Development of a highly efficient production process for recombinant protein expression in *Escherichia coli* NEB10β

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Abbreviations[1]

Abstract:

Recombinant protein expression in *E. coli* is well described, with multiple strains and process strategies available. However, strains used for cloning and molecular biology purposes are not generally considered for protein expression. Using these strains could result in a simplification of the production pathway of a newly cloned protein of interest. In this work, the *E. coli* strain NEB10β has been characterized for the expression of the complex fusion protein phosphite dehydrogenase-cyclohexanone monooxygenase (PTDH-CHMO), and a production process has been developed based on the P_{BAD} expression system. A fed-batch approach using a defined medium supplemented with amino acids, with glycerol as a carbon source, allows for an efficient recombinant protein expression process, incrementing 9.2-fold the production obtained in a complex medium batch and reaching around 2 g/L of product after 6 hours of induction. The process was successfully reproduced in a NEB10β strain for the production of the alcohol dehydrogenase (ADH) enzyme.

[1] **ADH** (Alcohol dehydrogenase); **AU** (Activity Unit); **DM** (Defined Medium); **PTDH-CHMO** (phosphite dehydrogenase - cyclohexanone monooxygenase); **TB** (Terrific Broth); **DCW** (dry cell weight).

1 Introduction

Recombinant protein production relies in several organisms as production hosts, being *Escherichia coli* one of the most widely used. Advantages of this gram-negative bacteria include high protein production yields, high growth rate, wide knowledge of its genome, possibility of high-density growing in inexpensive media [1] and the existence of several systems that allow for controlled induction of the expression of the protein of interest. Several *E. coli* production strains have been developed, mainly *E. coli* BL21 and K-12 derived strains [2] and are well established in the industry. In the development process of a new enzyme, these strains play an important role in allowing the obtention of high amounts of the protein of interest in the mid-to late stages of the protein development, before implementing an industrial production. However, constructing these production strains can imply a burden on these stages of the process development. This could be avoided by using molecular biology strains instead. They are often used in order to produce small amounts of the protein of interest in the early development stages, however, when higher amounts of product are needed, industrial strains are then employed. Nevertheless, using molecular biology strains to obtain high titers of a newly cloned protein of interest without needing to switch to a classical production strain would result in a simpler development of a recombinant protein production process.

There is a great number of different *E. coli* strains used for molecular biology, one of them being the NEB10β strain, for which there are very few reports of its use for producing recombinant protein, and all of them are at low scale [3], [4]. However, this strain holds a potential for recombinant protein production. Firstly, it is resistant to several bacteriophage infections, including T1 phage and ϕ80 phage. Bacteriophage contamination is an issue in bacterial culture [5]. Secondly, NEB10β strain contains the *araD139* mutation, which makes it unable to metabolize arabinose. This characteristic can be useful for applying arabinose as an inducer for protein expression, through the inducible *araBAD* promoter (P_{BAD}), a standard promotor used in *E. coli* expression systems [6]. This system presents several advantages, including tight regulation (very low levels of basal expression are achieved at uninduced state) [7], inexpensive induction and reaching moderately high levels of protein expression [6]. However, there is a lack of platform bioprocessing strategies to exploit the full potential of this system, therefore, there are few studies applying this system for fed-batch processes with *E. coli* [8]. Some examples of studies including *E. coli* fed-batch processes with this system include [8], [9] and [10].

This work focuses on the development of a lab scale bioreactor-based production process for the *E. coli* strain NEB10β, comprising its characterization in terms of temperature applied for production, inducer concentration, defined medium development and culture process strategy, implementing a fed-batch process with defined medium based on the P_{BAD} induction system, resulting in an efficient process

that improves the productivity obtained in a batch with complex medium. The complex fusion enzyme PTDH-CHMO has been used as a model product. This protein consists in the fusion of the enzyme cyclohexanone monooxygenase (CHMO), a Baeyer Villiger monooxygenase [11], with a phosphite dehydrogenase (PTDH) enzyme, which regenerates the cofactor NADPH necessary for CHMO activity [12]. The implemented process has then been successfully applied to another NEB10β strain producing the enzyme alcohol dehydrogenase (ADH), a protein with an array of industrial applications [13], in order to assess the reproducibility of the process platform. The developed process could be used as a platform for the obtention of high amounts of a new protein of interest without needing to construct a production strain.

2 Materials and methods

All reagents were obtained from Sigma-Aldrich (USA, MO) unless otherwise stated.

2.1.- *E. coli* strains and plasmids

NEB10β *E. coli* cells (New England Biolabs) containing the pBAD derived pCRE-CHMO plasmid [12] were used for PTDH-CHMO production; the pBAD plasmid containing the ADH sequence from *Saccharomyces cerevisiae* was used for ADH production. PTDH-CHMO and ADH sequences were optimized for the codon usage of *E. coli* using the software Gene Designer. The cells and the plasmids, containing 6xHis PTDH-CHMO and 6xHis ADH constructs, were generated by Marco W. Fraaije group at Groningen University and adapted to both complex Terrific Broth (TB) and defined medium (DM) (detailed in the next section) were generated.

2.2.- Culture in Erlenmeyer: Terrific Broth and Defined Medium

The experiments performed in Erlenmeyers, unless otherwise stated, were carried out at 24ºC and 135 rpm of agitation in 250 ml Erlenmeyers with 50 ml of medium, cultures were grown for 24h and were inoculated at an OD_{600} of 0.2.

Terrific broth was prepared following the standard recipe: KH_2PO_4 9.4 g/l, K_2HPO_4 2.2 g/l, glycerol 4 ml/l, yeast extract 23.6 g/l, tryptone 11.8 g/l. The composition of Defined Medium (DM) consisted in [14]: KH_2PO_4 11.9 g/l, K_2HPO_4 2.4 g/l, NaCl 1.8 g/l, $(NH_4)_2SO_4$ 3 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, $FeCl_3$ 0.02 g/l, and trace elements at 0.72 ml/l. Trace elements solution: $CaCl_2 \cdot 2H_2O$ 1.44 g/l, $AlCl_3 \cdot 6H_2O$ 0.041 g/l, $ZnSO_4 \cdot 7H_2O$ 0.87 g/l, $CoCl_2 \cdot 6H_2O$ 0.16 g/l, $CuSO_4 \cdot 7H_2O$ 1.6 g/l, H_3BO_3 0.01 g/l, $MnCl_2 \cdot 4H_2O$ 1.42 g/l, $NiCl_2 \cdot 6H_2O$ 0.01 g/l, $Na_2MoO_4 \cdot 2H_2O$ 0.02 g/l. Amino acids Leu (0.84 g/l), Ile (0.42 g/l) and Val (0.28 g/l) were also supplemented, as a specific requirement of NEB10β strain for protein production. Carbon source consisted in glycerol or glucose, depending on the experiment, both at 20 g/l. In both media, ampicillin at 50 μg/ml was added as selection marker, and arabinose at 0.02% (w/v) was added for induction.

In the case of the two-step production experiments, performed with defined medium, a first growth phase of 24h with glucose as a carbon source was followed by a medium switch to defined medium with glycerol as a carbon source and supplemented with arabinose. Media exchange was performed by means of a first centrifugation cycle at 5000 rpm for 10 minutes at 4ºC, followed by resuspension of the pellet with the medium of interest as a wash step, and a second centrifugation cycle with the same conditions as the previous one and resuspension of the pellet with the same initial 50 ml.

2.3.- Bioreactor culture: batch with TB and two-steps with defined medium

Bioreactor cultures were performed in a 2L Biostat B bioreactor (Sartorius Stedim) equipped with a pH, pO_2 and foam probes. pH was maintained at 7 by adding NaOH 30% and H_2SO_4 2M for TB cultures, whereas for DM culture NH_4OH 15% was used as base. An airflow of 1 vvm was applied, and oxygen levels were set to a pO2 of 30%, controlled through a cascade of stirring, which ranged between 200 and 1100 rpm. Foam formation was controlled by means of automatic antifoam DF204 20% (w/v) addition. 1 ml/L of antifoam DF204 20% was added to the medium prior to sterilizing the bioreactor. Experiments were performed at 24ºC. Precultures were performed as follows: 10 ml cultures were inoculated with 50 µl of a frozen stock and incubated at 24ºC for 8h, then 200 ml of culture were inoculated with the previous 10 ml and incubated overnight for 16h. Precultures media were not supplemented with arabinose. This culture was used to inoculate the reactor at an initial OD_{600} of 0.2 for a total volume of 2L in the case of the TB batch culture and 1L in the case of the two-step DM culture.

The TB batch culture had a duration of 22-24h and was supplemented with arabinose at 0.02% (w/v) from the beginning of the culture. In the case of the DM two-step culture, after 24h, once glucose had been depleted from the medium, an addition of 0.3 L of fresh medium with glycerol as carbon source and arabinose 0.02% (w/v) was added to the culture, which was stopped 24h later.

Glucose and glycerol concentrations were assessed using an automated Y15 analyser (BioSystems).

2.4.- Bioreactor culture: fed-batch in DM

Fed-batch experiments were performed in the same bioreactor than for batch cultures, and culture conditions were similarly set and controlled (see section 2.3), with the modification of pure oxygen addition after the maximum stirring was achieved in the cascade control for pO_2 maintenance.

Precultures were prepared as follows (all steps at 37ºC and 135 rpm): 10 ml of DM were inoculated with 50 µL of frozen stock and incubated for 8h. Then they were escalated to 2 Erlenmeyers of 250ml with 50 ml of DM and incubated overnight for

16h. After that, each 50 ml culture was transferred to a 500 ml Erlenmeyer adding an additional 50 ml of fresh DM medium, for a total 100 ml of culture.

The initial base medium was the same defined medium used in the batch experiments, using glucose as carbon source, and two different addition media were used: one for a first fed-batch growth phase (growth phase medium) and another for the induction phase (induction phase medium). Growth phase medium consisted in: glucose (478 g/l), $CaCl_2 \cdot H_2O$ (0.089 g/l), $MgSO_4 \cdot 7H_2O$ (9.56 g/l), $FeCl_3$ (0.49 g/l), trace elements (same solution described before) (62.94 ml/l), ampicillin at 50 μg/ml, and amino acids Leu (5.02 g/l), Ile (2.56 g/l), and Val (1.68 g/l). Induction medium had the same composition than growth medium with glycerol instead of glucose (at the same concentration of 478 g/l) and the addition of arabinose at 0.2 g/l.

The culture was inoculated at OD_{600} = 0.4 and consisted in a batch step for approximately 11h. It was followed by a first fed-batch growth phase for 8h at 37ºC, at a growth rate of 0.2 h^{-1}, in order to achieve high biomass levels. The medium feeding was performed through a pre-programmed exponential addition, using the following equation (Eq. 1):

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

Where F corresponds to the feeding flux (mL/min), μ to the set specific growth rate (h^{-1}), Δt to the time interval in which the feeding flux is applied (1h), X to the predicted biomass concentration in the bioreactor at the end of the time interval, V_0 to the culture volume at the beginning of the time interval, $Y_{x/s}$ to the biomass/substrate yield (set at 0.35 g/g) and S_0 to the concentration of substrate (either glucose or glycerol) in the feeding medium.

Then, a step of 1h without feeding and with a linear decreasing of temperature to 24ºC was applied. This was followed by a second fed-batch phase, in which induction medium was feeded, separated in two steps (one of 8 hours at a set growth rate of 0.05 h^{-1} and the other, overnight, of 14 hours at 0.03 h^{-1}). The induction was carried out by adding arabinose at a final concentration of 0.2 g/L in the bioreactor, approximately 2 hours into the first 8-hour step.

Once the induction phase started, the above mentioned NH_4OH 15% solution used as a corrector agent was switched by a NH_4OH 15% solution containing the amino acids Leu, Ile and Val at a concentration of 90, 45 and 30 g/l, respectively. These concentrations corresponded to the highest experimentally obtained concentrations for these amino acids in NH_4OH 15% solution, maintaining the same proportion of Leu:Ile:Val used in the base medium. Amino acids were added to the base solution since the proportion amino acid vs carbon source in the base medium (0.042 g/g for Leu:glucose) could not be met in the feeding medium due to solubility

issues. A maximum concentration of 5.02 g/l of Leu (and subsequent 2.56 g/l of Ile and 1.68 g/l of Val) could be achieved in the feeding media, representing a proportion of 0.0105 g/g with respect to glucose, 75% lower than in the reference base medium. Therefore, with the addition of amino acids to the base, which is added proportionally to the biomass growth during the culture, amino acid amount could be compensated without the need to externally add amino acids to the culture, thus simplifying the process.

2.5.- Recovery of the product:

The product was recovered starting with a first step of centrifugation at 5000 rpm for 10 minutes at 4ºC (centrifuge Avanti J20 (Beckman)) in 500 ml centrifugation tubes. The pellet was divided into several aliquots and kept frozen at -30ºC. Prior to measure the enzymatic activity or purify the enzyme, the aliquot of interest was resuspended in 50 mM Tris-HCl (pH 8.5) buffer and disrupted using a OneShot disruptor (Constant Systems), for 2 cycles at 1.47 kbar. This crude cell lysate was used for subsequent enzymatic assay of the product.

2.6.- Biomass measure

The cell density was measured in terms of absorbance (OD_{600}) using a Jenway 7315 spectrophotometer (Jenway, UK), diluting the samples in order to measure them in an absorbance range of 0.2-0.8. In order to convert it to dry cell weight (DCW, g/L), an equation relating dry cell weight and absorbance for *E. coli* cells was used. This absorbance/dry cell weight proportion had previously been determined, the resulting equation (Eq. 2) being:

$$DCW = 0.303*OD_{600} + 0.211 \quad (2)$$

2.7.- Product titer (g/L) determination: SDS-PAGE

The concentration of the product in culture of disrupted samples was determined by means of a SDS-PAGE. 15 µl of Laemmli buffer (Bio-rad) were added to 15 µl of each sample, the mixture was then incubated at 95ºC for 5 minutes and then 15 µl of it were loaded into the gel (MiniProtean TGX StainFree, Bio-rad), which was run for 25 minutes at 250V. 10 µl of molecular weight marker were also loaded to the gel. The concentration of the product in the loaded sample was performed by means of densitometry analysis comparing it to the 100 kDa band of the reference molecular weight marker (Precision Plus Protein Unstained, Biorad), using an ImageLab software (Bio-rad).

2.8.- Enzymatic activity product assessment

The activity of the produced PTDH-CHMO and ADH were assessed through an enzymatic assay, using the previously generated cell lysate (see section 2.5). In the case of PTDH-CHMO protein, CHMO activity was determined

spectrophotometrically by monitoring the decrease of NADPH at 340 nm (ε= 6.22 mM^{-1} cm^{-1}), using cyclohexanone as substrate, as described elsewhere (Valencia et al., 2018). ADH activity was measured spectrophotometrically following the formation of NADH at 340 nm. The reaction mixture contained ethanol at 543.6 mM, β-NAD at 7.5 mM, 20 mM of phosphate buffer (pH = 8.8), and the appropriate amount of enzyme sample. One unit of ADH activity was defined as the amount of enzyme activity required to catalyze the conversion of 1 μmol of NAD+ to NADH per minute at 25ºC.

Specific enzymatic activity (AU/mg enzyme) was calculated by dividing the volumetric activity (AU/ml) by the concentration of enzyme (mg/ml) for a given sample.

In Erlenmeyer experiments, results are presented as the mean of three independent experiments with 3 replicates run in parallel (n=3). Error bars correspond to the standard deviation.

3 Results

3.1.- NEB10β_PTDH-CHMO characterization: temperature and inducer concentration assessment

E. coli NEB10β strain was first characterized in terms of the temperature and inducer concentration used for PTDH-CHMO expression in Erlenmeyer culture with Terrific Broth medium. Expression at 24ºC resulted in the highest enzyme production (Figure 1A), with an increase of approximately two-fold with respect to production at 30ºC. In cultures at 37ºC, and at lower proportion at 30ºC, inclusion bodies were observed in the cells, causing a very low active enzyme expression. A concentration of 0.02% (w/v) of arabinose was applied for this assessment.

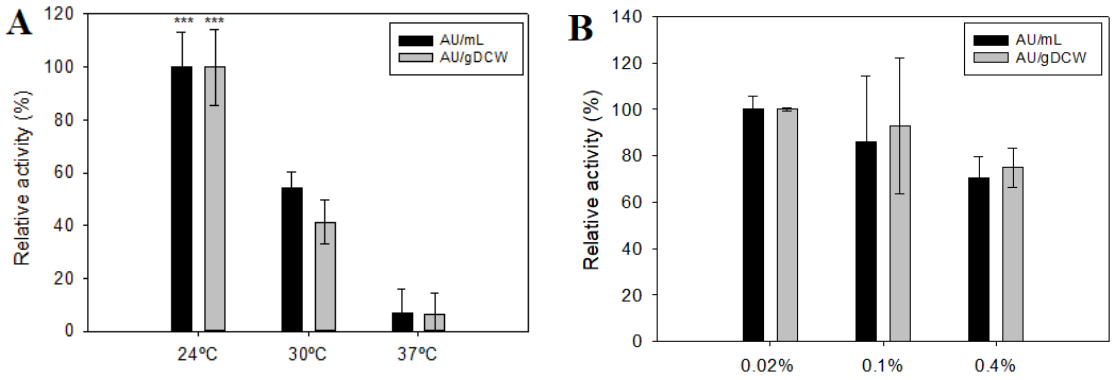
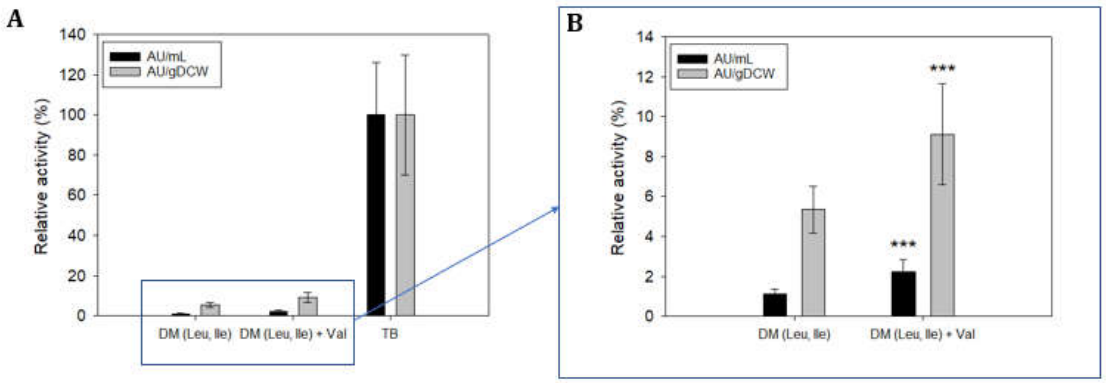


Figure 1: Assessment of temperatures (A) and inducer (arabinose) concentrations (B) for the expression of PTDH-CHMO in *E. coli* NEB10β. Significance of results was determined by running a paired *t*-student test (p-value < 0.05).

Inducer (arabinose) concentration was also assessed: three different inducer concentrations were tested, starting from 200 mg/L (0.02% (w/v)). By using higher inducer concentrations, no increase in enzyme production was observed (Figure 1B). In subsequent experiments, 24ºC and 0.02% (w/v) of arabinose concentration have been applied.

3.2.- Development of a defined medium: addition of valine

A defined medium [14] was used in order to later implement a fed-batch culture strategy, allowing for the increase of enzyme productivities. The medium contained glycerol as a carbon source, since, unlike glucose, it does not repress the induction of the P_{BAD} promoter by arabinose. It also had to be supplemented with the amino acids leucine and isoleucine, since the NEB10β strain is auxotroph for these two amino acids. Using this medium, the PTDH-CHMO productivities were very low compared to the complex TB medium in Erlenmeyer. Therefore, valine was added to the defined medium in an attempt to increase its productivity. Although *E. coli* NEB10β is not auxotroph for valine, the Δ(*ara leu*)7697 mutation that causes it to be auxotroph for leucine and isoleucine also affects genes that are involved in the biosynthesis pathway of valine: the genes b0077 and b0078, which are knocked out due to the Δ(*ara leu*)7697 mutation [15], codify for the acetolactate synthase enzyme, which is involved in the valine biosynthesis pathway. The addition of valine to the medium resulted in an approximate 2-fold increase of the product titer. However, both in terms of productivity and growth, it was still much poorer than those obtained with the complex TB medium (Figure 2).



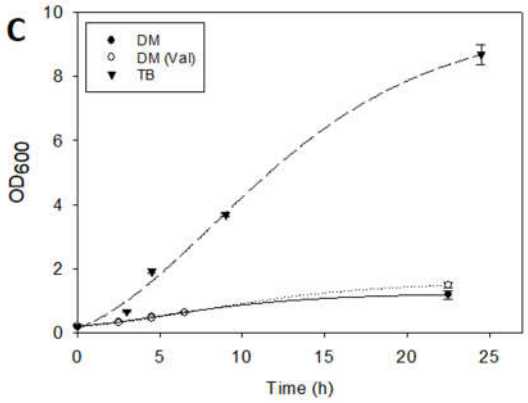


Figure 2: Assessment of the addition of valine to DM in terms of PTDH-CHMO expression in *E. coli* NEB10β. Comparison of the production obtained with DM with or without valine and also with terrific broth medium (A), comparison of the production obtained with DM with or without valine, amplified (B), and comparison of the culture growths obtained with the same media than in A (C). Significance of results was determined by running a paired *t*-student test (p-value < 0.05).

3.3.- Development of a culture strategy with defined medium: assessing a two-step production process with different carbon sources

In order to increase the productivity of the developed defined medium, and also in a further step to reach the implementation of a fed-batch process, alternative process strategies were assessed in Erlenmeyer cultures. These consisted in using different carbon sources in a simulation of a fed-batch: a first growth phase with a more biomass-producing efficient carbon source such as glucose was applied, followed by an induction phase with a non-repressing carbon source compatible with arabinose once glucose is completely exhausted. Sorbitol and glycerol were used as these carbon sources, glycerol yielding a higher productivity than sorbitol (Figure 3). In both cases, the product yield was higher than the control condition, in which the first growth phase was carried out with glycerol as a carbon source instead of glucose, and the induction phase was also carried out with glycerol. Therefore, the two-step process comprising a growth phase with glucose followed by an induction phase containing glycerol as carbon source showed the most promising result.

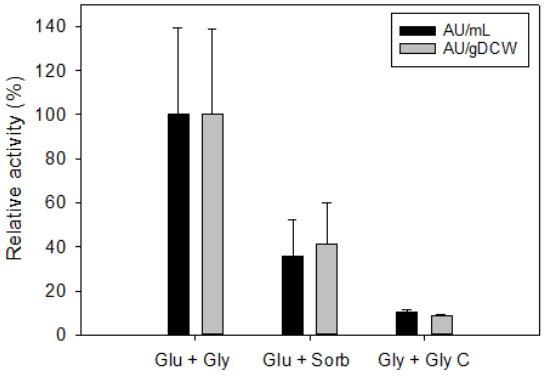


Figure 3: Assessment of the two-step process production of PTDH-CHMO in *E. coli* NEB10β. Comparison of the production levels in a glucose/glycerol, glucose/sorbitol, and glycerol/glycerol (control) strategy.

3.4.- Implementation into bioreactor: 2-step batch with defined medium with 2 carbon sources (glucose-glycerol) and comparison with TB batch

The developed two-step strategy with the defined medium was then implemented into a 2L bioreactor and compared to a batch culture with TB medium. In this case, in the two-step culture, when glucose was completely depleted from the medium (Figure 4A), medium with glycerol and arabinose was added to the culture as a pulse. The culture was then stopped 24h after induction, in order to have a total induction time comparable to the batch TB culture. The production (AU/mL) of PTDH-CHMO was similar for both approaches (Figure 4B), however, with the TB batch, this same product titer was achieved with less process time (24h vs 52h) and with less biomass, therefore yielding a higher volumetric and specific productivity. In the two-step strategy, after the addition of glycerol-containing medium, the consumption of glycerol was slow: an adaptation phase of glucose to glycerol consumption was observed.

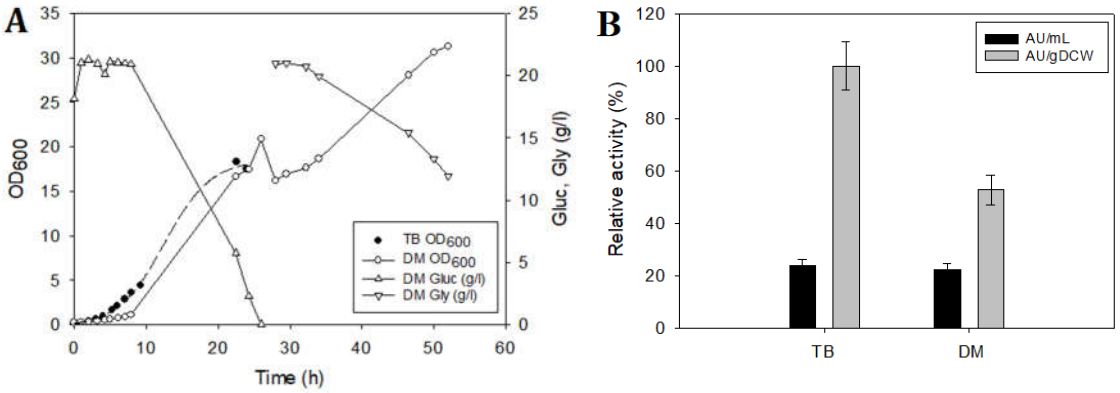


Figure 4: Assessment of the implemented two-step process with defined medium (DM) compared to the batch with terrific broth (TB), in 2L bioreactor. Depiction of the growth profiles of both cultures (A) and the production of enzyme (B).

3.5.- Implementation into bioreactor: fed-batch with defined medium

Once a production process in which the strain had been able to switch from glucose to glycerol consumption in a two-step batch process had been implemented, a more controlled fed-batch strategy was assessed, again with defined medium. In this case, a batch phase with glucose as a carbon source was followed by a fed-batch phase with glucose at a targeted specific growth rate (μ) of 0.2 h^{-1}, until an OD_{600} of approximately 100 was reached (corresponding to 30.5 g/L of biomass and 57 g of total biomass). This growth step was carried out at a temperature of 37ºC in order to achieve more quickly the desired biomass concentration. Then, the temperature

was lowered to 24ºC for the fed-batch phase with glycerol, as the induction of enzyme production was carried out in this step. This induction phase was performed at a $\mu = 0.05\ h^{-1}$, since glycerol had been consumed at a lower rate than glucose in the previous experiments. However, a slight accumulation of glycerol was observed at this growth rate (Figure 5), prompting the stopping of the feeding medium addition when this occurred. The induction was carried out adding arabinose 2 hours after the glycerol addition had started (indicated with an arrow in Figure 5), in order to ensure that the culture was adapted to grow consuming glycerol as a carbon source. After 6 hours of induction, the enzyme amount produced, in terms of activity, was 9.2-fold higher than for the previous TB batch. The culture was then continued for a further 14h (overnight) of fed-batch, at a $\mu = 0.03\ h^{-1}$ in order to avoid glycerol accumulation. At the end of this step the PTDH-CHMO production was 15.1-fold higher than the reference TB batch. The implemented fed-batch process was also favorable regarding the volumetric productivity, as well as in terms of specific activity of the produced enzyme, which was around 2-fold higher than for the TB batch (Table 1).

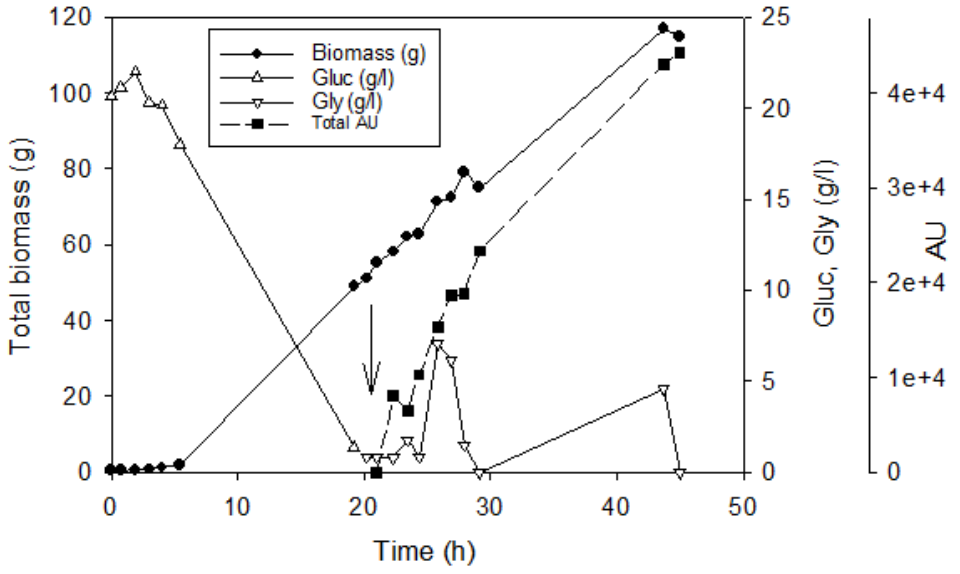


Figure 5: Biomass, substrate (glucose and glycerol) and PTDH-CHMO activity profiles of the *E. coli* NEB10β fed-batch culture in defined medium for PTDH-CHMO production. The arrow indicates the start of the induction.

Table 1: production parameters of PTDH-CHMO using *E. coli* NEB10β strain

	Batch TB	**Fed-Batch DM (6h ind.)**	**Fed-Batch DM (6h+overnight ind.)**
Activity (AU/mL)	1.35 ± 0.13	12.38 ± 1.19	20.42 ± 0.23

Volumetric productivity (activity) (AU/mL/h)	0.056 ± 0.005	0.44 ± 0.042	0.45 ± 0.005
Titer (g/L)	0.46 ± 0.05	1.99 ± 0.09	3.21 ± 0.07
Volumetric productivity (titer) (g/L/h)	0.019 ± 0.002	0.071 ± 0.003	0.071 ± 0.002
Specific productivity (AU/gDCW)	245.4 ± 22.60	310.27± 30.06	385.9 ± 4.33
Specific activity (AU/mg enzyme)	2.93 ± 0.419	6.22 ± 0.66	6.36 ± 0.16

3.6.- Validation of the developed process with a second enzyme: production of ADH

The fed-batch process implemented for the *E. coli* NEB10β strain for producing PTDH-CHMO was then applied to the production of a different enzyme, alcohol dehydrogenase (ADH), in order to assess its reproducibility and robustness. The process with the ADH-producing strain presented a similar growth profile to the reference PTDH-CHMO process. The improvement in terms of productivity of the defined medium fed-batch with respect to the TB batch was also on the same order than the reference process (24.3-fold increase at the end of the process, versus 15.1-fold for the PTDH-CHMO process).

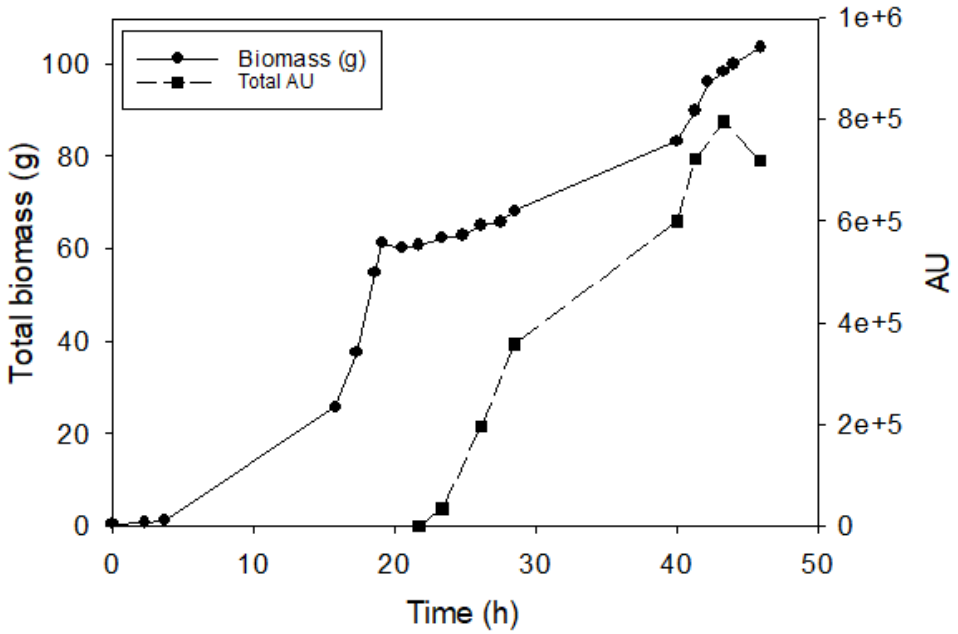


Figure 6: fed-batch with defined medium for ADH production: total biomass, total enzymatic activity and specific activity/biomass profiles.

Moreover, a similar pattern of higher specific activity of the produced enzyme in the defined medium fed-batch was observed for ADH (Table 2), duplicating the specific activity in the defined medium fed-batch process with respect to the complex medium batch, confirming again the behavior observed for the PTDH-CHMO process.

Table 2: production parameters of PTDH-CHMO and ADH using *E. coli* NEB10β strain

		Batch TB	FB DM (6h + overnight ind.)
Activity (AU/mL)	PTDH-CHMO	1.35	20.42
	ADH	16.53	402.1
Volumetric productivity (activity) (AU/mL/h)	PTDH-CHMO	0.056	0.45
	ADH	0.75	8.74
Titer (g/L)	PTDH-CHMO	0.46	3.21
	ADH	0.19	2.03
Specific productivity (AU/gDCW)	PTDH-CHMO	245	385.9
	ADH	2363	8056
Specific activity (AU/mg enzyme)	PTDH-CHMO	2.93	6.36
	ADH	86.6	198.6

4 Discussion

The obtention of high titers of a newly cloned recombinant protein with *E. coli* is a complex process normally requiring the use of well-established production strains. However, this could be simplified by using a strain compatible for both molecular biology and production purposes, therefore using only one strain for the protein development stage. Here, we report the ability of the molecular biology *E. coli* NEB10β strain as a host for the production of high amounts of recombinant protein, specifically for the enzymes PTDH-CHMO and ADH.

Initial characterization steps carried out in Erlenmeyer showed that low temperatures (24ºC) were found to enhance the production of an active product as well as to avoid the appearance of inclusion bodies. This was consistent with reports

of improved protein folding [16] and decreased hydrophobic aggregation related to lower temperatures [17], [18], [19]. The inducer concentration assessment revealed that an arabinose concentration of 0.2 g/L was found to be strong enough for inducing the protein expression, with higher inducer concentrations not improving the product titers. This arabinose concentration lays in the middle of a range of optimal arabinose concentrations reported in the literature for protein expression using the P_{BAD} system in other *E. coli* strains [6], [10].

Using a defined medium for protein production can be advantageous with respect to complex media, representing a cheaper, more reproducible and more scalable strategy, while also allowing a better control on process operations such as fed-batch. One of the main challenges that presents the NEB10β strain consists in the need for supplementation of the defined medium with leucine and isoleucine amino acids, since this strain is auxotroph for leucine and isoleucine. In this work it was found that adding valine, an amino acid that shares some of the steps of its biosynthesis pathway with leucine and isoleucine, could increment the production of product by 2-fold. During the protein induction phases glycerol was employed as a carbon source in order to avoid the repression of the P_{BAD} promoter. Using glycerol instead of glucose can be advantageous, since glucose has a high potentiality for generating acetic acid when oxygen is limited [10], and glycerol has also shown promising results in biomass/substrate yield in *E. coli* cultures [20].

Taking these several factors into account, a fed-batch process using a defined medium for the production of the PTDH-CHMO enzyme has been developed. This process consists in a batch phase and a first fed-batch growth phase in order to achieve high cell densities (both with glucose as a carbon source) followed by a second fed-batch induction phase with glycerol as a carbon source and induction with arabinose. In both fed-batch steps, feeding is controlled by a predetermined exponential profile, which is a robust and simple method. This approach represents an addition to the variety of arabinose-induced protein expression strategies reported in the literature. These include fed-batch processes that use defined medium with glucose kept at close to 0 g/L using a predetermined exponential feed rate [9]; fed-batch processes with complex TB medium and feeding controlled by a closed loop system depending on the oxygen concentration [10]; and fed-batch processes with defined media using glucose, employing *E. coli* strains that are able to consume arabinose [8].

The strategy developed in this work has allowed, for the PTDH-CHMO producing culture, after 6h of induction, an increase of 9.2-fold in product titer and 9.8-fold of volumetric productivity with respect to the culture batch with complex TB medium. The specific productivity in terms of enzymatic activity/biomass reached its maximum level after between 6 and 14h of the induction beginning, similarly to other reports of processes based on the P_{BAD} system [10]. Final product titers in the range of 2-3 g/L were achieved. The implemented production process has been

successfully replicated for the ADH enzyme production, showing its robustness and applicability. Furthermore, in both defined medium fed-batch processes, a higher specific activity of the produced enzyme was observed with respect to the batch with TB. This has been attributed to the difference of growth rate between the two processes, since lower growth rates have been described to enhance the production of the protein of interest in bacterial cultures [21]. Therefore, a product with a higher quality is obtained with the fed-batch process: for the same mass of protein, around two times more enzymatic activity is displayed.

Finally, the developed fed-batch strategy with defined medium for recombinant protein production with *E. coli* NEB10β strain, besides having a higher productivity than its batch with TB medium counterpart, is also more economically viable. The defined medium, despite the burden of including amino acids supplements, is cheaper than TB (around at least 30% cheaper in terms of cost per PTDH-CHMO activity unit), with costs calculated using small scale Sigma-Aldrich reagents (in the order of grams).

5 Conclusions

A production process for recombinant protein expression using the *E. coli* NEB10β strain has been established, to the best of the authors knowledge, for the first time in bioreactor. For the production of the complex fusion protein PTDH-CHMO, a fed-batch strategy using a defined medium supplemented with amino acids was found to have a 8-fold increase on volumetric productivity compared to a batch with complex TB medium after 20h of induction. This process was then validated through the production of the ADH enzyme with the same NEB10β strain, resulting again in a better performance (11.7-fold increase in terms of volumetric productivity) with respect to the batch with TB. The implemented fed-batch approach also results in a product with a high potency (around 2-fold higher than the batch) and it is also economically viable, rendering the NEB10β strain a suitable candidate for harboring the production process of newly cloned proteins, from the plasmid construction to the obtention of grams of product.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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