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Application of a Fed-Batch Bioprocess for the Heterologous Production of hSCOMT in *Escherichia coli*

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In this paper, a fed-batch cultivation process in recombinant *Escherichia coli* BL21(DE3) bacteria, for the production of human soluble catechol-*O*-methyltransferase (hSCOMT), is presented. For the first time, a straightforward model is applied in a recombinant hSCOMT expression system and distinguishes an initial cell growth phase from a protein production phase upon induction. Specifically, the kinetic model predicts biomass, substrate, and product concentrations in the culture over time and was identified from a series of fed-batch experiments designed by testing several feed profiles. The main advantage of this model is that its parameters can be identified more reliably from distinct fed-batch strategies, such as glycerol pulses and exponential followed by constant substrate additions. Interestingly, with the limited amount of data available, the proposed model accomplishes satisfactorily the experimental results obtained for the three state variables, and no exhaustive process knowledge is required. The comparison of the measurement data obtained in a validation experiment with the model predictions showed the great extrapolation capability of the model presented, which could provide new complementary information for the COMT production system.

Keywords: Human soluble catechol-*O*-methyltransferase, *Escherichia coli*, fed-batch bioprocess, protein production

Currently, recombinant human proteins as biological pharmaceuticals have become relevant targets in several medical domains. The combination of recombinant DNA technology and large-scale culture processes has enabled the production of sufficient active amounts that might otherwise not be obtained from natural sources [17]. The large-scale production of these proteins in the *E. coli* expression system is usually achieved using a two-stage process. In the first phase, cells are grown to a high cell density under favorable growth conditions in which protein synthesis is kept at a minimum [22], followed by a second step in which high-level expression of the recombinant protein is achieved upon induction.

The production is affected by numerous process factors, such as the cultivation mode, time of induction (with respect to cell mass concentration), duration of the production phase, and composition of the medium [2, 29]. Many data in literature showed that *E. coli* grows in salt-based chemically defined media [19, 39] as long as an organic carbon source is provided [22], as well as in rich complex organic media. Nevertheless, higher complexities in medium composition can lead to a lower reproducibility of the cell metabolic response and, therefore, lower the possibility of a well-controlled process [31].

In fact, recombinant protein production processes must be controlled by appropriately adjusting the cell environment [14], such as the type and concentration of macro- and micronutrients. It is frequently shown that cultivation medium composition directly dictates the amount of biomass produced [22], and therefore can dramatically influence the desired amount of the target protein at the end of the cultivation. Indeed, when *E. coli* is used as a host system to produce human proteins, detailed information on acetic acid formation should be obtained [40].

Although the improvement of the culture medium favors the attainment of high cell densities and recombinant protein yields, acetic acid production is also enhanced in complex media compared with semidefined and defined media [31]. Nevertheless, this main drawback in *E. coli* growth can be avoided by keeping low specific growth rates (*e.g.*, fed-batch control algorithms on dissolved oxygen tension and pH), by genetically altering the pathways involved in the formation of acetate [7, 28] or merely by selecting strains that have a particular genotype [27] and properly selecting the fermentation medium components [9]. For instance,

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recent results have demonstrated that glycerol is superior to glucose for reduced acetate and increased recombinant protein production when supplied post induction [20, 26].

Commonly, fed-batch cultivation approaches are sufficient to achieve this scope [8]. However, much work has to be done in order to establish the instant and profile addition of the growth-limiting carbon, often glucose [19, 32] or glycerol [38].

In the past decades, recombinant soluble catechol-*O*-methyltransferase (SCOMT) has been produced in *E. coli* [6, 25], in insect cells using a baculovirus expression system [34], and in mammalian cell cultures using expression vectors based on Epstein-Barr virus (a herpesvirus; [34]) and Simian virus 40 (a polyomavirus; [37]). In spite of all of the afore-mentioned systems having produced functional forms of the enzyme, the methods enable to produce up to 1 g of target protein. In fact, until now, insignificant research has been conducted in the upstream phase for human SCOMT (hSCOMT) production in order to improve the volumetric and mass productivity for several biopharmaceutical and neurological domains.

To our best knowledge, only one research group [35] described the improvement of hSCOMT expression in large scale, using a mineral modified medium with a substrate-limited feed strategy, by controlling the growth rate with a feed-forward algorithm. The overexpression makes possible to obtain a reasonable percentage of cell protein as the desired enzyme. Nevertheless, the enzymatic activity results are not specified, and this strategy imposes a significant metabolic burden on host cells.

Recently, there has been increasing awareness in the use of model predictive approaches for protein expression systems [10, 18]. Predictive control approaches are easy to understand and provide a straightforward way to explicitly handle constraints [14]. These approaches to protein process optimization and control rest on mathematical procedure models and their exploitation with numerical optimization methods. The models must be accurate enough to describe the relevant process features, and to exploit them such that the optimal values of the control variables can be determined within the constraints imposed by the real process.

With the aim of maximizing the recombinant hSCOMT productivity in *E. coli*, fed-batch cultures have been performed by employing several feeding strategies. This investigation hence centered on the effect of different feed profiles (pulses, exponential, exponential followed by constant rate) using a substrate-limited fed-batch strategy, designed to control the feed in order to maintain glycerol below the critical level for overflow metabolism. In addition, we show that the relevant features of production processes for recombinant hSCOMT protein can be described by a relatively simple process model. The mechanistic model proposed here describes the substrate consumption by the *E. coli* cells, the accumulation in the cells' biomass, and the production levels of the target protein within the bacteria. A comparison of the measurement data obtained in the validation experiment with the model predictions was performed in order to test the extrapolation capability of the model. The results described are expected to provide new complementary information for this production system; and regarding specific simulations, several feeding strategies can be designed and optimized with the aim of increasing the levels of biomass for hSCOMT synthesis.

MATERIALS AND METHODS

Chemicals

Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters) as the basis of the bacteria growth media formulation. Carbenicillin disodium salt, isopropylthiogalactosidase (IPTG), tryptone, yeast extract, lysozyme, dithiothreitol, glycerol, and glucose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade commercially available and used without further purification.

Construction of the Expression Vector and Bacterial Strain

The Champion pET101 Directional TOPO expression kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) was used for the expression of human SCOMT in its native form on *Escherichia coli* BL21(DE3) strains, and the process was carried out according to the manufacturer's instructions as described elsewhere [25].

Flask Experiments

Preliminary experiments were carried out in order to find the best concentration and carbon source (glucose or glycerol) to be incorporated in the developed semidefined medium (Table 1). The media under study had the following carbon range composition: glycerol (10, 20, 30, 40, and 60 g/l) and glucose (15, 20, and 30 g/l). In addition, the pH was close to 7.2 without adjustment before sterilization, and all the components were mixed and dissolved in deionized water and autoclaved for 15 min at 120°C. After autoclaving, a specific volume of a standard glucose solution was added under aseptic environment conditions to the medium in order to obtain the desired substrate concentration. For all flasks experiments and methods to obtain a suitable hSCOMT soluble preparation, the specific details of inoculation, fermentation, and recuperation steps can be found elsewhere [25].

Fed-Batch Bioprocess

A 3.5 l bioreactor (New Brunswick Scientific, Edison, NJ, U.S.A.) containing 1.250 l of semidefined medium (Table 1) was autoclaved at 121°C for 20 min. This medium was formulated according to specific yields of main nutrients to biomass for *E. coli* and reported general nutritional requirements for bacteria [41]. After sterilization, magnesium sulfate heptahydratate, carbenicillin, and the trace element solution (Table 1) were filter-sterilized and added separately and aseptically to the bioreactor, in order to avoid precipitation.

Unless otherwise stated, the bioreactor was inoculated with 250 ml of seed culture (1.250 l starting volume), and the temperature was kept at 37°C throughout the fermentation with an initial aeration and agitation rate of 0.2 vvm and 250 rpm, respectively. The dissolved

Table 1. Medium composition for batch, fed-batch, and nutrient feed solution.

Component	Concentration
Na_2HPO_4	5.50 g/l
NaCl	0.50 g/l
Citric acid monohydrated	1.64 g/l
Potassium citrate	2.00 g/l
Sodium citrate	0.67 g/l
Triptone	20 g/l
Glycerol	30 g/l
$MgSO_4 \cdot 7H_2O$	1.21 g/l
Carbenicillin	50 μg/ml
Trace elements	1.5 ml
Trace elements composition	
$FeCl_3 \cdot 6H_2O$	27 g/l
$ZnCl_2$	2 g/l
$CoCl_2 \cdot 6H_2O$	2 g/l
$Na_2MoO_4 \cdot 2H_2O$	2 g/l
$CaCl_2 \cdot 2H_2O$	1 g/l
$CuSO_4$	1.2 g/l
H_3BO_3	0.5 g/l
Feed Composition	
Glycerol	300 g/l
Triptone	40 g/l
$MgSO_4 \cdot 7H_2O$	15 g/l
Carbenicillin	250 mg/l
Trace elements solution	10 ml/l

oxygen tension (DOT) monitored with an Ingold pO_2 probe (Metter Toledo) was controlled at 30% of air saturation by an agitation cascade between 250 and 1,000 rpm and by varying the aeration rate between 0.2 and 1.7 vvm. Online, pH was measured by means of an Ingold pH probe (Metter Toledo) and was controlled to not drop below 7.0±0.1 by the automated addition of concentrated (2 M) NaOH/NH_4OH or (2 M) H_2SO_4/HCl. Furthermore, foaming was controlled by the automated addition of an antifoam 204 agent from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

A precultivation was carried out before each fermentation. Therefore, cells containing the expression construct were grown overnight at 37°C in agar plates with standard LB medium containing 50 μg/ml carbanecillin. A single colony was inoculated in 250 ml of a semi-defined medium (Table 1) with 50 μg/ml carbanecillin in 1 l shake flasks. The seed culture was incubated at 37°C and 250 rpm on a rotary shaker until the optical density at 600 nm (OD_{600}) of 2.6 was reached. Each preculture in an exponential growth phase was then used to inoculate the bioreactor with an inoculum size of 20% (v/v). After inoculation, the process was initially run as a batch (described in Results). Subsequently, the fed-batch strategy applied in this work could be projected as described elsewhere [4]: the glycerol feed ought to follow an exponential profile during the growth-phase until the maximum oxygen transfer capacity is met. At this time, one should also change the glycerol profile (*e.g.*, constant) once the maximum stirrer speed is reached in order to avoid oxygen depletion. Initially and in order to test the scheme depicted, after the batch phase, an exponential glycerol feed rate was compared with feed profiles that involved intermittent glycerol "pulses" during the growth phase, in order to access the influence of feed mode manipulations only on the cell growth. In all experiments, we established that the fed-batch phase started 1 h after the glycerol of the batch stage was exhausted.

Therefore, when the glycerol in the initial medium was depleted at 9 h after inoculation, the operation mode was switched to a fed-batch with an exponential profile, where a nutrient feed (Table 1) was added initially at a flow rate of 16 ml/h (exponential rate constant 0.4 h^{-1}) for 4 h. When the feed rate reached a rate of 53 ml/h, it was kept constant for 7 h until the end of the cultivation. Heterologous hSCOMT expression was induced with the addition of 1 mM IPTG after a predefined time. Moreover, the feed rate was adjusted to maintain a glycerol concentration between 4 and 10 g/l during the production phase of the fermentation. Samples were withdrawn as necessary and stored at −20°C for analysis.

Process Operation

Fed-batch simulations and fermentations were performed using an exponential feeding profile to keep the specific growth rate constant, followed by a constant feed profile. Glycerol was the limiting substrate and the exhaustion time was given by

$$t_{(\text{exhaustion of glycerol})} = \frac{\ln\left(\frac{Y_{X/S}S_0}{X_0}\right)}{\mu_{max}} \quad (1)$$

The feed solution was supplied using a peristaltic pump calibrate to impel an adequate flow specified by

$$F(L/h) = \frac{\mu_{set} X_0 V_0 \exp^{(\mu_{set} t)}}{Y_{X/S} S_F} \quad (2)$$

Analytical Methods

Cell growth determination. Dry cell weight (dcw) concentrations were estimated using a calibration curve between dcw concentration and optical density at 600 nm (OD_{600}). The dcw values were determined by weighing the pellet fraction from 1-ml culture samples centrifuged at 10,000 rpm for 15 min at 4°C. The pellets were washed twice and resuspended in 0.8% (p/v) NaCl isotonic solution, centrifuged again at the same conditions, and dried at 105°C for 48 h until constant weight. One unit of optical density was found to be equal to 0.3862 g dcw/dm^3. OD_{600} was obtained from the fermentation broth, measuring the absorbance at 600 nm in the range 0.2–0.9.

Glycerol, lactate, ammonium, and glucose assay. The determination of free glycerol in the supernatants of *E. coli* extracts (1 ml) was based on the method previously described by Bondioli and Bella [5]. An YSI7100 MBS analyzer (Yellow Springs, Ohio, U.S.A.) was employed to measure the concentration of lactate, ammonium, and glucose.

Human SCOMT activity assay. In general, the experiments of activity were designed to evaluate the methylation efficiency of recombinant hSCOMT, by measuring the amount of metanephrine, using epinephrine as substrate as previously described [24]. The hSCOMT activities were calculated as nmol of MN produced/h/mg protein.

Model Description

One model is presented for *E. coli* BL21(DE3) growth intended for the production of recombinant hSCOMT. The model proposed is

unstructured and nonsegregated, which means that all cells in the population are considered to have identical properties. Identification of the model structure and its parameters was based on experimental data obtained from fed-batch laboratory-scale cultivations. The feed strategy is designed to operate below the critical growth rate where acetate is accumulating. Specifically, the assumptions made in the model are as follows:

- The model does not take into consideration the complex components that are not used by the bacteria, since these complex components might accumulate to inhibitive concentrations.
- The keeping out of oxygen consumption.
- The dilution rate (D) was defined, in a specific time, as the ratio of substrate flow over bioreactor volume.

The model is based on mass balances for biomass X and substrate S. Additionally, an equation for the specific activity of the desired recombinant protein is considered. This balance must be distinguished from the balances of biomass and substrate around the reactor. Therefore, the model was formulated in terms of p_x [AU/cell mass], and does not refer to the protein concentration usually taken from the lysates extracts through the induction phase. The variables defining the state vector c are [X, S, P_x]. The following structure of the mathematical model based on specific mass balances of a fed-batch bioreactor is given by

$$\frac{dX}{dt}=(\mu - D)\cdot X \tag{3}$$

$$\frac{dS}{dt}=-q_S\cdot X - D\cdot (S - S_0) \tag{4}$$

$$\frac{dP_X}{dt}=q_P\cdot X - D\cdot P \tag{5}$$

For notation and values of the parameters, see Table 2.

The substrate uptake rate can be described by an adaptation of the Monod kinetics relation:

$$\mu=\mu_{max}\frac{S}{K_S+S}\left(1-\frac{X}{X_{max}}\right) \tag{6}$$

The specific substrate consumption rate q_S is assumed to be mainly dependent on the specific biomass growth rate μ [Eg (7)]. Additionally, maintenance requirements of the cells and substrate uptake to protein production were negligible in a first approach.

$$q_S=\frac{\mu}{Y_{XS}}+\frac{q_P}{Y_{PS}}+m_S \tag{7}$$

The approached maxima q_{max} is different at different specific biomass growth rates. Higher specific product concentrations were observed at lower specific growth rates, and vice versa, lower specific product concentrations were achieved at higher growth rates. In order to quantify these observations, the rate of change of the specific protein concentration q_p in Eq. (8) was described by a first-order dynamical process in the following way:

$$q_P=q_{pmax}\frac{S}{S+K_S}\frac{I}{I+K_P} \tag{8}$$

This approach can be considered as a form of self-inhibition effect, since the expression of q_p comprises two inhibitory factors: one from the substrate and a second from the inductor. Note that no recombinant protein is produced during the biomass growth phase (I=0), and hSCOMT production is only possible when I>0.1. Additionally, we assume that the maximal specific product concentration value, q_{max}, approached asymptotically to be dependent on μ.

Parameters Estimation

A program developed in MATLAB was used for simultaneous estimation of the model parameters, as developed by Teixeira *et al.* [33]. The program minimizes the mean of squared errors (MSE) using the Levenberg–Marquardt algorithm. The material balance equations for fed-batch operation along with the postulated kinetic equations were integrated using a $4^{th}/5^{th}$ order Runge–Kutta solver. The estimates of state variables concentration (biomass, substrate, and product) were compared with the correspondent off-line measurements. The final residuals and Jacobian matrix were used to calculate an approximation to the Hessian matrix, thereby assuming that the final solution is a local optimum. The Hessian matrix enabled the calculation of the parameters covariance matrix within 95% confidence intervals.

Table 2. Values of the model parameters, identified from preliminary experiments on flasks and bioreactor.

Abbreviation	Value	Dimensions
Operation parameters		
X_0	3.7	g/l
S_F	30	g/l
V_0	1.3	l
μ	0.3	h^{-1}
Model parameters		
$Y_{X/S}$	0.22±0.020	biomass per glycerol (g/g)
μmáx	0.54±0.071	h^{-1}
K_p [a]	0.1	AU/l
k_S	1	g/l
$q_{Pmáx}$	1.98±0.36	protein per biomass per h (AU/g/h)
$X_{máx}$	9.71±0.77	g/l
$Y_{P/S}$	1.23±0.30	protein per glycerol (AU/g)

[a]Reference values from Levisauskas *et al.* [18]

Results and Discussion

Expression Conditions on Shake Flask Experiments

In order to scrutinize the best growth conditions and expression levels of hSCOMT in the *E. coli* expression system applied in this work, a shaking culture evaluation (carbon source and concentration) was initially performed prior to the scale up in the bioreactor. To establish the best culture composition, the semidefined medium described in Table 1 was complemented with different compositions of a carbon source, glycerol or glucose, through several concentrations respectively ranging from 10 to 60 g/l for glycerol and 15, 20, and 30 g/l for glucose. These experiments were performed in order to provide suitable information for process model identification. Fig. 1 depicts the measurement data for biomass concentration and hSCOMT activity levels obtained for the two substrates at distinct concentrations.

It is well established that an excess of glucose in the media can lead to growth-inhibiting concentrations of acetate in different *E. coli* strains. Indeed, acetate is undesirable since it inhibits significantly the expression of heterologous proteins [13, 15] at considerably lower culture densities, and represents a diversion of carbon that might otherwise generate biomass or protein product [21]. In addition, previously conflicting results in the literature using glycerol dictates that growth rate and acetate formation can be highly dependent on the recombinant strain. Therefore, to overcome these points, we decided to employ a selective strain of *E. coli*, the BL21(DE3) Star from Invitrogen, which possesses a particular genotype that generates less acetate as a result of the activation of a glyoxylate shunt [31, 27].

As expected, the maximal specific hSCOMT activity attained was different for the several glucose concentrations, essentially as a result of the specific biomass growth rates achieved and the acetate accumulation. Specifically, in the presence of surplus of glucose (20 and 30 g/l), the cell growth seemed to be inhibited, accomplished with a "proportionally" decrease in the specific activity of the expressed protein and high levels of acetate (over 4 g/l). In contrast, for a lower glucose concentration, such as 15 g/l, the data obtained suggest that the specific activity of the recombinant protein approaches to a maximal value (Fig. 1). In spite of these analyses, this substrate concentration led to acetate accumulation, which promoted several adverse effects in the consequent scale-up.

Recently, several studies have demonstrated that glycerol is superior to glucose in reducing acetate formation during the growth phase, and interestingly, increased recombinant protein formation when supplied post induction [20]. In flask experiments, it was observed that an increase in the glycerol concentration did not affect considerably hSCOMT production and specific activity levels. Moreover, the comparison of the results in glycerol experiments with

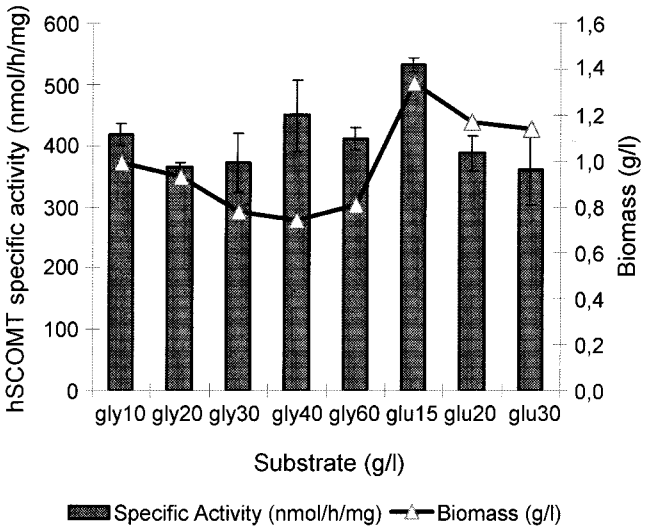

Fig. 1. Comparative studies of different carbon sources (gly, glycerol; glu, glucose) and concentrations in terms of hSCOMT specific activity (nmol/h/mg) and biomass (g/l) yields.

those obtained in glucose media demonstrated that the reduced growth rate of *E. coli* observed in fermentations with glycerol could be more profitable, while reducing acetate (less than 1 g/l) and maintaining hSCOMT specific activities over all the range checked (Fig. 1). Under these conditions, using glycerol as the exclusive carbon source at low levels in the semidefined medium, it seems that the inhibitory action of several metabolites including acetate can be more straightforwardly manipulated in the bioreactor. Therefore, for the delineate scope, the best performance on flasks scale in terms of growth rate, biomass, and volumetric hSCOMT productivity was obtained when 10 g/l of glycerol was supplement in the medium.

Scale-Up in a Batch Bioprocess

Subsequently, in order to examine the applicability of the system described in the flasks experiments, the growth of *E. coli* cells was performed without protein induction in two ordinary batch modes with a working volume of approximately 1.250 l. At this phase, only two glycerol concentrations were tested (10 and 30 g/l) in order to determine the maximal specific biomass growth rate (μ_{max}), the approximate yield coefficient of biomass produced as a function of the glycerol consumption, and the exhaustion time of the main carbon source, to establish the beginning of the fed-batch mode and consecutive profile feed. The choice of these glycerol concentrations was based on Fig. 1, whereas the levels of hSCOMT activity obtained were similar for the glycerol range concentration tested. In fact, for the scale-up, testing a glycerol concentration of 30 g/l rather than 40 g/l could be advantageous; for instance, it could decrease the acetate levels and reduce the lag phase in the bioreactor.

In terms of the kinetic parameters, such as specific cell growth rate, higher glycerol concentrations in the broth medium led to lower μ (0.3 h^{-1}), contrasting with the 0.5 h^{-1} estimated for 10 g glycerol/l. In both experiments, the Y_{xs} was found to be roughly 0.4 g cells/g glycerol, and it was also verified that *E. coli* stops growing when the initially added amount of glycerol is consumed, 10 and 15 h respectively for 10 and 30 g glycerol/l. This detail is enough to expand the lag phase at 30 g glycerol/l and increase the batch time. This determination was confirmed by application of Eq. (1). As a result, when a missing substance is known in advance, it can be used as a limiting factor in the biological system. Hence, in order to develop a suitable fed-batch strategy, it was established that the batch phase should be performed at 10 g/l of glycerol during 9 h, followed by a feed profile strategy.

Production of hSCOMT by a Fed-Batch Bioprocess

In a first approach, the yield coefficient ($Y_{X/S}$) was estimated from the fed-batch experiments through the use of glycerol balance measurements. In fact, this parameter is close to the estimation done during the batch experiment: around 0.35 and 0.5 g cells/g glycerol, respectively, for exponential and pulse feeding modes. These experiments also showed that supplementation of glycerol by an intermittent feed mode operation (three additions) was an inadequate strategy, since unusually, the glycerol concentration was constant at the predefined set-point (10 g/l). The results reveal that a good alternative was to follow an exponential glycerol profile during the exponential stage, to restore gradually and overtime the glycerol contents in the bioreactor around 10 g/l.

From bibliographical data, one way to prevent oxygen depletion is to lower the fermentation temperature after induction, since low efficiency in active recombinant protein production can occur when maintaining the temperature of 37°C, for example, because of inclusion bodies formation related to high growth rate during recombinant expression [11, 30]. In this work, the majority of the target protein was found in an active and soluble form, and no reduction of temperature was necessary because the growth rate was partially controlled during the fed-batch phase owing to the imposition of lower glycerol levels.

Therefore, to maximize recombinant hSCOMT production by achieving a maximal final culture density (g dcw/dm^3) and also maximal specific activity (U/g dcw) at the end of the growth, we decided to establish an exponential profile during the growth phase followed by a constant feed over the induction stage. In this phase, the maximum oxygen transfer capacity of the system is attained, and the biomass levels could decrease mainly because of oxygen limitations. Consequently, a control between agitation and oxygen dissolved levels had to be done in order to ensure a maximum stirrer speed in the medium without possible oxygen depletions.

After several experiments, it was established that the present feeding strategy [exponential followed by constant feed; Fig. 2 and Eq.(8)] provided the best results in terms of biomass (10 g dcw/ l in 19 h). The specific growth rate, μ_{set}, was fixed to 0.3 h^{-1} (Table 2). This value was low enough to allow proper folding of recombinant protein, to avoid high oxygen transfer rates, and to keep low acetate concentrations, since the acetic acid consumption can be done preferentially by the glyoxylate pathway when the glucose is not present [31].

Depending on the fixed production system, metabolic stress in *E. coli* is often proposed as a reason for the decrease in yields of a target protein during its gene expression, which can result in the redirection of cellular metabolism [12] and a drop off in the growth rate [1]. These effects have been reported in some biological systems, essentially due to the addition of the inducer IPTG [3, 16]. Once growth conditions and criteria for inductor quantity have been fixed, the induction time is the manipulated variable to maximize production yields.

In this work, we circumvented the need to determine the effect of the induction time on cell growth and recombinant protein yields, since as described previously [35], the final yield of SCOMT was not dependent on the culture density at the moment of the induction. Hence, although the concentration of the produced protein was to a great extent proportional to cell concentration, and processes designed to yield high cell densities are beneficial, we decided to

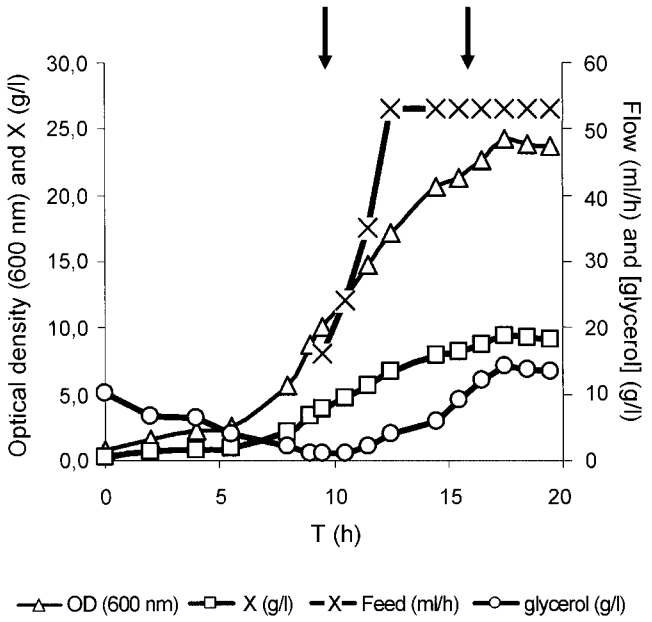

Fig. 2. The behavior of cultivation parameters during fed-batch procedure of hSCOMT expression. The starting of the feeding and induction are indicated by the left and right arrow, respectively. OD, optical density [dry cell weight (g/l)=0.3863 s OD]; X, biomass concentration (g/l).

perform the recombinant protein induction by one pulse IPTG addition at a dry cell weight of 8.8 g/l at the late feeding phase (Fig. 2).

It is well known that a high-energy demand for a nongrowth-related process (in this case, the synthesis of the recombinant protein) can decrease the observed biomass yield coefficient [23, 36]. This behavior was observed in the fed-batch tested (Fig. 2), and in spite that the culture reached automatically in the stationary phase, the volumetric hSCOMT accumulation increased significantly from 92,187 to 583,975 (U/l) in three hours of induction (Fig. 3); approximately 41% higher than in a routine flask experiment for the same experimental conditions. Another fact is that we could prolong the induction time (e.g., for three more hours), since the reduction of biomass concentration with the associated pseudo inhibitory effects of the induction stage were not dramatically observed as in the literature for other recombinant proteins in high cell density systems.

Model Design

A model was designed based on Monod kinetics that incorporates biomass inhibition, and substrate and inductor limitation with a straightforward approach for protein production, which was used to predict biomass, glycerol, and hSCOMT levels over the fermentation run. In Fig. 4, a simulation of the model of *Escherichia coli* BL21(DE3) with an exponential/constant (4A) and a pulse (4B) glycerol feed is demonstrated. In a first approach, we decided to neglect the lag phase data in terms of biomass and substrate concentrations in order to improve the results between experimental values and model approach. The curves for biomass and the substrate depict the corresponding modeling results obtained after fitting the model to all three data sets with the same parameter set (see Table 2).

The resulting fits show that the agreement among model and measured data is satisfactory, particularly if one takes into account the significantly different operational feeding conditions under which the process was examined. In particular, the abrupt increase in glycerol concentration shown in the model (Fig. 4A) was not confirmed by the experimental data, probably owing to an initial accumulation of the substrate at the earlier growth phase. However, after the protein induction instance the glycerol concentration dropped off quickly, since the growth and metabolic activity of the host cells were influenced by the expression of the heterologous protein [39]. For the experiment described in Fig. 4B (intermittent pulses of glycerol), our measurement data were satisfactorily accurate when compared with the representation proposed by the developed model.

Validation Experiments

Finally, analysis of the process performance was tested in a validation experiment accomplished with the production of recombinant hSCOMT protein (Fig. 5). A good agreement

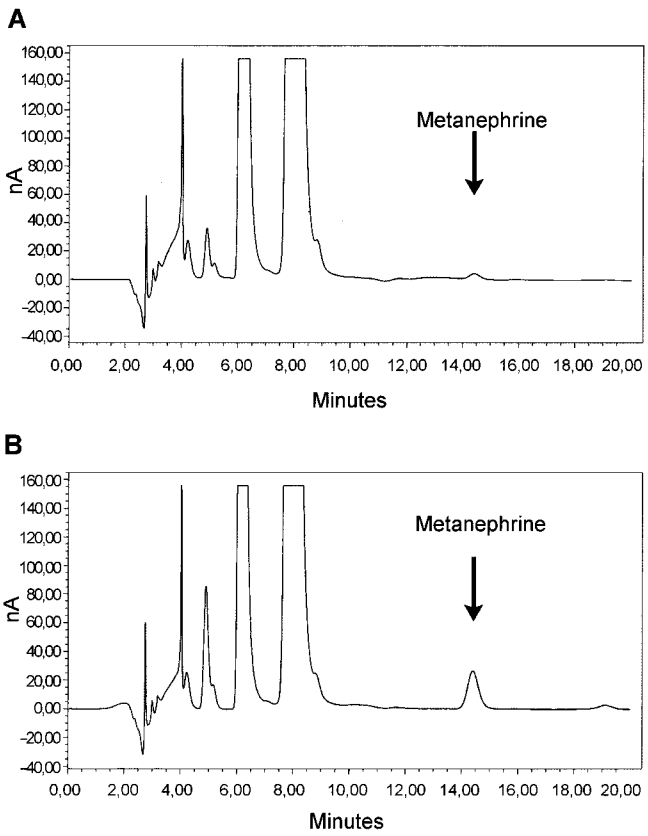

Fig. 3. Chromatograms obtained from the incubation medium for recombinant hSCOMT activity assay: (**A**) 1 h of induction in a bioreactor and (**B**) 3 h of induction in a bioreactor.

between prediction and experimental data was observed, indicating the high-quality prediction properties of this straightforward model. Furthermore, the comparison of hSCOMT yields in shake flasks with this fed-batch bioreactor strategy led to a significant improvement in the process performance. Fig. 5 also shows that a maximum of specific activity can be reached after 3 h of induction; however, this final concentration could be increased if induction were prolonged four hours more in a higher biomass concentration. Nevertheless, specific protein activities in the cell mass were found in the predicted range, and the total productivity of the process also met the predicted values.

Concluding Remarks

In this work, a straightforward knowledge of (a) the maximum allowable biomass concentration in the fermentation system, (b) the growth and protein evolution behavior after induction, and (c) the inductor concentration assayed by the final expected biomass, show how to apply an exponential followed by a constant fed-batch strategy to get satisfactory yields in recombinant hSCOMT expression systems. The mathematical model proposed for simple parameters prediction and further optimization of the operational procedure in

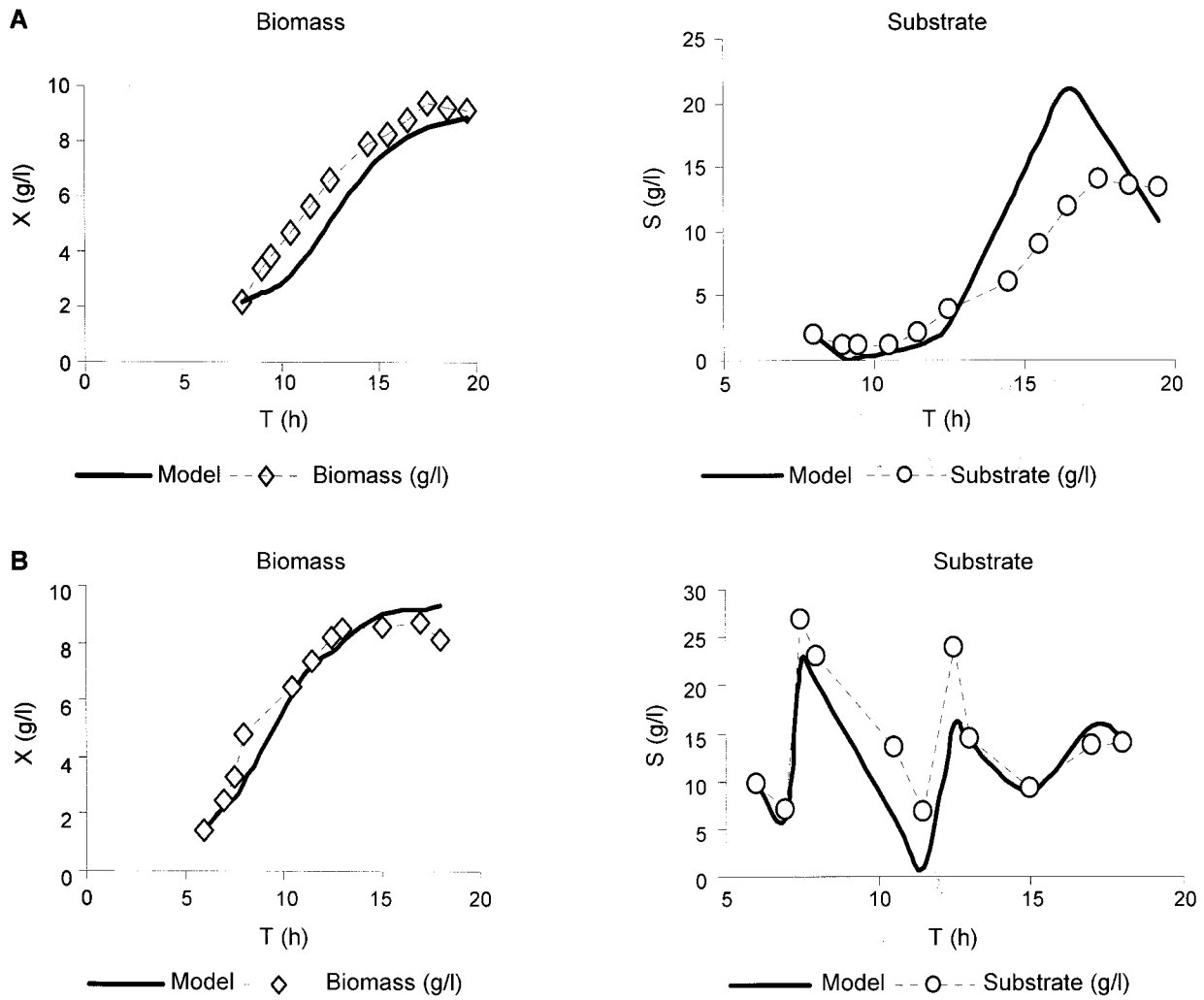

Fig. 4. Biomass levels and glycerol concentrations during experimental bioreactor trials using (**A**) exponential followed by constant glycerol fed-batch operation mode and (**B**) intermittent glycerol profiles.
The plots were accomplished with results predicted by simulations using the model proposed for biomass and substrate concentration.

recombinant hSCOMT production processes reliably predict the experimental data. The above methodology was used in the determination of the proper conditions for high-level hSCOMT production, achieving a final volumetric accumulation after 3 h of induction around 581,780 U/l.

The results described are expected to provide new complementary information for this specific production system, and regarding these simulations, a feeding strategy can be designed and optimized with the aim of reducing the levels of substrate in order to increase the levels of biomass and hSCOMT protein.

List of Symbols

μ: specific biomass growth, 1/h
$\mu_{máx}$: maximal specific biomass growth, 1/h
Y_{XS}: biomass/substrate yield, g/g
Y_{PS}: product/substrate yield, g/g
X: biomass amount in fermenter, g
$X_{máx}$: maximal biomass amount in fermenter, g;
S_F: substrate concentration in feeding solution g/l
S: substrate concentration, g/l
S_0: substrate concentration in the feed, g/l
q_S: specific substrate consumption rate, g/g/h
q_P: specific protein accumulation rate, AU/g/h
q_{Pmax}: maximal specific protein accumulation, AU/g/h
K_S: Monod constant for substrate consumption, g/l
D: Dilution rate, 1/h
I: Inductor concentration; g/l

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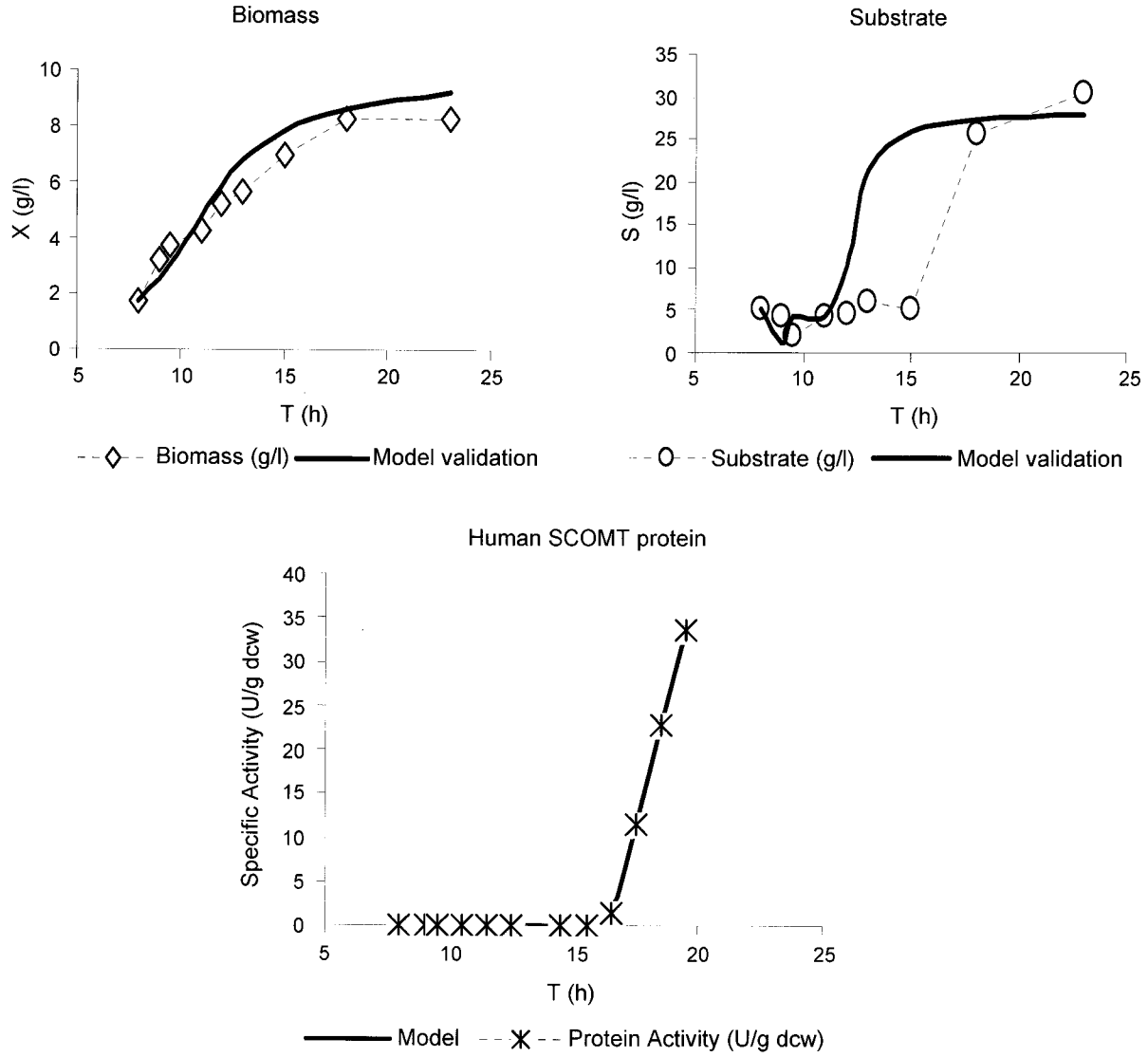

Fig. 5. Validation profiles of the process variables in a fed-batch experiment (exponential followed by constant profile) with the production of the recombinant hSCOMT protein.

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