) for 2 min.

### 2.11. Fed batch cultures

A seed culture of *C. glutamicum* ATCC13869 harboring pTGR100 was prepared in a 1-L Erlenmeyer flask containing 0.1 L of semi-defined medium cultivated at 30 °C and 200 rpm in a shaking incubator. The semi-defined medium consists of 9 g of $K_2HPO_4$, 4 g of $KH_2PO_4$, 2 g of urea, 10 g of $(NH_4)_2SO_4$, 2 g of yeast extract, 2 g of $MgSO_4$, 200 μg of biotin, 5 mg of thiamine, 10 mg of $FeSO_4$, 1 mg of $MnSO_4$, 1 mg of $ZnSO_4$, 200 μg of $CuSO_4$, and 10 mg of $CaCl_2$ per liter, supplemented with 10 g of glucose. Kanamycin was added at a concentration of 25 mg/L to maintain the plasmid stability.

Fed-batch fermentation was carried out in a 2-L bioreactor (New Brunswick BioFlo 115, USA) containing 1 L of the same medium. The temperature and stirring was maintained at 30 °C and 1200 rpm,

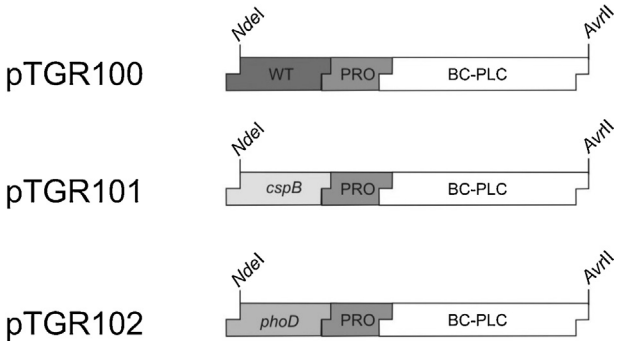


**Fig. 1.** Schematic representation of the DNA constructions expressed in this work. Signal sequences from the *C. glutamicum cspB* gene and *B. subtilis phoD* gene were used to replace the native secretory sequence in the *B. cereus* PLC ORF. PRO states 14-AA long propeptide sequence.

respectively. The pH was maintained at 7.0 by addition of 25% $NH_4OH$ and dissolved oxygen level was controlled at 30% of air saturation by changing the pure oxygen percentage when necessary. The feeding process was initiated when the glucose initially present in the medium was exhausted. A solution containing 600 g/L glucose, 2 g/L $MgSO_4 \cdot 7H_2O$ and 24 g/L of yeast extract was added using an exponential feeding rate strategy. The feeding rate ($F$, mL/h) was determined by Eq. (1) (Lee, 1996) in order to maintain the specific growth rate at 0.13 1/h.

$$F = \frac{\mu X_0 V_0 e^{(\mu t)}}{S_0 Y_{X/S}} \quad (1)$$

$X_0$ is the biomass concentration (g/L) when the feeding is started, $V_0$ is the initial volume (L), $\mu$ is the desired specific growth rate (1/h), $S_0$ is the glucose concentration in the feeding solution (g/L) and $Y_{X/S}$ is the substrate yield.

Expression of BC-PLC gene was induced when the $OD_{600}$ reached 80 by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Genbiotech, Argentina) at a final concentration of 0.5 mM. Afterwards, the feeding rate was maintained at 10 mL/h.

## 3. Results

### 3.1. Expression vector construction and secretion engineering

The ORF encoding the mature BC-PLC preceded by proenzyme and its secretory presequence was PCR-amplified from *B. cereus* genomic DNA. *Nde*I and *Avr*II sites were incorporated overlapping the starting ATG codon and immediately downstream the stop codon respectively to facilitate manipulation of the DNA. Two additional constructions were created where the secretory sequence was replaced by the one from the CspB protein from *C. glutamicum* (secreted by the Sec system), or the one from PhoD enzyme from *B. subtilis* (secreted using the Tat system); for which a high secretion efficiency in *C. glutamicum* was previously reported (Fig. 1) (Itaya and Kikuchi, 2008; Meissner et al., 2007; Salim et al., 1997; Tateno et al., 2007). The three ORFs were cloned into the *Nde*I-*Avr*II sites of the pTGR5 (Ravasi et al., 2012), a synthetic vector which contains

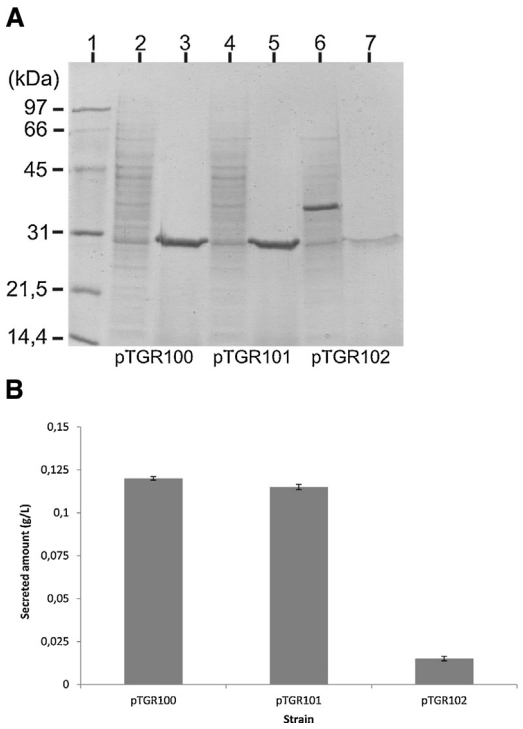


**Fig. 2.** Analysis and quantitation of BC-PLC expression. (A) SDS PAGE of pellet fractions and culture supernatants from *C. glutamicum* pTGR100 (lanes 2 and 3), pTGR101 (lanes 4 and 5), pTGR102 (lanes 6 and 7) and molecular weight marker (lane 1). (B) Densitometric analysis using GelPRO Analyzer software was used for quantification of secreted PLC driven by the different secretion signals (lanes 3, 5 and 7).

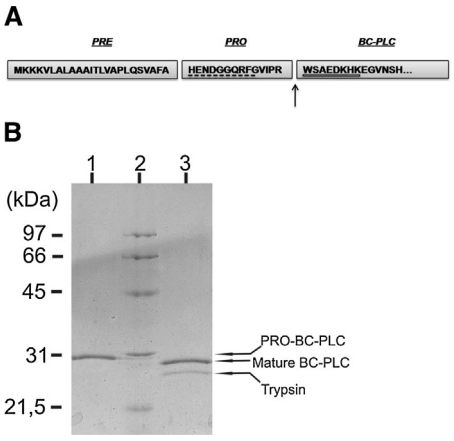


**Fig. 3.** N-terminal sequence analysys of BC-PLC expressed in C. glutamicum ATCC 13869. (A) N-terminal amino acid sequence was analyzed by Edman degradation method. Dashed line indicates the N-terminal sequence obtained for the secreted PLC and double line indicates the aminoacid sequence for trypsin digested samples. Trypsin cleavage site is marked with an arrow. (B) SDS PAGE analysis of induced culture supernatant of *C. glutamicum* pTGR100. Lane 1 Secreted Pro-BC-PLC. Lane 2 MWM. Lane 3 BC-PLC (without its pro sequence) after trypsin treatment.

the IPTG-inducible *tac* promoter and the RBS from the *C. glutamicum sod* gene, to obtain the pTGR100, pTGR101 and pTGR102 plasmids respectively. The *tac* promoter and *sod* gene RBS were chosen based on previous experiments that demonstrated high expression of a reporter gene when these regulatory elements were used (Ravasi et al., 2012).

The three expression plasmids were used to transform *C. glutamicum* ATCC13869, a strain widely used for industrial fermentations (Bona and Moser, 2004; Kimura et al., 1999; Ohnishi et al., 2003; Ohnishi and Ikeda, 2006; Tateno et al., 2007). The recombinant clones were grown in shake flask on BHI-Sorbitol medium and induced by the addition of 0.5 mM IPTG. SDS-PAGE analysis of the culture supernatants showed a single band with a molecular weight close to that of BC-PLC for the three expression strains. Plasmid pTGR100, where the secretion of BC-PLC is driven by its native sequence, provided the highest amount of BC-PLC. The low titers observed for the pTGR102 plasmid, could be explained by poor secretion efficiency, since the majority of the full length unprocessed protein was accumulated in the intracellular fraction (Fig. 2A).

To further increase the production of BC-PLC, a codon optimized version of the gene was designed using a codon randomization strategy and a codon frequency table for *C. glutamicum* (Liu et al., 2010; Menzella, 2011). This synthetic gene was used to replace the wild type BC-PLC in pTGR100, to give plasmid pTGR103. When it was expressed in *C. glutamicum* ATCC13869, the amount of protein produced was similar to that obtained with the wild type gene (data not shown), suggesting that codon usage is not a barrier for the heterologous expression of BC-PLC in *C. glutamicum*.

The ATCC13869 strain harboring the pTGR100 plasmid reached an average final $OD_{600}$ of 20 after 24 h of cultivation, with BC-PLC titers of 0.12 g/L as determined from gel scanning analysis using the GelPro software (Fig. 2B) and this strain was chosen to further develop a manufacturing process.

### 3.2. Enzyme purification and activity assays

The enzyme was recovered from pTGR100 culture supernatants, concentrated by ammonium sulfate precipitation as described in Section 2. The purified preparations did not display phospholipase activity in the initial assays using the chromogenic substrate ONPC. N-terminal sequencing of the recombinant BC-PLC protein revealed that the 14 amino acids propeptide portion remained uncleaved, preventing the enzyme to display its catalytic activity (Fig. 3A). This result is in agreement with early observations indicating that the N-terminal tryptophan residue of the mature enzyme is essential for BC-PLC activity (Hough et al., 1989; Johansen et al., 1988).

Proteolysis with trypsin or clostripain has been reported to cleave the propeptide from BC-PLC (Lazarowski and Lapetina, 1990; Tan et al., 1997). Addition of trypsin to purified BC-PLC provided an enzyme preparation with a specific activity of 195 U/mg and and $V_{max}/K_m$ = 0.017 1/min, identical to the values obtained for a pure *B. cereus* PLC standard. N-terminal sequencing of the digested protein (Fig. 3A) and SDS-PAGE analysis (Fig. 3B) clearly confirmed the complete and precise cleavage of the propeptide to give the mature enzyme.

### 3.3. Enzymatic oil degumming evaluation

To evaluate the performance of the recombinant BC-PLC produced in *C. glutamicum* for oil degumming, laboratory scale reactions were set up mimicking the conditions used for enzymatic oil degumming on industrial scale. 1 kg of crude soybean oil containing 1.75% phospholipids (700 ppm phosphate) was treated with 5 mg of recombinant mature BC-PLC. The mixture was emulsified and incubated at 50 °C, the optimal temperature reported for BC-PLC (Otnaess et al., 1977). The course of the reaction was followed over 3 h by measuring the concentration of 1,2-DAGs, a product of the hydrolysis of PC and PE. Fig. 4A shows that after 1.5 h the curve reached a plateau and that the maximum concentration of 1,2-DAGs generated is 1.01%, corresponding to the amount expected from the complete hydrolysis of PC and PE.

To confirm the specificity of the reaction, the phospholipid fraction was extracted and analyzed by NMR. Fig. 4B shows the NMR

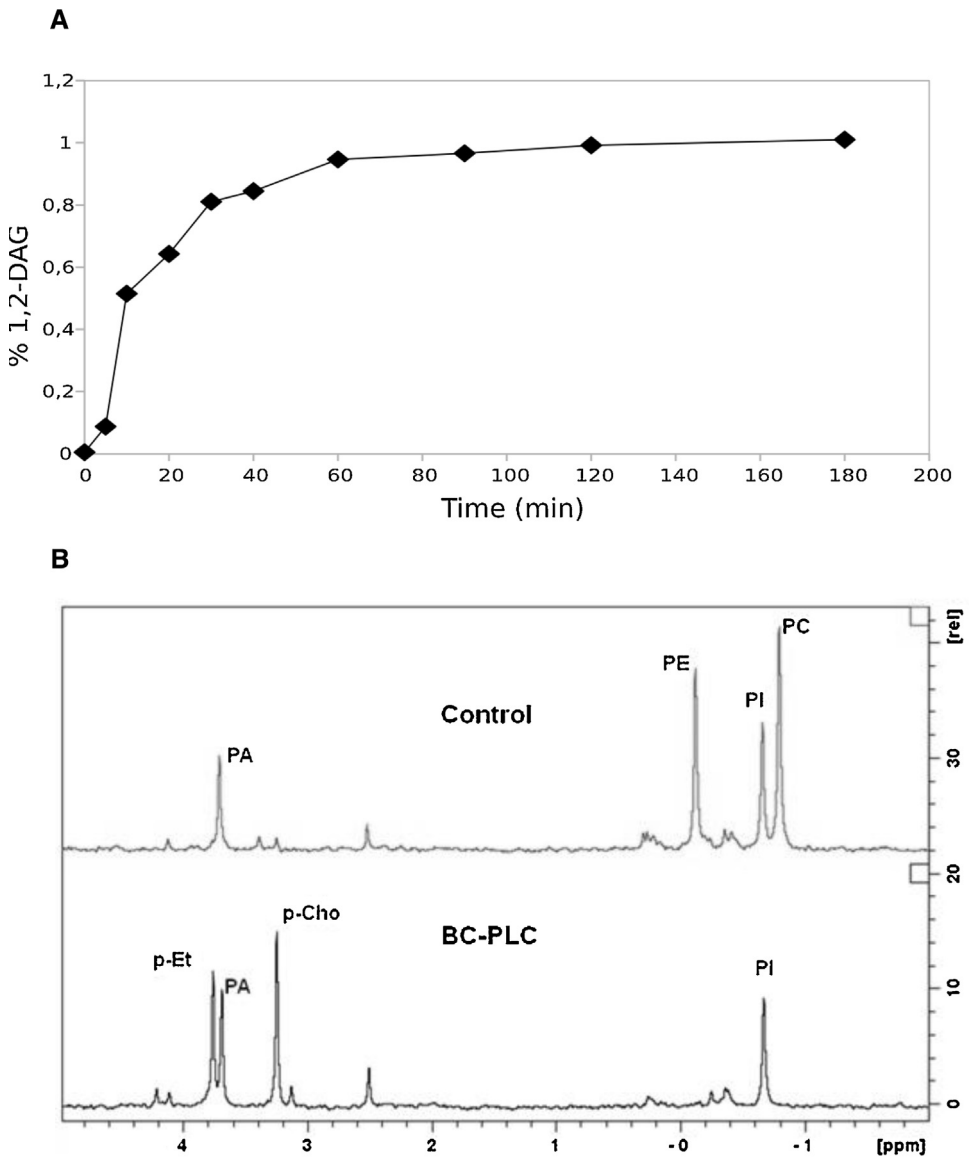


**Fig. 4.** Analysis of phospholipid hydrolysis in soybean oil by by BC-PLC. (A) Diacylglicerol determination by HPLC-ELSD of crude oil treated with BC-PLC at different reaction times. (B) NMR spectra for treated oil and a control where no enzyme was added. BC-PLC produced in *C. glutamincum* was able to completely hydrolyze PC and PE in oil treated samples, generating phosphocoline (pCho) and phosphoethanolamime (pEt) as determined by RMN peak profile. PI and PA indicates phospatidylinositol and phosphatidc acid respectively.

spectra for treated oil and a control where no enzyme was added. After the enzymatic treatment the peaks corresponding to PC and PE are no longer detectable while peaks for phosphocoline (pCho) and phosphoethanolamime (pEth) are generated. Taken together, these results clearly show that PC and PE are completely hydrolyzed by BC-PLC to generate 1,2-DAG.

### 3.4. High cell density fermentation process development

Considering the current oil price of ~600$ per ton and an extra-yield of oil of 1% generated by the enzymatic degumming using 5 g of PLC per ton; the profit obtained per gram of PLC is ~$1.25. Assuming a fermentation cost of $1 per liter, our goal was to obtain at least 2 g/L of BC-PLC to achieve a cost-effective manufacturing process. Thus, a fed batch fermentation process was developed in a 2-L lab scale bioreactor. The recombinant strain was cultivated in a semi-defined medium using glucose as a carbon source. After depletion of the initial amount of glucose, a feeding strategy using a balance mass equation was used to maintain the specific growth rate at 0.13 (Lee, 1996; Menzella and Gramajo, 2004). When cultures reached an $OD_{600}$ of 80, 0.5 mM IPTG was added to induce production of BC-PLC and the feeding rate was kept constant at a rate of 10 mL of feeding solution per L.

Fig. 5 shows that production of BC-PLC was detectable 7 h after the induction, and its production continuously increased until a maximum titer of 5.5 g/L, achieved at 40 h after induction. A cell mass yield of 1 $OD_{600}$/g of glucose remained constant throughout the entire fermentation, indicating that the production of BC-PLC does not affect the growth of the recombinant strain. Among three different fermentation runs, the BC-PLC productivity and growth profile were very similar; which provides an indication of the robustness of the fermentation process.

Finally, the cell mass was separated by centrifugation and 1 L of the supernatant incubated with 2 mg of trypsin for 1 h at 37 °C. A fraction of this preaparation was futher purified as mentioned in Section 2 and 5 mg of this enzyme were added to 1 kg of crude

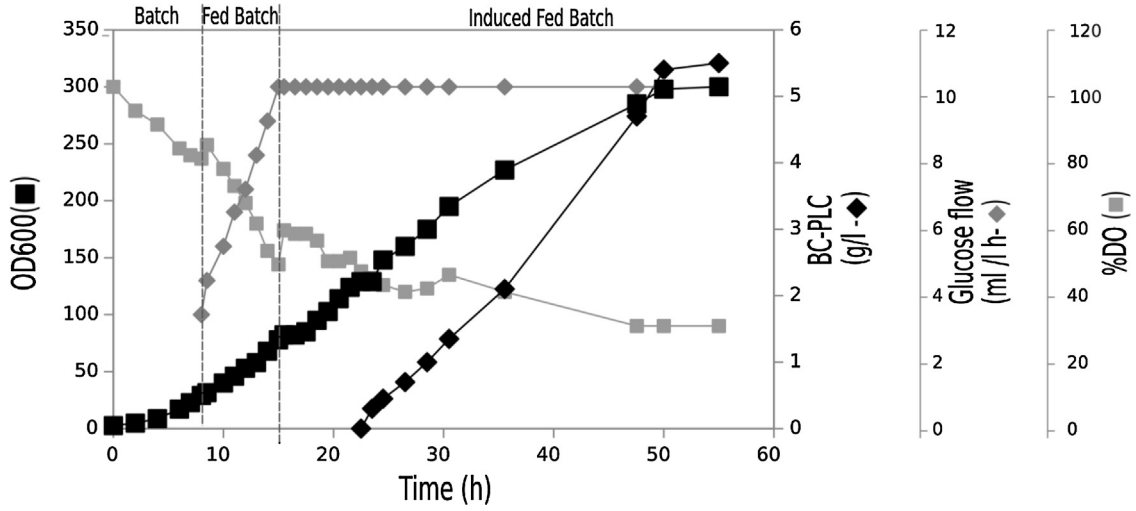


**Fig. 5.** Analysis of fed batch fermentation of C. glutamicum ATCC 13869 expressing BC-PLC. Time course of BC-PLC total protein concentration (black diamond), biomass (black square), glucose flow (grey square) and dissolved oxygen (grey diamonds) during fed-batch fermentation in the 2-L fermenter with a semi-defined medium. Glucose was used as the sole carbon source and IPTG was used as inductor. Values shown are means of three independent experiments. The standard deviations were in all the cases less than 10% of the corresponding means.

oil. The mixture was emulsified and maintained at 50 °C with continuous stirring in order to remove the phospholipids as described above. The results of this degumming experiment were identical to those obtained with the enzyme produced in batch cultures, obtaining 1.05% of 1,2-DAGs produced after a 180 min treatment, showing that the quality of the enzyme obtained from the fed batch process is not altered.

## 4. Discussion

Enzymatic oil degumming is an environmentally friendly process that is quickly replacing traditional aqueous degumming in oil refining processes. Thus, there is a need for novel enzymes that can efficiently eliminate phospholipids and for efficient processes for their production. Currently, the preferred enzymes are PLCs that convert the phospholipids into 1,2-DAGs. PLCs provide an extra-yield of oil both as a result of the generation of 1,2-DAGs and from the release of a fraction of TAGs that are normally trapped in the gums.

*B. cereus* PLC is an attractive candidate naturally produced by a pathogenic microorganism. Its heterologous production in *C. glutamicum*, a GRAS (Generally Recognized As Safe) host widely used in large scale fermentation processes, is therefore an appealing strategy.

Two alternative secretion pathways Sec- and Tat- have been successfully used to produce enzymes in *C. glutamicum* (Itaya and Kikuchi, 2008; Kikuchi et al., 2008). Thus, we decided to initially evaluate the two of them using secretion sequences for which high level secretion of heterologous proteins were previously reported (Itaya and Kikuchi, 2008; Meissner et al., 2007; Salim et al., 1997), along with the wild type secretion sequence of BC-PLC. The secretion sequence from *C. glutamicum* CspB protein, secreted by the Sec system, showed an efficiency to promote the secretion of BC-PLC close to the obtained for the native sequence from *B. cereus*, and both provided about eight fold more secreted protein than the Tat secretion sequence from PhoD from *B. subtilis*. Since *B. cereus* naturally uses the Sec system for the secretion of PLC, it seems that this secretion system is the preferred one for this enzyme.

BC-PLC obtained from *C. glutamicum* cultures did not display activity unless activated by trypsin proteolysis. The natural mechanism for the removal of the propeptide has not been described for *B. cereus*. However, for a homologous PLC from the related bacterium *Listeria monocytogenes*, an extracellular protease was found to be involved in the proteolysis of the propeptide (Domann et al., 1991; Slepkov et al., 2010). This may explain why the protein is not processed in *C. glutamicum*, since this microorganism does not possess any homologous extracellular protease. A similar limitation was previously reported by Kikuchi et al. (2003) for the production of a transglutaminase pro-enzyme. In that work they co-expressed a protease to overcome this limitation, obtaining an active enzyme. Our efforts to express a construction connecting the coding sequence for the mature enzyme to the secretion sequence from *B. cereus*, and thus omitting the pro-sequence, were unsuccessful; suggesting that this protein may be toxic for the host. Beyond these theoretical considerations, the use of trypsin in our preparations is affordable at industrial scale since only 2 mg are required to activate a preparation containing 5 g of the BC-PLC proenzyme.

Recombinant BC-PLC showed a high efficiency to hydrolyze PC and PE (as shown by NMR spectra) which together represent 70% of the phospholipids present in soybean oil. HPLC-ELSD analysis showed a complete conversion to DAGs which could represent a significant contribution to the productivity and economy of the oil degumming process. This remarkable efficiency was achieved in conditions suitable for refining plants, providing therefore a scalable treatment of soybean oil using recombinant BC-PLC.

To develop a cost-effective process for the large scale manufacturing of BC-PLC, a high cell density fermentation process was designed with a fed batch strategy. Using a semi-defined medium, a final concentration of 5.5 g/L was obtained which correspond to a volumetric productivity of 0.1 g/(L h), the highest reported for *C. glutamicum* to the best of our knowledge. The fact that the production of BC-PLC does not affect the growth of the recombinant strain suggests that a continuous production process might be implemented, which would further reduce the cost of the production process.

## 5. Conclusion

To our knowledge, we have achieved the highest recombinant protein production ever reported in *C. glutamicum*. We also demonstrated the potential of BC-PLC for industrial oil degumming and provided an efficient process for its large scale production.

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