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Influence of specific growth rate over the secretory expression of recombinant potato carboxypeptidase inhibitor in fed-batch cultures of *Escherichia coli*

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ABSTRACT

A high cell density cultivation protocol was developed for the secretory production of potato carboxypeptidase inhibitor (PCI) in *Escherichia coli*. The strain BW25113 (pIMAM3) was cultured in fed-batch mode employing minimal media and an exponential feed profile where the specific growth rate was fixed by limitation of the fed carbon source (glycerol). Plasmid loss rates were found to be proportional to the specific growth rate. Distribution of PCI along the cell compartments and the culture media was also dependent on the fixed growth rate. When specific growth rate was kept at $\mu = 0.10\,h^{-1}$, 1.4 g PCI L^{-1} were obtained when adding the product present in periplasmic extracts and supernatant fractions, with a 50% of the total expressed protein recovered from the extracellular medium. This constituted a 1.2-fold increase compared to growth at $\mu = 0.15\,h^{-1}$, and 2.0-fold compared to $\mu = 0.25\,h^{-1}$. Last, a cell permeabilization treatment with Triton X-100 and glycine was employed to direct most of the product to the culture media, achieving over 81% of extracellular PCI. Overall, our results point out that production yields of secretory proteins in fed-batch cultures of *E. coli* can be improved by means of process variables, with applications to the production of small disulfide-bridged proteins. Overall, our results point out that control of the specific growth rate is a successful strategy to improve the production yields of secretory expression in fed-batch cultures of *E. coli*, with applications to the production of small disulfide-bridged proteins.

1. Introduction

Secretory expression of heterologous proteins in *Escherichia coli* has a number of advantages over more common cytosolic expression. First, secretion of the recombinant product is attractive form a downstream processing stand-point, since no cell-disruption steps are needed and contamination with other proteins is reduced both in the periplasm and culture media [1,2]. Secondly, the formation of disulfide bridges is actively catalyzed in the periplasmic space [3,4]. Also, for proteins that are toxic to the host, secretion may palliate their detrimental effect over culture growth [2].

Several proteins have been successfully produced in the periplasmic space and culture supernatants of high cell density cultures of *E. coli* [5,6], but since the capacity of the bacterial secretion machinery is limited and there are several factors that affect protein expression and translocation [7,8], achieving high protein yields of protein exported through the inner membrane can be a complex task. In this sense, it has been proven that translational and translocation levels have to be properly coupled to reach a state where most of the expressed heterologous protein is secreted [9,10]. This can be achieved by manipulations of genetic parameters like the promoter strength [11], the nature of the signal sequence [12,13] or the plasmid copy number [14], but optimization of the culture protocols is also necessary. Previous studies show the influence of culture media composition, growth kinetics, induction moment and temperature over secretory protein yields [1,2,5].

Potato carboxypeptidase inhibitor (PCI) is a small protein naturally occurring in leafs and stems of *Solanum tubesorum* [15]. Composed by 39 residues and three disulfide bridges, it has potential biomedical applications given its proven antitumoral properties [16,17]. PCI had previously been produced in *E. coli* using the pIN-III-ompA-derived plasmid pIMAM3, which allows for the translocation of the protein to the periplasmic space where formation of its disulfide bonds was successfully achieved and the active form could be recovered from culture supernatants [18,19]. Excretion of PCI out of the cell envelope is probably favored by its small

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size and compact structure. A fed-batch procedure had previously been designed for the overexpression of PCI in high cell density cultures in semi-complex media, but relatively low levels of biomass (15 g DCW L^{-1}) were achieved, and the process was not automated, with feedstock additions not responding to any monitored variable. The aim of this work was to design a robust, automated and repeatable fed-batch process at bench-top level in order to increase the production of biologically active PCI by maximizing both the biomass concentrations and the expression-secretion of the inhibitor. Since it was observed that the specific growth rate (μ) had a major influence in the amounts of excreted PCI, a series of fermentations at different fixed growth rates were carried out. The dynamics of the PCI concentration profiles in the cytosol, periplasmic space and culture media was analyzed in order to identify and overcome the bottlenecks in the secretory production of this protein.

2. Materials and methods

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) under otherwise stated.

2.1. Strains and plasmid

E. coli strain MC1061 (hsdR2 hsdM$^+$ hsdS$^+$ araD139 Δ(ara-leu)7697 Δ(lac)X74 galE15 galK16 rpsL (StrR) mcrA mcrB1) and plasmid pIMAM3 were used in previous works [18,19]. BW25113 (Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lambda$^-$, rph-1, Δ(rhaD-rhaB)568, hsdR514) was obtained from the Coli Genetics Stock Center at Yale.

2.2. Shake-flask cultivation conditions

For shake-flask experiments, either LB media or MDE media supplemented with 100 μg mL^{-1} ampicillin were used. The composition of LB was, per liter: 10 g peptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl; whereas the composition of MDE media was, per liter: 5 g glucose or glycerol, 11.9 g K_2HPO_4, 2.4 g KH_2PO_4, 1.8 g NaCl, 3.0 g $(NH_4)_2SO_4$, 0.11 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeCl_3$, 0.03 g thiamine and 0.72 mL of trace elements solution. Trace element solution composition was, per liter: 1.44 g $CaCl_2{\cdot}2H_2O$, 42 mg $AlCl_3{\cdot}6H_2O$, 50 mg $ZnSO_4{\cdot}7H_2O$, 160 mg of $CoCl_2{\cdot}6H_2O$, 1.6 g $CuSO_4$, 10 mg H_3BO_3, 1.42 g $MnCl_2{\cdot}4H_2O$, 10 mg $NiCl_2{\cdot}H_2O$ and 20 mg of $Na_2MoO_4{\cdot}H_2O$.

Seed cultures were prepared in 50 mL culture tubes by inoculating 10 mL of LB broth with a single colony from a fresh transformation plate, followed by incubation overnight at 37 °C and 200 rpm in an orbital shaker. Shake-flask cultures were typically prepared in 500 mL shake-flasks using 1 mL of the seed culture to inoculate 100 mL of LB or MDE media, which were then incubated under the same conditions as seed cultures. To induce protein expression, IPTG from a 100 mM stock was aseptically added to the desired final concentration. After induction, cells were allowed to grow into stationary phase for 7–8 h.

2.3. Bioreactor cultivation conditions

Fed-batch cultivation experiments were carried out using a 2 L jar and a standard Biostat B® digital control unit. A flux of 1.5 vvm of air was injected trough the fermentor to satisfy the respiratory needs of the cultured strain. Additions of 15% (w/v) NH_4OH were made to keep pH at a set point of 7.00. Temperature was set at 37 °C. Dissolved oxygen levels were kept at 60% of the saturation concentration by means of the stirring speed and/or by mixing the inlet gas with pure oxygen in increasing proportions.

Seed cultures of the strain of interest were grown in LB media as described previously for shake-flask experiments. Inocula cultures were prepared in 500 mL shake-flasks by adding 5 mL of seed culture into 95 mL of fresh MDE media, then incubating at 37 °C and 200 rpm in an orbital shaker. Once these cultures reached $OD_{600\,nm}$ of 1–1.2, 80 mL of them were added into the fermentation jar containing 720 mL of MDF media. MDF contains per liter: 20.0 g glucose, 4.5 g yeast extract, 2.0 g NaCl, 4.1 g $(NH_4)_2SO_4$, 13.2 g K_2HPO_4, 2.6 g KH_2PO_4, 0.5 g $MgSO_4$, 0.03 g $FeCl_3$, 25 mL of trace elements solution and 100 mg ampicillin. Once glucose was depleted, the feeding part of the fermentation was started with the addition of FS feeding solution, which contained per liter: 450 g glycerol, 9.6 g $MgSO_4$, 0.5 g $FeCl_3$, 0.5 g $CaCl_2{\cdot}2H_2O$, 0.3 g thiamine, 32 mL trace elements solution and 500 mg ampicillin. Addition of the feedstock was done according to an open loop method described before in previous works (20). This exponential feeding protocol allows for the control of the specific growth rate of the bacterial culture by limitation of the carbon source (21). No source of phosphate was included in the feedstock in order to avoid the precipitation of $Ca_3(PO_4)_2$ and $Fe(PO_4)$; however two punctual additions of a concentrated phosphate solution were done, each equivalent to 5 g of PO_4^{3-}.

2.4. Biomass and metabolites analyses

Bacterial growth was followed by optical density measurements at 600 nm using a spectrophotometer (KONTRON Uvicon 941 plus Spectrophotometer). Optical density was correlated to dry cell weight through a calibration curve constructed by standard methods [21]. Evaluation of plasmid stability was accomplished by plating properly diluted amounts of culture samples on plain LB-agar plates and on then LB-agar plates supplemented with 100 μg mL^{-1} ampicillin. The colony count on plain plates represented the total cells, while the count on the antibiotic containing plates stood for plasmid bearing cells. For the determination of glycerol, glucose, phosphate, ammonium and acetic acid, 1 mL samples of culture were centrifuged at 9000 × *g* for 3 min in a tabletop centrifuge (Haereus). The supernatant was then filtered through a 0.22 μm syringe filter (Millipore) and purified by HPLC (Hewlett-Packard 1050) on an Aminex HPX-87H column (Biorad), with H_2SO_4 15 mM as the mobile phase at a flow rate of 0.60 mL min^{-1}. Analysis was done with an IR detector (Hewlett-Packard 1047) at room temperature. Phosphates and ammonia were determined using commercial colorimetric kits (Hach Lange).

2.5. Cell fractionation and culture supernatant preparation

Before cell fractionation, $OD_{600\,nm}$ of each culture was determined to determine the broth volume from were 2.4 mg DCW could be isolated. The sample volume was centrifuged at 3000 × *g*, 4 °C for 10 min, and periplasmic extracts were obtained by osmotic shock as described elsewhere [22]. The resulting pellet containing spheroplasts was resuspended in 300 μL PBS buffer and sonicated to release soluble cytoplasmic proteins using a Vibracell® model VC50 (Sonics & Materials). Cell lysates were centrifuged at 12,000 × *g*, 4 °C for 15 min to recover the soluble cytosolic contents. Culture supernatants were separated from the bacterial pellet by centrifugation at 5000 × *g*, 4 °C for 10 min, followed by filtration with a syringe-driven 0.22 μm filter device (Millipore). Clear supernatants (40 mL) were then loaded on a SepPak C18 (1 g) Reverse Phase column (Waters), previously equilibrated with acetonitrile and rinsed with ultrapure water. Columns were washed with 4 mL 10% acetonitrile and 4 mL ultrapure water previous to elution with 4 mL of 30% isopropanol. When needed, supernatants were concentrated using Amicon or Minicon centrifugal filter devices (Millipore).

2.6. Protein electrophoresis and estimation of protein content

Total protein in samples content was assessed in triplicate using a commercial kit for the Bradford assay (Biorad) with Bovine Serum Albumin (BSA) as a standard. Separation and visualization of proteins over electrophoresis gels was carried out using 12% Bis-Tris gels from the Novex system (Invitrogen) using MES-SDS as running buffer. Upon staining with colloidal Coomassie [23], the bands of interest were quantified by gel densitometry (Kodak Digital Science); this type of quantification was mainly used for the estimation of pre-PCI in the cytosol.

2.7. Reverse phase HPLC quantification

Reverse phase liquid chromatography was employed to separate and quantify active PCI in periplasmic and supernatant fractions. An Ultimate 300 HPLC system (Dionex) and a C_{18} cartridge (Waters) were employed, using a sample volume of 100 μL containing 1% TFA. Elution of PCI was done over a gradient of acetonitrile (pH = 1.00) from 20% to 80%. Using standards of purified protein, a calibration curve was constructed to estimate the concentration of active PCI in the injected samples.

2.8. Enzymatic assay

The inhibitory activity of PCI samples was determined using a commercial kit (Sigma–Aldrich). This kit allows to measure the activity of carboxypeptidase A (CPA), as well as the screening of inhibitors of this enzyme, as described elsewhere [24]. Non-induced culture samples of BW25113 or MC1061 (pIMAM3) were used as blanks.

3. Results

3.1. Shake-flask preliminary experiments

3.1.1. Comparison of expression between the strains MC1061 and BW25113

As previously mentioned, the expression system MC1061 (pIMAM3) had successfully been used for the production of PCI in both shake-flask and high-density cultures in complex and semi-complex media [19]. However, MC1061 is a leucine auxotroph and hence its culture in defined media requires the addition of this amino acid. In small scale cultures this did not constitute a relevant inconvenient, but it was found to be a major handicap for high-density cultivation, since the amino acid needs can be several