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Development of fed-batch profiles for efficient biosynthesis of catechol-*O*-methyltransferase

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ABSTRACT

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) plays a crucial role in dopamine metabolism which has intimately linked this enzyme to some neurodegenerative diseases, such as Parkinson's disease. In recent years, in the attempt of developing new therapeutic strategies for Parkinson's disease, there has been a growing interest in the search for effective COMT inhibitors. In order to do so, large amounts of COMT in an active form are needed, and the best way to achieve this is by up-scaling its production through biotechnological processes. In this work, a fed-batch process for the biosynthesis of the soluble isoform of COMT in *Escherichia coli* is proposed. This final process was selected through the evaluation of the effect of different dissolved oxygen concentrations, carbon and nitrogen source concentrations and feeding profiles on enzymatic production and cell viability, while controlling various parameters (pH, temperature, starting time of the feeding and induction phases and carbon source concentration) during the process. After several batch and fed-batch experiments, a final specific COMT activity of 442.34 nmol/h/mg with approximately 80% of viable cells at the end of the fermentation were achieved. Overall, the results described herein provide a great improvement on hSCOMT production in recombinant bacteria and provide a new and viable option for the use of a fed-batch fermentation with a constant feeding profile to the large scale production of this enzyme.

1. Introduction

Catechol-*O*-methyltransferase (COMT, E.C. 2.1.1.6) is a methyltransferase enzyme that catalyses the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to one of the hydroxyl groups of the catechol substrate (including catecholamine neurotransmitters and catechol estrogens) in the presence of Mg^{2+} [1]. This methylation reaction is a sequentially ordered mechanism, with SAM being the first to bind to the enzyme, followed by the Mg^{2+} ion and finally the substrate [1]. The enzyme exists as two isoforms: a soluble, cytosolic protein (SCOMT) and a membrane-bound protein (MBCOMT) [2], both coded by the same gene (located in chromosome 22) from two promoters. The general function of COMT is the elimination of biologically active or toxic catechols and other metabolites, while playing a particularly important role in the metabolism of catecholamines, especially in the breakdown of the neurotransmitter dopamine [3].

Over the last decades, the lack of dopamine has been linked to Parkinson's disease (PD) [4], and so, levodopa and dopamine agonists are currently the drugs of choice for PD when a significant symptomatic effect needs to be achieved [5]. However, the use of COMT inhibitors plus levodopa is more effective at reducing PD symptoms when compared to the use of levodopa alone [6]. In the present, only two COMT inhibitors are currently available, namely tolcapone, which use is restricted; and entacapone, a safer but less efficient compound [7]. In order to develop new COMT inhibitors, a high quantity of enzymatically active COMT is needed, either for crystallization studies based on structural-based inhibitors interactions [8], or to perform in vitro experiments required for the development of a new drug formulation.

The best strategy to obtain considerable amounts of human proteins is by applying recombinant technology. In the case of recombinant human SCOMT (hSCOMT), it has been produced via different expression systems, such as transfected mammalian cells [9], insect cells (via mammalian and baculovirus vectors) [10], plant cells (via a potyvirus) [11] and prokaryotic cells, such as

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Escherichia coli. *E. coli* is a Gram-negative bacterium and is the most commonly used organism for heterologous human protein biosynthesis [12–16], allowing the establishment of large scale production systems due to its ability to quickly reach high cell densities in inexpensive media.

For routine protein expression, *E. coli* B and K strains, along with their derivatives, are the most frequently used, with BL21 being the most suitable strain for protein production due to the lack of two specific proteases (*lon* and *ompT*), thus avoiding heterologous protein degradation. One particular BL21 derivative strain, *E. coli* BL21 (DE3), has been used to successfully express thousands of homologous and heterologous soluble proteins to high levels [16,17], including COMT [18–20]. Apart from the optimization of growth conditions, to achieve high quantities of recombinant protein, a large-scale culture processes have to be applied, mostly based on fed-batch mode cultures [14,21,22].

A fed-batch culture is generally started with an inoculum growing at the maximum specific growth rate that can be sustained using the nutrients initially present in the bioreactor, followed by the imposition of a specific regime of nutrient feed until fermentation is complete [14]. These methods are based on mathematical models that describe growth patterns and the expected demand for nutrients [22]. Regarding the pattern of nutrient addition, three main types of pre-determined feeding profiles can be considered: constant, exponential and stepwise feeds [14]. Another feeding approach that can be used is based on the direct or indirect feedback control systems for the controlled addition of nutrients. Indirect control is based on online monitoring of parameters such as pH, dissolved oxygen, CO_2 evolution rate and cell concentration. Direct feedback is based on monitoring the concentration of the major carbon substrate [14,22].

In this work, a fed-batch bioprocess was developed, via an up-scaling of hSCOMT production. Initially, several batch fermentations were carried out, in order to establish the ideal culture conditions, for instance batch phase and bioreactor operation for the fed-batch fermentations. After this stage, several fed-batch fermentations with different feeding profiles were tested in order to maximize biomass production and to improve protein activity levels, without compromising cell viability.

2. Materials and methods

2.1. Chemicals

Ultrapure reagent-grade water was obtained from a Mili-Q system (Millipore/Waters). Carbenicillin disodium salt, calcium chloride dihydrate, magnesium sulfate heptahydrate, lysozyme, cobalt(II) chloride hexahydrate, dithiothreitol (DTT), SAM chloride salt, DNase, epinephrine (bitartrate salt), disodium ethylenediamine tetraacetic acid (EDTA), sodium octyl sulfate (OSA), bovine serum albumin (BSA), LB-Agar, IPTG, tryptone, glycerol and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium chloride, sodium chloride, boric acid were supplied by Fluka (Buchs, Switzerland). Sodium phosphate dibasic and potassium dihydrogen phosphate monobasic were obtained from Panreac (Barcelona, Spain). Bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) was obtained from Molecular Probes®, Invitrogen, part of Life Technologies (Carlsbad, CA, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Expression vector and bacterial strain

The Champion pET101 Directional TOPO expression kit (Invitrogen Corporation, Carlsbad, CA, USA) was used for the expression of hSCOMT on *E. coli* BL21(DE3) strain kindly provided by Bial (Departamento de Investigação e Desenvolvimento, São Mamede do Coronado, Portugal).

2.2.2. Escherichia coli pre-cultivation, batch and fed-batch fermentations

In this study, except for tryptone and glycerol concentrations, all media components for the semi-defined medium were kept constant (5.5 g/L Na_2HPO_4, 0.5 g/L NaCl, 1.64 g/L citric acid monohydrate, 2 g/L potassium citrate, 1.21 g/L $MgSO_2{\cdot}7H_2O$, 50 μg/mL carbenicillin and 1.5 mL/L trace elements solution) for the pre-cultivations, batch, and batch phase of fed-batch experiments. The trace elements solution consisted of 27 g/L $FeCl_3{\cdot}6H_2O$, 2 g/L $ZnCl_2$, 2 g/L $CoCl_2{\cdot}6H_2O$, 2 g/L $Na_2MoO_4{\cdot}2H_2O$, 1 g/L $CaCl_2{\cdot}2H_2O$, 1.2 g/L $CuSO_4$ and 0.5 g/L H_3BO_4, prepared in 1.2 M HCl. LB agar plates supplemented with 50 μg/mL carbenicillin were inoculated from a cell bank aliquot and grown overnight at 37 °C. Afterwards, a pre-cultivation was performed inoculating a single colony into a 500 mL shake flask containing 125 mL of semi-defined medium, and grown at 37 °C and 250 rpm until an optical density at 600 nm (OD_{600}) of approximately 2.6 was reached. Batch and fed-batch processes were carried out in 750 mL bench-top parallel mini-bioreactors (Infors HT, Switzerland) with 250 mL of semi-defined medium. In our research group, three physical culture conditions for the production of hSCOMT in shake flasks were already optimized [20], namely temperature (40 °C), pH (6.5) and stirring rate (351 rpm) and this was the starting point for the strategy described in the present work. So, the bioreactors were inoculated from the pre-cultivation to obtain a starting OD_{600} of approximately 0.2. Temperature and pH were kept constant throughout the batch and fed-batch phases at 40 °C and 6.5, as previously optimized, with the pH value controlled by the automatic addition of 0.75 M H_2SO_4 and 0.75 M NaOH through two peristaltic pumps. The dissolved oxygen percentage (pO_2) was controlled by a two-level cascade of stirring (between 250 and 900 rpm) and air flow (between 0.2 and 2 vvm).

In general, the feeds consisted of different concentrations of tryptone and glycerol dissolved in deionized water and their addition was maintained by automated peristaltic pumps controlled by IRIS software (Infors HT, Switzerland).

2.2.3. Cell lysis

Intracellular SCOMT was obtained via a combined lysis process. Typically, 2 mL of samples from fermentations were centrifuged at 4 °C and 16,000 × *g* for 5 min, resuspended in 500 μL of a standard buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, 5 μg/mL leupeptin and 0.7 μg/mL pepstatin), transferred to lysis tubes and kept on ice. The lysis process was then carried out as previously described [20]. The resulting supernatant, containing the solubilized SCOMT, was used as sample for the enzyme activity and protein quantitation assays.

2.2.4. Flow cytometry assays

In order to assess cellular viability during the fermentation runs, samples were retrieved at specific times and treated for the flow cytometry assays, according to a previously developed protocol [23]. The samples' OD_{600} was measured and a dilution with PBS buffer was prepared to obtain a final OD_{600} of 0.2 (approximately 1×10^8 cells/mL and further diluted in PBS with 4 mM NaEDTA to a cell concentration of about 1×10^6 cells/mL). To this cell suspension, the appropriate volumes of PI and BOX were added in order to attain final concentrations of 10 and 2.5 μg/mL, respectively. The samples were incubated for 15 min at room temperature in the dark, centrifuged for 5 min at 5000 rpm and resuspended in PBS prior to analysis in a CyAn ADP flow cytometer (Beckman Coulter Inc., California, United States). Acquisition and analysis were performed with the Summit Software (Beckman Coulter

Inc., California, United States). The acquisition was based on light scatter and fluorescence signals resulting from 25 mW solid state laser illumination at 488 nm Fluorescence signals were collected by FL1 (530/40 nm, BOX) and FL4 (680/30 nm, PI) bandpass filters. Light scattering, BOX and PI fluorescence measurements were acquired logarithmically. Threshold was set on SSC to exclude noise, other particles and debris. Cells were gated according to their light scatter parameters. Sample acquisition was operated at flow rate of no more than 300 events per second and a total of 5000 cells were gated and analyzed for each sample.

2.2.5. Analytical methods

2.2.5.1. Glycerol. For glycerol determination, samples were retrieved at specific times and centrifuged at 4 °C and 16,000 × *g* for 5 min The resulting supernatant was then filtered through a 0.22 μm filter (Millipore) for subsequent HPLC analysis onto an Agilent 1290 Infinity LC HPLC system (Waldbronn, Germany) coupled with a Refractive Index Detector (RID) (Agilent 1260 Infinity). Compound separation was achieved using a Hi-Plex H ion-exchange analytical column (Agilent, Santa Clara, CA, USA) with a 7.7 × 300 mm and 8 μm pore size. The mobile phase consisted of a 5 mM H_2SO_4 solution prepared with ultrapure water, filtered through a 0.2 μm pore membrane and degassed for 15 min before use. Flow rate was set to 0.6 mL/min and column temperature was set to 65 °C.

2.2.5.2. Enzymatic activity. The enzyme activity was measured via the quantity of metanephrine produced as a result of the reaction between recombinant hSCOMT and the substrate epinephrine, with samples being processed as described elsewhere [24]. The resulting metanephrine was measured via an HPLC system with coulochemical detection as previously described [25], applying a total protein concentration of 150 μg/mL. Specifically, the injections were performed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, CA, USA) equipped with an autosampler and quaternary pump coupled to an ESA Coulochem III (Milford, MA, USA) coulometric detector. Chromatographic separation was achieved on an analytical column Zorbax 300SB C_{18} reverse phase analytical column (250 mm × 4.6 mm i.d. 5 μm) (Agilent, Santa Clara, CA, USA). The mobile phase (0.1 M sodium dihydrogen phosphate, 0.024 M citric acid monohydrate, 0.5 mM OSA and 9% acetonitrile, v/v), pH 2.9, was filtered under vacuum (0.2 μm hydrophilic polypropylene filter) and degassed in ultrasonic bath before use. Column effluent was monitored with an electrochemical detector by a coulometric mode, which was equipped with a 5011 high sensitivity dual electrode analytical cell (electrodes I and II) using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: −350 mV). The flow rate applied was 1 mL/min. Column temperature was optimized to 30 °C. The chromatograms were obtained by monitoring the reduction signal of the working electrode II.

2.2.6. Total protein

The protein determination was carried out using a Pierce BCA Protein Assay kit (Thermo Scientific, USA) on a 96 well plate according to manufacturer's instructions, after which the absorbance at 570 nm was measured and the values applied to a previously calculated calibration curve.

3. Results

3.1. Batch fermentations

Two batches were performed at 30% dissolved oxygen to determine the typical growth curve under these conditions. The medium composition used was the one previously described in Section 2.2.2, with a concentration of glycerol and tryptone of 30 and 20 g/L,

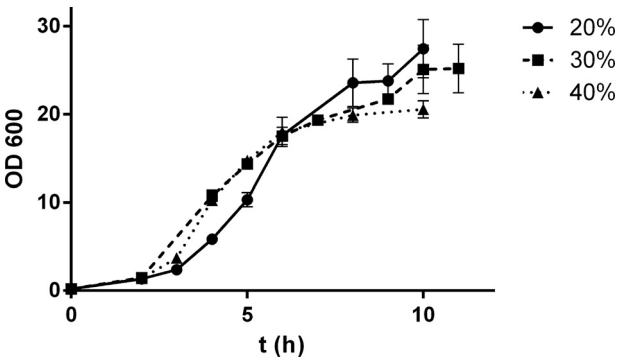

Fig. 1. Growth curves of *E. coli* in a batch process with 20, 30 and 40% dissolved oxygen ($n = 2$).

respectively. These fermentations showed that the stationary phase of growth is reached after approximately 8 h of fermentation. Under these conditions the maximum OD attained is of about 28 (data not shown).

Subsequently, the next step was to evaluate the effect of dissolved oxygen concentration on COMT production, testing three set-points for dissolved oxygen concentrations (20, 30 and 40%) and performing recombinant COMT induction. The three different dissolved oxygen set-points (20%, 30% and 40%, Fig. 1) were tested in duplicates and the results for each set-point were averaged. All fermentations were stopped 4 h after induction, according to the experiments. For the activity assays, cell samples were retrieved at the end of the fermentation. The results from Fig. 1 show that a dissolved oxygen concentration of 20% gives better results than the other two concentrations tested in terms of maximum OD reached.

The following step was the assessment of the most appropriate carbon (glycerol) and nitrogen (tryptone) source concentrations in the batch phase stage for the fed-batch process, in order to reduce time, and also to increase cell density at the end of the batch phase. It is extremely relevant to reduce batch and fed-batch times in order to avoid, or at least minimize, nutrients/oxygen depletion. To achieve this, the concentration of glycerol and tryptone were varied, according with three formulations: 1st formulation (20 g/L glycerol and 20 g/L tryptone), 2nd formulation (10 g/L glycerol and 15 g/L tryptone) and 3rd formulation (20 g/L glycerol and 30 g/L tryptone) (growth curves were depicted in Fig. 2).

3.2. Growth rate and time of fed-batch initiation

The last parameters to be assessed before initiating fed-batch experiments were this strain's growth rate and the time at which to initiate the feeding process under these conditions. The growth rates, μ (h^{-1}), obtained for the 1st, 2nd and 3rd formulations, depicted previously, were 0.51, 0.49 and 0.55 h^{-1}, respectively,

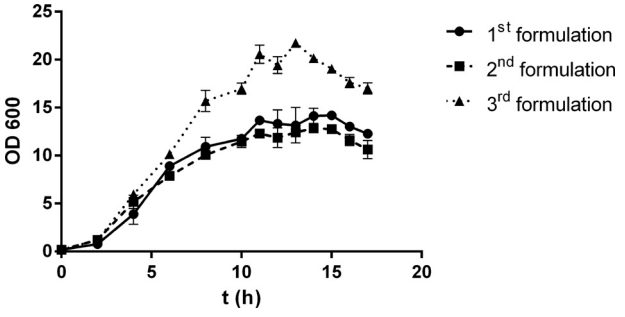

Fig. 2. Growth curves of the assays corresponding to the three formulations ($n = 2$). 1st formulation, 20 g/L tryptone and glycerol; 2nd formulation, 10 g/L glycerol and 15 g/L tryptone; 3rd formulation, 20 g/L glycerol, 30 g/L tryptone.

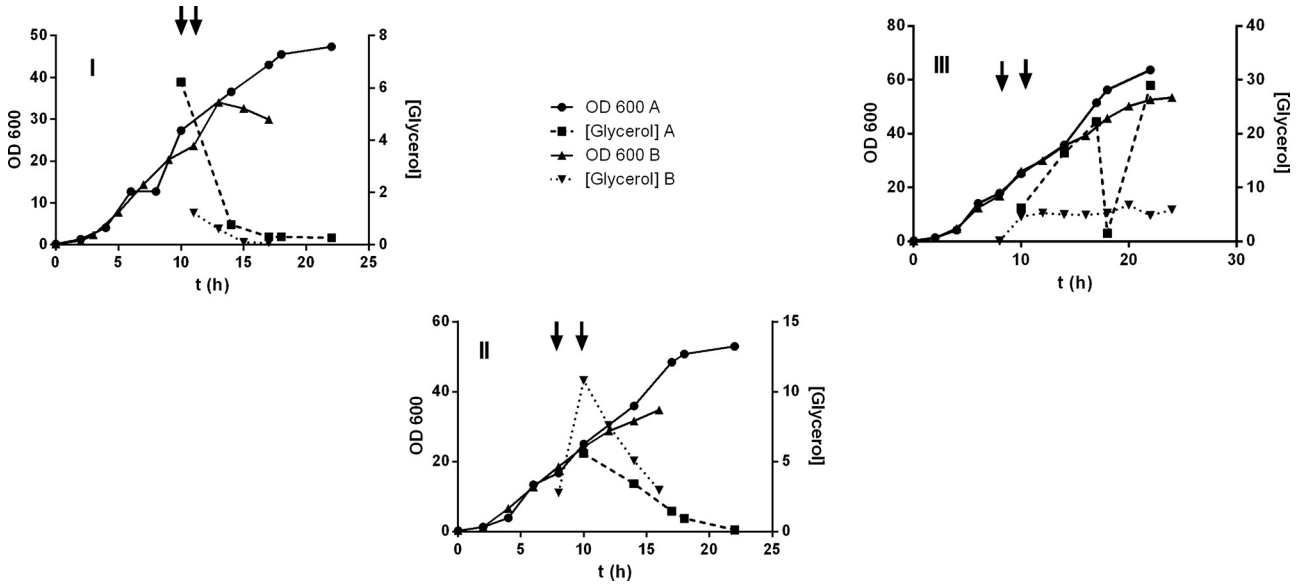

Fig. 3. Growth curves and glycerol concentration profiles of the assays for the constant feeding profiles. I – 1 g glycerol/L/h, left and right arrows indicate the starting of the feeding for replicates A and B, respectively. II – 3 g glycerol/L/h, right and left arrows indicate the starting of the feeding for replicate A and B, respectively. III – 6 g glycerol/L/h, right and left arrows indicate the starting of the feeding for replicate A and B, respectively.

indicating that these glycerol and tryptone concentrations allowed similar growth profiles. In theory, the fed-batch process should be initiated when the carbon source is completely depleted, to ensure nutrient limitation. Given this, it is relevant to know exactly when the carbon source is completely depleted. So, glycerol concentration was measured every 2 h for the three formulations mentioned in the previous subsection. Results are consistent with the initial glycerol concentrations in each fermentation. The 1st and 3rd fermentations were started at an initial glycerol concentration of 20 g/L, and the 2nd at 10 g/L, and after 4 h of fermentation, only a small amount of that initial glycerol was consumed (data not shown).

3.3. Fed-batch trials

Given all the previous assays, the fed-batch fermentations were initiated with a batch phase containing glycerol and tryptone at a concentration of 20 g/L and a dissolved oxygen rate of 20%. The feeding profiles were initiated at 10.5 h of batch fermentation. For the preliminary fed-batch studies, two predetermined feeding profiles, namely exponential and constant feeding were preferred. For each feeding profile, three feeding rates were evaluated: 1, 3 and 6 g glycerol/L/h for constant feeds and 0.1, 0.2 and 0.3 h^{-1} for exponential feeds.

3.3.1. Constant feeding profiles

To achieve the desired rates (1, 3 and 6 g glycerol/L/h), several feed mediums with different glycerol concentrations were prepared. For these assays, the three feeding rates were tested as duplicates (A and B), without induction, so that the growth profiles could be established (Fig. 3). In these fed-batch experiences, glycerol was measured as mentioned in Section 2.2.4 until the end of the feeding process. The growth curves for these profiles (Fig. 3) show a maximum OD of about 50 which, as expected, is considerably higher than those obtained in the batch experiments. For the 1 g/L/h constant feeding profile, glycerol concentration was kept close to zero until the end of the fed-batch process, meaning that these cultures were able to consume all of the glycerol provided by the feeding solution. For the 3 g/L/h constant feeding profile, glycerol concentration reached close to zero values only after about 10 h of fed-batch, meaning that limiting concentrations are not reached during most of the fed-batch process. However, the maximum OD reached (52) was very similar to that of the 1 g/L/h feeding profile. Finally, for the 6 g/L/h feeding profile, glycerol concentrations either increased throughout the experiment (replicate A) or were kept constant at relatively low levels (replicate B). Since glycerol concentrations during the fed-batch phase of the feeding profiles evaluated were very different (from almost 0 g/L to as high as 30 g/L), cytometry assays were used to see if the feeding profile of 1 g/L/h was, in fact, the best choice among the three constant feeding profiles tested.

In order to assess cell physiology during the fed-batch experiments, flow cytometry assays were carried out using a PI/BOX dual staining. Dead cells will be stained with both BOX and PI, cells with depolarized membrane will be stained only with BOX and viable cells will not be stained. The results (not shown), indicate that as fermentation time increases, the percentage of dead cells (stained with PI and BOX) also increases. This effect is heightened at higher feeding rates, possibly because of the higher glycerol concentrations, which can hamper *E. coli* growth. In fact, at the end of the fermentation, the average percentages of viable cells were 79.43, 65.84 and 75.61% for 1, 3 and 6 g/L/h, respectively.

3.3.2. Exponential feeding profiles

The three chosen specific growth rates for exponential feeding profiles were 0.1, 0.2 and 0.3 h^{-1} with feed medium addition speed being calculated according to an equation previously described [14]. For this set of experiments, the three specific growth rates were also performed in duplicates (A and B) without induction (Fig. 4). Glycerol concentration and cellular viability assessments were performed as described for the continuous feeding profiles.

The results showed that all three specific growth rates tested yielded approximately the same maximum ODs (40–50), which were also similar to those obtained with the constant feeds. In these experiments, glycerol concentrations were generally high from the start of the feeding, due to higher feeding speeds, indicating that cells are not able to exhaust all the glycerol added to the culture medium. Taking into account the results of both feeding profiles, the selected feeding profile for hSCOMT induction fermentation was a constant feed of 1 g glycerol/L/h.

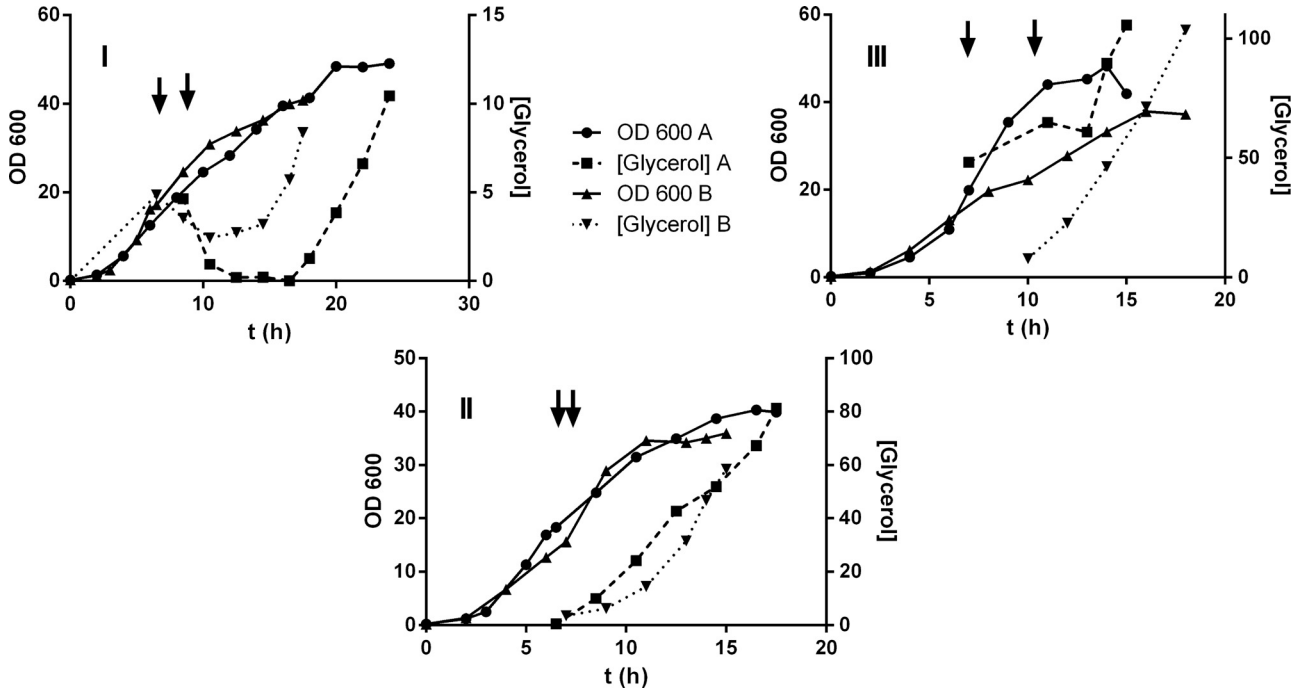

Fig. 4. Growth curves and glycerol concentration profiles of the assays for the exponential feeding profiles. I – 0.1 h^{-1}, right and left arrows indicate the starting of the feeding for replicates A and B, respectively. II – 0.2 h^{-1}, left and right arrows indicate the starting of the feeding for replicates A and B, respectively. III – 0.3 h^{-1}, left and right arrows indicate the starting of the feeding for replicates A and B, respectively.

3.4. COMT production in fed-batch fermentations

As mentioned above, for the final fermentations a constant feed of 1 g glycerol/L/h was used, with a higher (50 g/L) initial concentration of tryptone in order to compensate the possible tryptone limitation during the fed-batch phase. All other bioprocess parameters remained unaltered. Firstly, a fermentation without induction was performed, in order to determine the starting point of the stationary phase with this new medium formulation, and consequently the start of the feeding (Fig. 5). As seen in Fig. 5, the stationary phase was reached at about 8 h into the fermentation, and that was the time chosen to initiate the feeding. However, and since there was no significant increase in cell growth after this point, we decided to initiate the feeding 1 h earlier (at 7 h) in the subsequent experiment, with IPTG induction.

The induction was carried out 1 h after starting of the feeding, for 4 h. In this fermentation, glycerol quantitation assays were carried out as mentioned above and as expected, glycerol consumption profile was very similar to the previous assays carried out with this feeding profile, however in this case, glycerol concentration was low just from the beginning, and after 2 h of feeding, the concentrations remained the same in both replicates (data not shown). Cytometry assays were carried out as explained above, and the results for these fermentations can be seen in Fig. 6 (only for the first replicate). As the results show, the percentage of viable cells at the end of the fermentation are relatively high, between 84% and almost 90%.

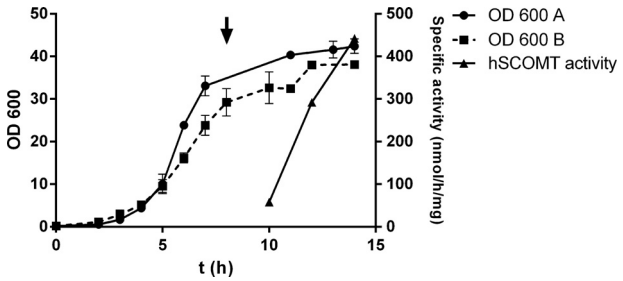

Fig. 5. Growth curve for the final fermentations, with (B) and without (A) IPTG induction ($n = 4$). Arrow indicates the starting of the feeding for curve B.

For the enzymatic assay, samples were taken every 2 h after induction (until 6 h of induction), and treated according to the method described in Section 2.2.3. Specific activity results are plotted in Fig. 5, and as we can observe an increment in activity is achieved during 6 h after induction from 56 nmol/h/mg to 442.34 nmol/h/mg.

4. Discussion

In recent years, several attempts have been performed to obtain a large quantity of active and pure hSCOMT. One of the most effective ways of enhancing recombinant protein production is the application of a fed-batch process, which highly increases cell density and, subsequently, protein production. In this work, a fed-batch bioprocess was developed for hSCOMT biosynthesis. Initially, several batch fermentations were carried out, in order to establish and optimize culture conditions, batch phase and bioreactor operation for the fed-batch fermentations. After this stage, a series of fed-batch fermentations with different feeding strategies were tested in order to obtain the maximum biomass production.

4.1. Batch fermentations

Firstly, dissolved oxygen concentration in culture media was studied, as it is one of the most difficult variables to reproduce, due to the combination of low oxygen solubility in water and the requirement for pure oxygen supplementation when cell density increases [26].

As mentioned in Section 3, two batches were performed at 30% dissolved oxygen [19] to determine the typical growth curve under these conditions. A maximum OD of 28 was obtained in these assays, which was significantly higher than the value

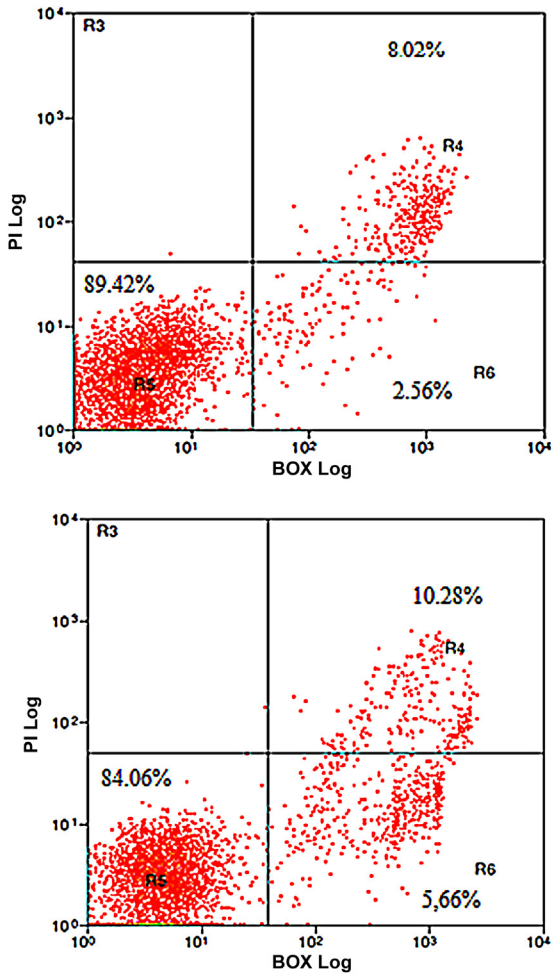

Fig. 6. Cell samples taken at (A) 0 h immediately after induction and (B) 6 h after induction, for the first replicate. Cells were stained with PI/BOX, and up to three main subpopulations of cells can be distinguished, corresponding to healthy polarized cells (R5), not stained; depolarized cells (R6), stained with BOX; and cells with permeabilized and depolarized cytoplasmatic membranes, (R4), stained with both PI and BOX.

previously obtained [19] for fed-batch fermentations applying the same expression system, culture medium and dissolved oxygen concentration. In fact, just by applying the physical parameters optimized by [27] to a mini-bioreactor platform, maximum OD values reached were very promising.

Afterwards, three standard set points for dissolved oxygen concentration (20, 30 and 40%) were tested. Based on the maximum OD reached, these results showed that a batch at 20% oxygen gives better results than 30% and 40%. This may not correspond to the expected results as higher percentages of dissolved oxygen should allow increased cell growth. However, the maintenance of the set value of dissolved oxygen is not possible throughout the whole batch process using agitation and airflow cascade, indicating that oxygen supplementation might be needed for these fermentations. Subsequently, two more fermentation runs at 20% dissolved oxygen were performed, with samples for enzymatic activity assay being withdrawn every hour after induction, to verify whether there was a peak of activity during this 4 h period. Therefore, we concluded that the best time for enzymatic activity was, in fact, 4 h after induction, due to the fact that those times corresponded to the highest values of specific COMT activity (316.16 and 237.20 nmol/h/mg for each assay, respectively), what is in agreement with previous results [19,20].

The next step in this study was to test carbon and nitrogen source concentrations in the batch phase. Regarding carbon source, it is known that, when compared to glucose, glycerol could be a better choice as it yields reduced acetate levels, low growth inhibition at high concentrations [13,14,19,28] and higher heterologous protein expression levels in *E. coli* [19,29]. Lower concentrations of glycerol (10–20 g/L) were proven to be preferable for higher hSCOMT specific activity results [19], and so, this was the concentration range chosen. Tryptone concentration variations were kept around the 20 g/L concentration present in the semi-defined medium, as it was previously optimized. From Fig. 2, it appears that tryptone greatly influences cell growth, as the fermentation with the higher tryptone concentration (3rd formulation) yielded higher optical densities, whereas the fermentation with the lowest tryptone concentration (2nd formulation) showed a decreased cell density when compared with the other two formulations. Glycerol does not seem to have such a great impact in cell growth at the lower concentrations used in these experiments, since the two formulations with different glycerol concentrations (1st and 2nd) led to similar growth profiles and cell densities, which meets the results previously obtained [19]. Since the main aim of these experiments was to reduce the batch phase time, the selected formulation was glycerol and tryptone at a concentration of 20 g/L, the first formulation, due to the fact that nutrient exhaustion occurred at a lower fermentation time (data not shown).

4.2. Fed-batch trials

To initiate the fed-batch trials, the growth rates for each glycerol/tryptone combination had to be assessed, and we verified that these were very similar and consistent with previously estimated values [19] (about 0.50 h^{-1} for a glycerol concentration of 10 g/L). It is important to determine the specific growth rates for each formulation for the establishment of the feeding profiles, namely exponential feeding profiles, as these are normally set to fall below the maximum specific growth rate of the expression system, thus minimizing acetate formation [14,30]. Results showed that, for the selected formulation of 20 g/L of glycerol and tryptone (1st formulation), after 11 h of fermentation almost all of the glycerol present in the culture is consumed. This was the time selected to initiate the feeding process. With all aspects determined, the feeding profiles were chosen, based on previously described feeding profiles [19], on the typical growth rates for exponential feeding [14], and on the maximum specific growth rates obtained for the batch fermentations, since the growth rates selected for the feeding should be lower than the maximum value obtained, in order to guarantee complete glycerol consumption.

4.2.1. Constant feeding profiles

In a constant feeding strategy, a predetermined constant rate of glycerol is fed to the reactor [14]. The results obtained for the fermentations with constant feed profiles suggested that the amount of glycerol fed to the bioreactor was significantly higher than what *E. coli* could consume.

From the three feeding profiles tested, the one that had a greater reproducibility was 1 g/L/h, and since all three of them achieved similar maximum ODs (around 50), this seemed the best option to perform a constant feeding profile.

4.2.2. Exponential feeding profiles

Typically, exponential feeding allows cells to grow at predetermined specific growth rates, usually between 0.1 and 0.3 h^{-1} [14], and so three exponential feeding rates falling between these limits were chosen (0.1, 0.2 and 0.3 h^{-1}). The results for the exponential feeding profiles suggested that cell growth was not visibly hampered by the high glycerol concentrations observed in

feeding stage, as these feeding profiles were able to achieve the same OD level of that obtained for the constant feeding profiles. From the three growth rates, the lower rate used ($0.1\ h^{-1}$) seems to be preferable, taking into account its reproducibility and the ability of cells to consume the glycerol provided by the feed in the early stages of the fermentation.

Comparing these results to those obtained with constant feeds, both allowed the achievement of very similar maximum ODs (between 50 and 60, approximately), and because the feeding solutions for the exponential feeds require much larger quantities of glycerol, constant feeds seem preferable, considering the lower costs associated in a further scale-up strategy.

Similarly to the results obtained for constant feeding experiments, cellular viability results in exponential feeding showed that the number of dead cells increased throughout the fed-batch phase. Since glycerol concentration did not seem to have a great influence in cell growth and viability, it seems that other aspect may be affecting cell growth in late stages of the fermentation. One of the possibilities is the accumulation of toxic byproducts during the process, that has been reported in fed-batch processes [14,22,27]. Another possible factor that might be influencing these results is tryptone concentration, which might be hampering *E. coli* viability as a limiting substrate.

4.3. COMT production in fed-batch fermentations

Maximum OD reached in these fermentations was a little lower (about 40), which can be associated with IPTG induction, since this inducer is known to be toxic and promote metabolic stress [13,17]. The comparison of cytometry results from the fermentations at constant feeding with the same feeding rate (1 g/L/h) showed overall lower percentages of permeabilized and dead cells. This may be possibly due to the higher concentration of tryptone present in these fermentations, confirming the above mentioned possible effect of low tryptone concentrations in cell viability. Another reason for these seemingly better results might be related with process duration. In these last assays, the whole process (batch and fed-batch) only took 13 h to develop, against the 17 and 22 h of the processes that used the same feeding rate. This shorter period was probably due to the early implementation of the fed-batch technique (7 h of batch fermentation, against 9 and 10 for the other assays). With lower fermentation times, possibly toxic by-products are less likely to accumulate, or they do so at lower levels, and so their effect on cell viability is not so evident.

From Fig. 5, we can see that specific hSCOMT activity enhances progressively after induction, with the highest value (442.34 nmol/h/mg) being achieved 6 h after induction, since the promoter had more time to act.

5. Conclusions

In this study, several fermentation conditions were tested to increase SCOMT production in *E. coli* BL21 (DE3) strains, with the aim of developing a fed-batch strategy suitable for COMT production and further scale-up. This study indicates that a fed-batch process as a good option for recombinant human SCOMT production in *E. coli* BL21 (DE3), and it was verified that a constant feeding process is preferable to exponential feeding strategies. An OD_{600} of about 40 was achieved via a constant feeding profile of 1 g glycerol/L/h, with a maximum specific hSCOMT activity of 442.34 nmol/h/mg. Finally, we verified that a high percentage of viable cells was maintained at the end of the fermentation. The combined results of high optical densities reached in comparison with previous work with this protein in this expression system, the high specific hSCOMT activity and high cell viability at the end of the fermentation suggest that further optimization of this particular expression system is a great option for human SCOMT production, and a scale-up process could be extremely promising, giving even better results in terms of cell growth and protein productivity.

Conflict of interest

The authors have declared no conflict of interest.

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