Diffusion and Inhibition Processes in a Hollow-fiber Membrane Bioreactor for Hybridoma Culture. Development of a Mathematical Model

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The performance of a hollow-fiber membrane bioreactor (HFBR) (molecular weight cut-off 30 kD, fiber surface area 2050 cm^2) containing a culture of hybridoma cells has been investigated. Experimental data were used as basis to develop a model of general application. Concentrations of fundamental nutrients (glucose and glutamine), inhibitory products (ammonium and lactate), and monoclonal antibodies (MAb) against bovine lactoferrin (IgG_1) were monitored over time. Exchange of nutrients and products occurred across the capillary surface, whereas cells and MAb remained in the extra-capillary space (ECS). A protein-free culture medium (Hybrimax) with and without antibiotics was used. In both cases, the final MAb concentration was the same; however, antibiotic presence slowed down the time to achieve this concentration. Diffusion assays have been carried out in order to support the development of a mathematical model that describes the performance of the HFBR, including mass transfer and reaction terms. Inhibition by ammonium and lactate has been considered in the kinetics, providing model results consistent with experimental data. Further research with other cell lines and/or culture media will allow to broaden the field of application of this model for general use in HFBR systems.

Key words:
hollow-fiber membrane bioreactor, diffusion, inhibition, hybridoma, modelling

Introduction

Hybridoma technology was developed to overcome the limitations of polyclonal antibodies (broad specificity, possible cross-reactivity or variability among immune serum batches)[1]. Monoclonal antibodies (MAb) are currently used in many applications, such as the diagnosis and treatment of certain diseases and the purification of substances. The growing demand for monoclonal antibodies at reasonable prices means that it is necessary to carry out an optimization of their production processes[2].

Since the development of cell hybridoma technology, production of ascites in mice has been the primary method available for the production of large amounts of monoclonal antibodies[3]. However, as *in vivo* assays are controversial and criticized for both practical and ethical reasons, manufacturers and Official Medicines Control Laboratories are encouraged to develop and implement alternative methods to limit the use of such methods[4]. Thus, in the last decade, researchers have developed numerous *in vitro* methodologies for hybridoma culture. The development of these assays offers the benefit of replacing mice ascites, enabling the production of large amounts of monoclonal antibodies under well-controlled conditions[5].

Analysis of the evolution of substrate and product concentrations is fundamental in the determination of hybridoma kinetics. Glucose and glutamine are key substrates for hybridoma growth. Glucose is mainly transformed into pyruvate, while pyruvate is partially converted to lactic acid. Part of the glutamine is deaminated, yielding ammonium and glutamate, which is later transformed into other amino acids for biosynthesis purposes. Whereas glucose and glutamine are fundamental nutrients, ammonium and lactate are products of cell metabolism that can act as inhibitors when their concentrations are high enough[6]. Regarding the culture medium, the use of a protein-free medium facilitates subsequent purification of the monoclonal antibodies. Additionally, due to increasing safety concerns as well as cost issues, the requirements for biotechnological

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processes now include the use of media free from animal-derived components[7].

As regards *in vitro* techniques, hybridoma culture has been traditionally carried out in bags, flasks, or bottles. However, cell densities and MAb concentrations obtained in batch culture are generally low[8]. This disadvantage is overcome by the use of hollow-fiber membrane-based bioreactors (HFBR), which allow higher antibody concentrations and relatively lower operating costs. For these reasons, the use of hollow-fiber cell culture technology has greatly expanded worldwide[9,10]

Hollow-fiber modules provide a high surface to volume ratio, as much as 200 cm^2 per mL. This allows a large number of cells to aggregate in a very small volume, thus obtaining high cell densities. Jain and Kumar[11] have reported cells yields up to 10^7–10^9. These authors have also stated that HFBR systems are well suited for the production of antibodies in semi-continuous operation in concentrations higher than 2 mg mL^{-1} day, using a protein-free medium. The main disadvantage of using a hollow-fiber bioreactor for an extended period is that the system is prone to be contaminated during the replacement of the culture medium and recovery. Antibiotics are usually added to *in vitro* cell cultures to avoid this problem[12,13]. It is essential to know how antibiotics affect MAb production, however there are limited works focused on this subject[14,15,16], and the operative mechanisms of antibiotics with cell metabolism are still not clear[17,18]. One example of the existing works is the study developed by Barnabé and Butler[14] who reported that MAb specific production rates were not affected by low concentrations of tunicamycin (0.01–0.1 μg mL^{-1}), whereas higher antibiotic concentrations (1 μg mL^{-1}) made cell viability decrease and specific production rates increase, probably due to passive antibody release by non-viable cells.

Furthermore, the production of monoclonal antibodies by means of hybridoma cell lines has become an important biotechnological task. The efficiency of these processes can be further enhanced by developing macroscopic models, which can constitute valuable tools to reduce time and costs of bioprocess development[19]. The goal in modelling complex systems, such as MAb production by hybridoma cells, is the development of simple models that estimate system performance with an acceptable level of accuracy.

Thus, antibodies are well established in mainstream clinical practice as well as in research areas[20]. Additionally, in recent years, hollow-fiber membrane-based bioreactors have been reported as apt cell culture systems for therapeutic applications[9], such as tissue engineering[21] or growing red blood cells[22]. Therefore, in this paper, the performance of hybridoma cells in a HFBR system was analyzed as a case study of cell culture in a membrane reactor. Modelling is increasingly being used to understand bioreactor behaviour, specifically some attempts to model hybridoma growth[23,24] and MAb production[25,26] in HFBR have been carried out. Moreover, different authors have recently proposed sophisticated mathematical equations to describe hybridoma metabolism and antibody production in batch cultures[19,20]. However, as far as we know, there are virtually no published studies that consider both hybridoma metabolism and MAb production in HFBR systems. This paper has developed an easily applicable reaction-diffusion model, assuming certain simplifications based on additional experiments, to assess the performance of the system, i.e. conversion of substrates (glucose and glutamine) into products (lactate and ammonium), as well as MAb production with and without antibiotics. Hence, the aim of this work was to contribute to the understanding of hybridoma culture performance in HFBR systems and, at the same time, develop a model of general application for cultures carried out in similar membrane bioreactors.

Materials and methods

Cell line and cell culture

The hybridoma cell line studied here was HB-8852 (American Type Culture Collection) that produces IgG_1 monoclonal antibodies against bovine lactoferrin. A protein-free culture medium (Hybrimax) was employed.

The cell culture was developed following the procedure described by Legazpi *et al.*[6] Hybridoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 % (v/v) fetal bovine serum (FBS), and subsequently a gradual adaptation to DMEM with 10 % (v/v) FBS was carried out. The cells were then passed to Hybrimax with 10 % (v/v) FBS and adapted so as to grow in Hybrimax with 4 % (v/v) FBS. A buffer of MOPS (3-(N-morpholino)propanesulfonic acid) and bicarbonate were employed to maintain the pH of the culture medium close to 7.2. This adaptation process was carried out in T-flasks and, once adapted to grow in Hybrimax with 4 % (v/v) FBS, $3.4 \cdot 10^7$ viable cells were inoculated into the HFBR extra-capillary space (ECS 12 mL volume). The cells were then gradually adapted to Hybrimax without serum in four steps (3 %, 2 %, 1 %, and 0 % (v/v) FBS). This process lasted approximately 5 weeks. All the reagents were supplied by Sigma-Aldrich.

The HFBR (CELLMAX) (Thermo Fisher Scientific Inc., Waltham, USA) consisted of a bundle of 2565 cellulose fibers with a molecular weight

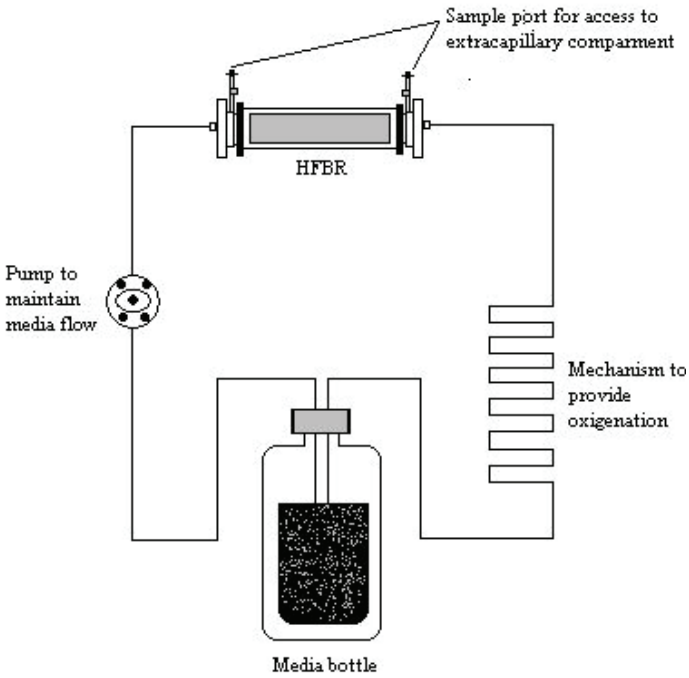

Fig. 1 – *Schematic representation of the HFBR system*

cut-off value of 30 kD, and a length of 13 cm. The fiber wall thickness was 15 μm, the internal diameter 180 μm, and the external diameter 210 μm. The medium was contained in a reservoir (a 500-mL bottle containing 300 mL of medium), and circulated within the capillaries while the cells were grown in the ECS. Exchange of nutrients and products occurred across the capillary surface, whereas cells and MAb remained in the ECS (IgG size 150–170 kD). The pumping medium was continually oxygenated, since passive gas exchange was realized by a silicone tubing device connected to the HFBR system. The flow was approximately 17 mL min^{-1}. The operation was semi-continuous, the HFBR worked in a closed-circuit system, although the exhausted medium was regularly replaced by fresh Hybrimax (when the lactate concentration reached 1000–1200 ppm) (Fig. 1).

The cells were cultured in an incubator maintained at 37 ºC with 5 % CO_2 in air. The experiments were performed with and without antibiotics (SIGMA Penicillin-Streptomycin Solution with 10000 units penicillin and 10 mg mL^{-1} streptomycin; 1:50 dilution) (Sigma-Aldrich Corp., St. Louis, USA). When the experiment with antibiotics was completed, the medium was replaced by fresh Hybrimax and, after a lapse of 24 hours, the experiment without antibiotics commenced. As explained model development section, the cell growth during the experiment was quite low and the order of magnitude in the concentration of cells was maintained.

Analysis

Samples of 4 mL of medium were taken with a syringe from the bottle, filtered through 0.45 μm pore membranes, divided into aliquots, and frozen at –20 ºC for later analysis. This sampling size was chosen so that the change in the medium volume (~12 %) did not affect kinetic results. Glucose concentration was determined by the dinitrosalicylic acid method (S.D. < 3 mM). Glutamine concentration was determined using an enzymatic kit (SIGMA, Ref.: GLN-2) (S.D. < 0.1 mM). Lactate was determined by ion exchange chromatography (IonPac AS4A-SC column, IonPac AG4A-SC guard column) (S.D. < 0.3 mM). Ammonium concentration was determined by ion exchange chromatography (IonPac CS-10 column, IonPac CG10 guard column) (S.D. < 0.1 mM). To quantify antibody IgG_1, 120 μL samples were taken through the ports with a syringe from the bundle and analyzed using an enzyme-linked immunosorbent assay (ELISA) (S.D. < 3 μg mL^{-1}). Ion exchange chromatography columns were supplied by Thermo Fisher Scientific Inc. and reagents by Sigma-Aldrich. These analysis methods are detailed in Legazpi *et al.*[6]

At the end of the culture, all cells were harvested with a syringe through the sample ports of the cartridge. Dry weight was employed to quantify the final concentration of cells in the ECS.

Statistical analyses

In order to evaluate the effect of antibiotics on HFBR performance, experimental data for substrates (glucose and glutamine) and products (lactate and ammonium) were analysed by running contrast of hypothesis tests at a 95.0 % confidence level. "The antibiotics do not affect the bioreactor performance" is the *null hypothesis* (H_0), whereas the *alternative hypothesis* (H_1) is "the antibiotics affect the bioreactor performance". Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess that all the samples came from normal distributions. These analyses were performed using IBM SPSS Statistics 20 (International Business Machines Corp., New York, USA).

Diffusion assays

Nutrients passed through the wall of the fibers from the capillaries to the ECS where hybridoma cells were immobilized, whereas products diffused from the extra-capillary space inside the lumen fibers. With the aim of determining the hydrodynamic parameters needed to model the system, diffusion assays were performed with the main compounds involved in the process (glucose, glutamine, lactate, and ammonium) without cells.

For the analysis of glucose diffusion, the cartridge was loaded with distilled water and the medium bottle was filled with a solution of glucose at a concentration similar to those employed in the experiments with hybridoma cells (initial concentration 4.0 mg mL^{-1}). Samples were then taken regularly from the ECS to evaluate the rate of diffusion of glucose from the capillaries. Glutamine diffusion was assessed in the same manner (initial concentration 0.34 mg mL^{-1}).

For the products (lactate and ammonium), the medium bottle was filled with distilled water, and the cartridge was loaded with a solution of lactate or ammonium in a concentration similar to those obtained in the experiments with hybridoma cells (initial concentrations 1.0 mg mL^{-1} and 0.046 mg mL^{-1}, respectively). Samples were also taken from the ECS.

Model development

Calculation of mass transfer coefficients

Diffusion assays allowed the estimation of the mass transfer coefficient values (K'_L) required to solve the model equations. In the absence of cells, the material that appears/disappears in the ECS is equal to the material that passes through the fiber walls.

$$V_e \frac{dC_{e,GLC}}{dt} = K'_{L,GLC} A (C_{i,GLC} - C_{e,GLC}) \quad (1)$$

$$V_e \frac{dC_{e,GLN}}{dt} = K'_{L,GLN} A (C_{i,GLN} - C_{e,GLN}) \quad (2)$$

where V_e is the volume of the ECS, K'_L the mass transfer coefficient for glucose (GLC) or glutamine (GLN), A the total fiber surface area, C_i the substrate concentration in the intra-capillary space (equal to the concentration in the bottle), and C_e the substrate concentration in the extra-capillary space.

The ECS volume was 12 mL and the total fiber surface area was 2050 cm^2. As no reaction is taking place, C_i can be expressed as a function of C_e:

$$C_{i,GLC} = \frac{(C_{i,GLC})_{initial} V_i - C_{e,GLC} V_e}{V_i} \quad (3)$$

$$C_{i,GLN} = \frac{(C_{i,GLN})_{initial} V_i - C_{e,GLN} V_e}{V_i} \quad (4)$$

where V_i is the volume of medium in the bottle.

Substituting Equations (3) and (4) in Equations (1) and (2) and integrating, we obtain:

$$\underbrace{\frac{-1}{\left(\frac{1}{V_i}+\frac{1}{V_e}\right)} \ln \frac{\left[\frac{(C_{i,GLC})_{initial}}{V_e} - C_{e,GLC}\left(\frac{1}{V_i}+\frac{1}{V_e}\right)\right]}{\left[\frac{(C_{i,GLC})_{initial}}{V_e} - (C_{e,GLC})_{initial}\left(\frac{1}{V_i}+\frac{1}{V_e}\right)\right]}}_{H_{GLC}} = K'_{L,GLC} At \quad (5)$$

$$\underbrace{\frac{-1}{\left(\frac{1}{V_i}+\frac{1}{V_e}\right)} \ln \frac{\left[\frac{(C_{i,GLN})_{initial}}{V_e} - C_{e,GLN}\left(\frac{1}{V_i}+\frac{1}{V_e}\right)\right]}{\left[\frac{(C_{i,GLN})_{initial}}{V_e} - (C_{e,GLN})_{initial}\left(\frac{1}{V_i}+\frac{1}{V_e}\right)\right]}}_{H_{GLN}} = K'_{L,GLN} At \quad (6)$$

where $(C_i)_{initial}$ and $(C_e)_{initial}$ are the substrate concentrations of glucose (*GLC*) or glutamine (*GLN*) at the beginning of the diffusion experiments, and t the experiment time. Plotting H_{GLC} or H_{GLN} vs. t, a line is obtained for each substrate, and the corresponding mass transfer coefficient can be calculated from the slope of this line. The mass transfer coefficients of products were calculated in a similar way.

Assumptions of the model

Seeing as the goal was to develop an easily applicable model for a complex system, a number of significant simplifications were introduced. Several initial assumptions normally considered by different researchers were assumed: isothermal process; Newtonian uncompressible fluid with constant physical properties; flow rates and pressures being uniformly distributed over the module cross-section; fiber bundles being regarded as a collection of parallel rods, which means that the packing-density distribution and flow distribution were consistent along the module length; the fibers had the same diameter, were rigid and could not be deformed; and entrance and exit effects in the lumen as well as the end effects in the shell were ignored[28].

Other approximations and simplifications are listed and justified below:

1) In the HFBR, the inflow concentration of any compound ($C_{i,\ inlet}$) was approximately the same as the outflow concentration ($C_{i,\ outlet}$).

Assuming perfect mixing in the bottle and not taking into account the time that the medium takes to circulate from the HFBR to the bottle, the amount of substance that appears/disappears in the bottle during an increment of time is the same as the amount of substance that disappears/appears in the bundle of fibers:

$$V_i \Delta C_{inlet} = Q(C_{i,\ outlet} - C_{i,\ inlet})\Delta t \qquad (7)$$

where Q is the flow through the ICS, V_i the volume of medium in the bottle, and t the time.

If the difference between C_{inlet} and C_{outlet} is a percentage of C_{inlet}, α, it can be written as:

$$(C_{i,\ outlet} - C_{i,\ inlet}) = \alpha C_{i,\ inlet} \qquad (8)$$

From Equation (1) and being $\tau = V_i / Q$:

$$\frac{\mathrm{d}C_{i,\ inlet}}{\mathrm{d}t} = \frac{\alpha C_{i,\ inlet}}{\tau} \qquad (9)$$

$$\alpha = \frac{\tau}{C_{i,\ inlet}} \frac{\mathrm{d}C_{i,\ inlet}}{\mathrm{d}t} \qquad (10)$$

As Q = 1020 mL h^{-1} and V_i = 300 mL, then τ = 0.29 h. The values obtained for α will be commented in Results and discussion and this assumption will be justified.

2) The time that the medium takes to circulate from the bottle to the HFBR and from the HFBR to the bottle was negligible in the context of the experiment. It is known that the medium took 2.3 min to reach the cartridge from the bottle, whereas the products took 0.9 min to get from the cartridge to the bottle. This time (3.2 min in all) is insignificant with respect to the rate of change of the concentrations in the studied compounds. It is important to remark that, taking into account the experimental data (shown in Results and discussion section), the variation of substrates and products concentrations during these 3.2 min was always below 5 %.

3) The cell growth that took place during the experiments was negligible with respect to the high cell concentrations in the cartridge.

The final cell biomass was determined at the end of the experiment, being 16 mg mL^{-1} dry weight (~5·10^7 cell mL^{-1}). The initial concentration was calculated considering the kinetic values for hybridoma growth in a T-flask[6] and assuming similar behavior, obtained was a value of the same order of magnitude, only 20 % lower than the final concentration.

4) The volume of culture medium contained in the bottle was virtually constant throughout the experiments. The initial volume was 300 mL. As 9 samples of 4 mL were taken, the final volume was 264 mL, just 12 % lower.

Model equations

Considering the assumptions postulated in the previous section, simple equations can be established to describe the system behavior based on mass balance in the bottle and in the intra-capillary space for substrates as well as products.

It was considered that the void spaces among cell layers around neighboring hollow fiber membranes give negligible contribution to flow in the ECS. Likewise, the flow resistance and the possible change in membrane properties caused by the adhesion of cells to the fiber membranes were also supposed negligible[9,28].

The material that disappears/appears in the bottle is equal to the material that passes through the fiber walls:

$$V_i \frac{\mathrm{d}C_{i,\ GLC}}{\mathrm{d}t} = -K'_{L,\ GLC} A(C_{i,\ GLC} - C_{e,\ GLC}) \qquad (11)$$

$$V_i \frac{\mathrm{d}C_{i,\ GLN}}{\mathrm{d}t} = -K'_{L,\ GLN} A(C_{i,\ GLN} - C_{e,\ GLN}) \qquad (12)$$

$$V_i \frac{\mathrm{d}C_{i,\ LAC}}{\mathrm{d}t} = K'_{L,\ LAC} A(C_{e,\ LAC} - C_{i,\ LAC}) \qquad (13)$$

$$V_i \frac{\mathrm{d}C_{i,\ AM}}{\mathrm{d}t} = K'_{L,\ AM} A(C_{e,\ AM} - C_{i,\ AM}) \qquad (14)$$

where V_i is the volume of medium in the bottle, K'_L the mass transfer coefficients for glucose (*GLC*),

glutamine (*GLN*), lactate (*LAC*) or ammonium (*AM*), C_i the concentration in the intra-capillary space (equal to the concentration in the bottle), C_e the concentration in the extra-capillary space, and A the fiber surface area.

The material that appears/disappears in the extra-capillary space is equal to the material that passes through the fiber walls minus/plus the consumption/production by the hybridoma cells:

$$V_e \frac{\mathrm{d}C_{e,GLC}}{\mathrm{d}t} = K'_{L,GLC} A(C_{i,GLC} - C_{e,GLC}) - V_e r_{GLC} \quad (15)$$

$$V_e \frac{\mathrm{d}C_{e,GLN}}{\mathrm{d}t} = K'_{L,GLN} A(C_{i,GLN} - C_{e,GLN}) - V_e r_{GLN} \quad (16)$$

$$V_e \frac{\mathrm{d}C_{e,LAC}}{\mathrm{d}t} = K'_{L,LAC} A(C_{i,LAC} - C_{e,LAC}) + V_e r_{LAC} \quad (17)$$

$$V_e \frac{\mathrm{d}C_{e,AM}}{\mathrm{d}t} = K'_{L,AM} A(C_{i,AM} - C_{e,AM}) + V_e r_{AM} \quad (18)$$

where V_e is the volume of the extra-capillary space, r_{GLC} and r_{GLN} the rates of consumption of glucose and glutamine, and r_{LAC} and r_{AM} the rates of formation of lactate and ammonium, respectively.

The rates of substrate consumption and product generation were considered proportional to the viable cell concentrations. In addition, two inhibitory terms for lactate and ammonium were introduced in order to take into account the reduction in consumption and production rates observed during the final hours of the experiment. Equations 15–18 can be rewritten as follows:

$$V_e \frac{\mathrm{d}C_{e,GLC}}{\mathrm{d}t} = K'_{L,GLC} A(C_{i,GLC} - C_{e,GLC}) - V_e q_{GLC} X_v \left(1 - \frac{C_{e,LAC}}{\left(C_{e,LAC}\right)^*}\right)^{n_{LAC}} \left(1 - \frac{C_{e,AM}}{\left(C_{e,AM}\right)^*}\right)^{n_{AM}} \quad (19)$$

$$V_e \frac{\mathrm{d}C_{e,GLN}}{\mathrm{d}t} = K'_{L,GLN} A(C_{i,GLN} - C_{e,GLN}) - V_e q_{GLN} X_v \left(1 - \frac{C_{e,LAC}}{\left(C_{e,LAC}\right)^*}\right)^{n_{LAC}} \left(1 - \frac{C_{e,AM}}{\left(C_{e,AM}\right)^*}\right)^{n_{AM}} \quad (20)$$

$$V_e \frac{\mathrm{d}C_{e,LAC}}{\mathrm{d}t} = K'_{L,LAC} A(C_{i,LAC} - C_{e,LAC}) + V_e p_{LAC} X_v \left(1 - \frac{C_{e,LAC}}{\left(C_{e,LAC}\right)^*}\right)^{n_{LAC}} \left(1 - \frac{C_{e,AM}}{\left(C_{e,AM}\right)^*}\right)^{n_{AM}} \quad (21)$$

$$V_e \frac{\mathrm{d}C_{e,AM}}{\mathrm{d}t} = K'_{L,AM} A(C_{i,AM} - C_{e,AM}) + V_e p_{AM} X_v \left(1 - \frac{C_{e,LAC}}{\left(C_{e,LAC}\right)^*}\right)^{n_{LAC}} \left(1 - \frac{C_{e,AM}}{\left(C_{e,AM}\right)^*}\right)^{n_{AM}} \quad (22)$$

where q and p are the maximum specific rates for substrates glucose (*GLC*) or glutamine (*GLN*) and products lactate (*LAC*) or ammonium (*AM*), X_v the cell concentration, $(C_{e,LAC})^*$ and $(C_{e,AM})^*$ the maximum concentrations of lactate and ammonium that can be obtained (extrapolated from experimental data), and n_{LAC} and n_{AM} the exponents of the inhibitory terms.

It has been reported that byproducts such as ammonium and lactate affect cell physiology and metabolism.[29] For instance, Schneider *et al.*[30] reported inhibition of glutamine uptake by elevated ammonia concentrations. Besides, it is a well-known fact that accumulation of these cellular metabolism byproducts may inhibit hybridoma MAb production.[11,29]

The produced antibody remained in the extra-capillary space; so, in this case, only the production term is necessary. Two inhibitory terms were once again considered:

$$\frac{\mathrm{d}C_{e,MAb}}{\mathrm{d}t} = p_{MAb} X_v \left(1 - \frac{C_{e,LAC}}{\left(C_{e,LAC}\right)^*}\right)^{n'_{LAC}} \left(1 - \frac{C_{e,AM}}{\left(C_{e,AM}\right)^*}\right)^{n'_{AM}} \quad (23)$$

where $C_{e,MAb}$ is the antibody concentration in the extra-capillary space, p_{MAb} the maximum specific production rate for antibody, and n'_{LAC} and n'_{AM} the exponents of the inhibitory terms.

Results and discussion

Hollow-fiber bioreactor performance

Cell metabolism and antibody production

Fig. 2 shows the evolution of glucose and glutamine concentrations, while Fig. 3 shows the evolution of lactate and ammonium concentrations. Glucose and glutamine concentrations decreased throughout the duration of the experiment, although the consumption rate was slower during the last hours of experimentation. In a previous study carried out with the same cell line in T150-flasks containing 100 mL of protein-free medium[6], it was observed that these substrates decreased only during the exponential growth phase. At the end of the experiment, the concentration of cells measured in the cartridge was $5 \cdot 10^7$ cell mL^{-1}, approximately a quarter of the concentration reported for the Alps 25-3 hybridoma cell line in the extra-capillary space of a dual hollow-fiber bioreactor[29]. Lactate and ammonium likewise increased throughout the experiment, as their production is associated with glucose and glutamine consumption. In contrast, antibody concentration was almost constant after 4 hours of culture in the medium without antibiotics (see Fig. 4). It has been previously observed that MAb production for this cell line is not totally associated with cell growth[6]. Cell growth and antibody generation are conditioned by the availability of amino acids in the medium[31]. For example, the presence of glutamine is a necessary condition for MAb production. Although, in this case, glutamine was available in the medium even during the final stages of the experiment, it is possible that other essential amino acids may have been depleted. Another possibility is that lactate and/or ammonium inhibit antibody production. In fact, a considerable amount of literature exists on the inhibitory or toxic effects resulting from lactate and ammonia accumulation in mammalian cell cultures[31]. Specifically, an inhibitory effect on cell growth and antibody production has been reported for these metabolic by-products[11,19,29].

The final antibody concentration was 0.22 mg mL^{-1} (approximately 4.4 pg cell^{-1}). This value was similar to that reported by Kurkela *et al.*[32] in one day for a HFBR system (0.3 mg mL^{-1}), and lower than that obtained by Kessler *et al.*[33] in one day for 6C10 cell line culture in a Tricentric bioreactor (1.8 mg mL^{-1}). Street *et al.*[34] reported a maximum antibody concentration of 2.4 mg mL^{-1} for the CB. Hep-1 hy-

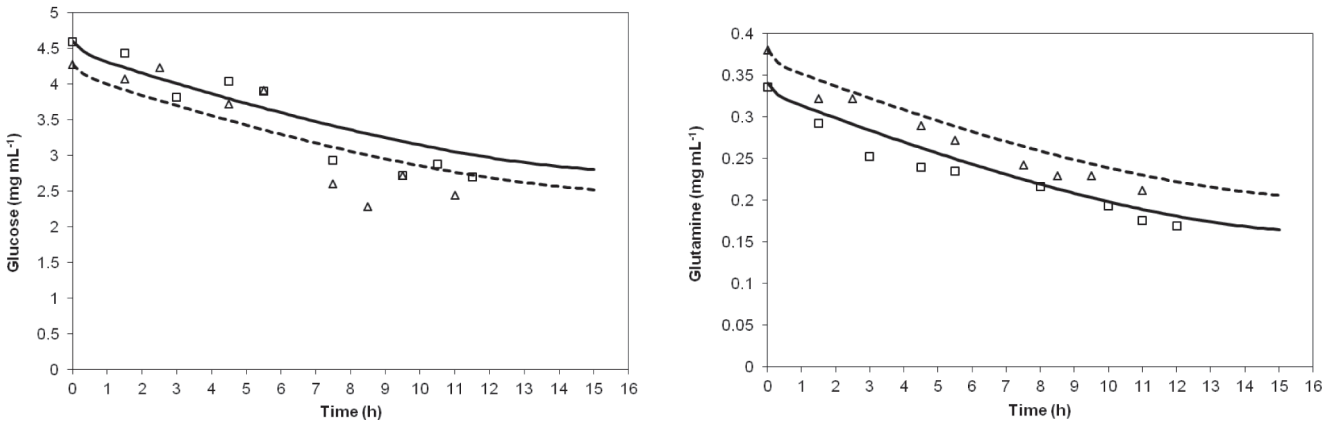

Fig. 2 – *Evolution of substrate concentrations (samples taken from the bottle). Experimental data with (□) and without (Δ) antibiotics, and the results of modelling with (continuous line) and without (discontinuous line) antibiotics (eq. 11 to 14, and 19 to 22).*

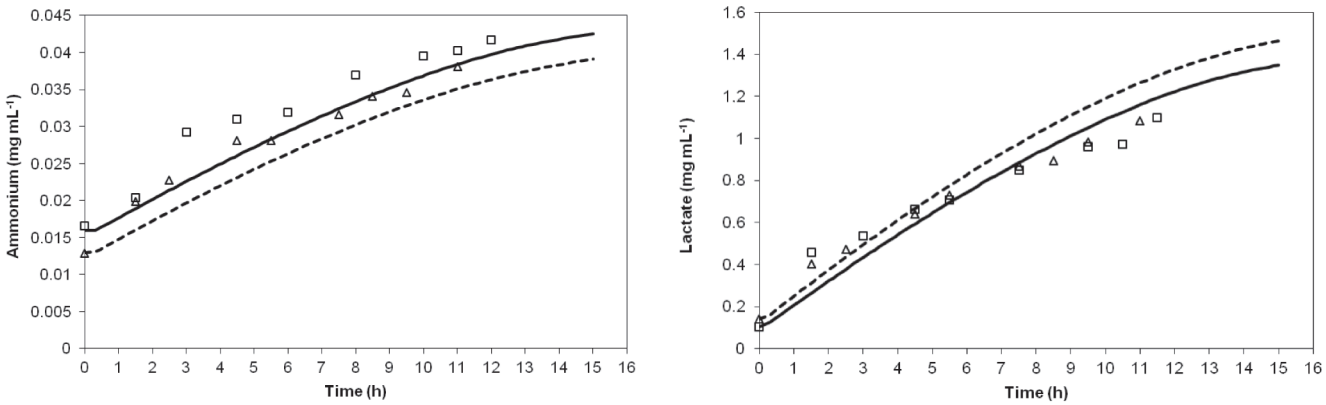

Fig. 3 – *Evolution of product concentrations (samples taken from the bottle). Experimental data with (□) and without (Δ) antibiotics and, the results of modelling with (continuous line) and without (discontinuous line) antibiotics (eq. 11 to 14, and 19 to 22).*

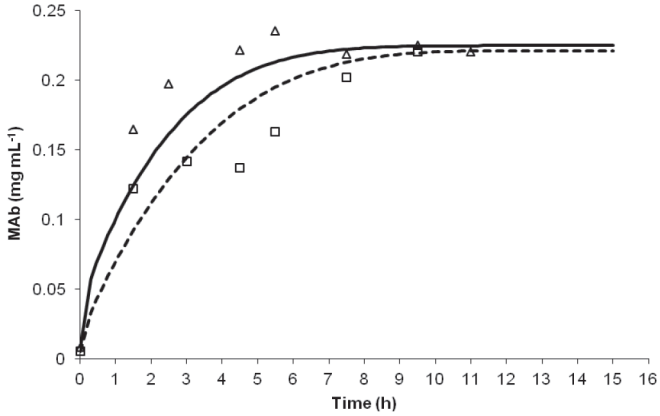

Fig. 4 – *Evolution of antibody concentration (samples taken from the bundle). Experimental data with (□) and without (Δ) antibiotics and, the results of modelling with (continuous line) and without (discontinuous line) antibiotics (eq. 11 to 14, and 19 to 23).*

bridoma cell line in a HFBR employing protein-free culture medium, whereas other authors obtained concentrations for the same cell line that did not exceed 0.015 mg mL^{-1}. In a recent study employing 5A8 hybridoma cells immobilized in calcium alginate, the maximum concentration obtained was 0.006 μg mL^{-1} [35]. It is thus essential to note that antibody production strongly depends on the characteristics of the medium and the cell line employed, as well as on the culture technique developed.

Comparison of cell metabolism and antibody production with and without antibiotics

The evolution of substrates (glucose and glutamine) and inhibitors (lactate and ammonium) with and without antibiotics can be seen in Figs. 2 and 3. In both cases, the glucose-lactate yield was 1.0, this yield is approximately half the value obtained in T-flask culture[6]. These results are similar to those reported by other authors with a different cell line (AB2-143.2) in a continuous reactor[36]. As regards glutamine consumption and ammonium production rates, once again, these were almost the same with and without antibiotics, with yields of 1.2 in both cases. This value is approximately double the glutamine-ammonium yield obtained in T-flask culture[6]. Schmid *et al.*[37] reported similar values for the AB2-143.2 cell line in small-scale cultures. Statistical tests confirmed that consumption of substrates and production of products were not affected significantly by the presence of antibiotics.

Fig. 4 shows the evolution of antibody concentration with and without antibiotics. Although the final concentration obtained was the same in both cases, the presence of antibiotics increased the time needed to reach this concentration. Whereas 4 h was sufficient time to obtain the maximum antibody concentration, approximately double this time was required when using antibiotics (8 h). The average productivity decreased from 0.002 mg mL^{-1} h^{-1} to 0.001 mg mL^{-1} h^{-1}. However, it should be noted that the use of antibiotics avoids system contamination and ensures the possibility of reusing hybridoma cells in several cycles with fresh medium. According to Da Silva[17], almost all antibiotics interfere in a lesser or greater extent with the production of antibodies as they form complexes with the DNA molecules, impeding the formation of antibodies. More recently, Kallala *et al.*[18] suggested that antibiotics exert direct effects on mitochondrial physiology within mammalian cells, disrupting mitochondrial function and cell activity. The effect of antibiotics on MAb production is determined by different factors, such as antibiotic nature and concentration, cell lines, culture medium…[14,15,16]

Comparison of HFBR performance with other in vitro hybridoma culture techniques

Table 1 shows the comparison of MAb production data obtained in the HFBR with data obtained for the same cell line and culture medium employing other *in vitro* hybridoma culture techniques, i.e., i-MAb bags and T-flasks. The use of a HFBR enabled much higher MAb concentrations to be obtained, as was also reported by other authors[11,18]: approximately 7 times higher than that achieved with T-flask culture, and 3 times higher than that achieved with i-MAb bags. This high MAb concentration facilitates the subsequent concentration and purification steps. Moreover, the time needed to obtain the maximum MAb concentration was much shorter using a HFBR than with the other *in vitro* techniques. Consequently, the productivity of the HFBR was much higher than when employing the other two techniques, with a value (0.0021 mg mL^{-1} h^{-1}), the same order of magnitude as that reported in other hybridoma cell lines cultured in HFBR[29]. Despite these advantages, it should be not-

Table 1 – *MAb production data employing different culture techniques*

	i-MAb bag* (500 mL)	T150-flask*	HFBR
MAb maximum concentration (mg mL^{-1})	0.074	0.030	0.220
Time to achieve the maximum MAb concentration (h)	720	50	4
Productivity (mg mL^{-1} h^{-1})	0.0001	0.0006	0.0021**
Medium yield (MAb obtained /culture medium consumed) (mg mL^{-1})	0.074	0.030	0.009

*Authors´ own data, in part reported by Legazpi *et al.*[6]
**Calculated considering the volume of culture medium consumed

ed that the medium yield (MAb produced in relation to consumed medium) was more than 3-fold higher in T-flask culture than in the HFBR, and 8-fold higher in the case of i-MAb bags. The drawback of i-MAb bags is obviously the time needed to obtain the maximum concentration of antibodies (approximately one month).

The medium yield data reported in Table 1 were calculated considering the production process to begin with fresh medium and end when the maximum MAb concentration is obtained. The actual operation with T-flasks and a HFBR differs slightly, as MAb is periodically harvested by replacing the used medium with fresh medium. The amount of MAb harvested in one month was calculated assuming that 40 % of the medium contained in the T-flask was replaced daily with fresh medium, and that all the medium in the HFBR was replaced every 12 hours. MAb production would be 37 mg per i-MAb bag (500 mL capacity), 12 mg per T150-flask, and 96 mg for the HFBR. Therefore, if high amounts of antibody were required, it would be recommendable to employ an HFBR instead of other culture techniques. This culture system not only enables higher MAb production, but also allows working for several months, obtaining a much more concentrated product.

Experimental system analysis and assessment of the model

Fig. 5 shows the evolution of substrates and products during the diffusion assays described in Materials and methods. As can be seen, it took 30–40 min for the glucose, glutamine, lactate, and ammonium concentrations to be equal in the bottle and in the ECS. These results allowed the estimation of the mass transfer coefficient values (explained in model development section) shown in Table 2. The validity of this estimation can be tested by comparing the good agreement between experimental and model data (Fig. 5).

Table 2 – *Mass transfer coefficients calculated from experimental data*

	r^2	$K_{Li}'A$ (mL h^{-1})	K_{Li}' (cm h^{-1})
Glucose	0.959	41.0	$2.0 \cdot 10^{-2}$
Glutamine	0.992	67.7	$3.3 \cdot 10^{-2}$
Lactate	0.996	87.3	$3.8 \cdot 10^{-2}$
Ammonium	0.986	76.4	$3.7 \cdot 10^{-2}$

From the experimental data obtained in the diffusion assays, α values were calculated in each sampling time for lactate, ammonium, glucose, and glutamine. Ninety-four percent of the obtained values for α were lower than 0.06. This means that the difference between the inflow and the outflow concentrations was less than 6 % practically throughout the experiments. This justified the approximation 1 commented in model development section.

The data used to solve the model can be seen in Table 3. In order to calculate the q_{GLC}, q_{GLN}, p_{AM}, and p_{LAC} values, the specific rates of consumption and production obtained in the T-flask experiments were

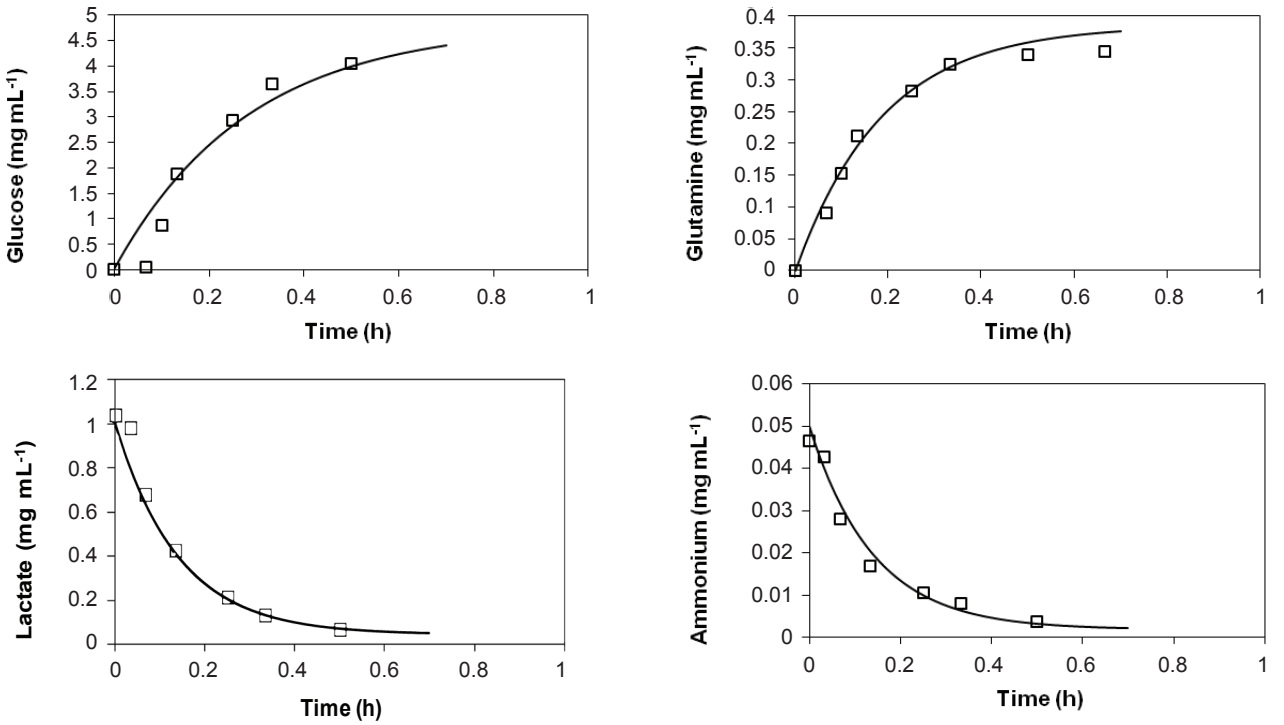

Fig. 5 – *Evolution of substrate and product concentrations in diffusion assays (samples taken from the bundle). Experimental data (□) and the results of modelling (continuous line) (eq. 11 to 18; reaction term being null).*

Table 3 – *Parameter values, with and without antibiotics, used to solve the model*

	Initial concentrations in the ICS (mg mL^{-1})	Initial concentrations in the ECS (mg mL^{-1})	K_L' (cm h^{-1})	A (cm^2)	V_i/V_e (mL)	X_v (mg mL^{-1})	q_{GLC}, q_{GLN} p_{AM}, p_{LAC} (h^{-1})	$(C_{e,Lac})^*$ (mg mL^{-1})	$(C_{e,AM})^*$ (mg mL^{-1})	n_{LAC} n'_{LAC}	n_{AM} n'_{AM}
Glucose	4.3 (without ant.) 4.6 (with ant.)	4.3 (without ant.) 4.6 (with ant.)	$2.0\cdot10^{-2}$	2050	300/12	16	0.30	1.45	0.047	0.10	0.15
Glutamine	0.38 (without ant.) 0.34 (with ant.)	0.38 (without ant.) 0.34 (with ant.)	$3.3\cdot10^{-2}$	2050	300/12	16	0.03	1.45	0.047	0.10	0.15
Lactate	0.14 (without ant.) 0.11 (with ant.)	0.14 (without ant.) 0.11 (with ant.)	$3.8\cdot10^{-2}$	2050	300/12	16	0.25 (without ant.) 0.23 (with ant.)	1.45	0.047	0.10	0.15
Ammonium	0.013 (without ant.) 0.016 (with ant.)	0.013 (without ant.) 0.016 (with ant.)	$3.7\cdot10^{-2}$	2050	300/12	16	0.005	1.45	0.047	0.10	0.15
Antibody	–	0.008 (without ant.) 0.005 (with ant.)	–	2050	300/12	16	0.023 (without ant.) 0.010 (with ant.)	1.45	0.047	1.30	0.50

assumed as the starting point[6]. A good fit was obtained for glucose employing the T-flask value (q_{GLC} = 0.3 h^{-1}). For lactate and ammonium, the values of maximum specific production rate obtained in the HFBR were very similar to the specific rates of production measured in T-flask (p_{LAC} = 0.25 and p_{AM} = 0.005 h^{-1}, respectively). However, the maximum specific consumption rate of glutamine in the HFBR was half the specific consumption rate value obtained in T-flasks (q_{GLN} = 0.03 and q_{GLN} = 0.06 h^{-1}, respectively). Comparing the results with and without antibiotics in the HFBR, again it can be concluded that the presence of antibiotics in the medium had no significant effect on the reaction rates of substrates and products. Changes in hybridoma metabolism determined by culture conditions may be responsible for the differences observed for glutamine consumption. Cell confinement makes hybridomas grow, forming biofilms and conglomerates. This different environment interacts with cell development, altering morphology and membrane permeability, as well as surface tension and osmotic pressure[31,35,39]. Maximum specific rates found in this work were slightly higher than lactate and ammonium specific production rates and glucose specific consumption rate reported by Ozturk and Palsson[40] (0.13, 0.002, and 0.14 h^{-1}, respectively). On the contrary, the maximum specific consumption rates for glutamine were very similar (≈ 0.03 h^{-1}). Additionally, Amribt *et al.*[12] reported values for the maximum specific uptake rate for glutamine of the same order of magnitude.

As explained in model development section, inhibition by lactate and ammonium was included in the kinetic equations (see eq. 19–22). The exponents of the inhibitory terms were assumed to be the same for glucose/glutamine consumption and lactate/ammonium production, both values being found to be quite similar (n_{LAC} = 0.10 and n_{AM} = 0.15, respectively).

As shown in Figs. 2 and 3, the agreement between experimental and model data for substrates and products is quite good, despite the fact that the model overestimates actual values in the case of the lactate after 8 hours.

Fig. 4 shows the evolution of antibody concentration (experimental data and model results) with and without antibiotics. The value obtained for p_{MAb} was higher without antibiotics, indicating that the presence of antibiotics negatively affects the production of MAb. In both cases, the production rate of MAb slowed down after approximately 3 hours. This behavior, also observed in T-flasks[6], might be explained by the inhibition of antibody production due to the accumulation of toxic byproducts, such as lactate and ammonia[11]. For these reasons, inhibitory terms were also considered for MAb production. In this case, inhibition by lactate was found to be stronger than inhibition by ammonium, with exponents of n'_{LAC} = 1.3 and n'_{AM} = 0.5, respectively. Model and experimental data have been compared with good agreement ($r^2 > 0.98$ in all cases).

Conclusions

The use of antibiotics (streptomycin/penicillin) did not affect glucose and glutamine consumption or lactate and ammonium production. Although the same final concentration of antibody was obtained

with and without antibiotics, twice the time was needed to obtain the maximum MAb concentration when antibiotics were added.

When antibody production in a HFBR was compared with other systems, i.e. T-flasks and i-MAb bags, the highest antibody concentration was obtained using a HFBR, and the time needed to obtain this concentration was the shortest. However, the waste of medium per mg of MAb obtained was maximal in this case.

It was possible to simulate the system employing a simplified model that considered reaction and mass transfer terms. Inhibition by lactate and ammonium was considered in the model for substrate consumption and product generation. The model accurately described the evolution of substrates and products, and even antibody production, which was found to be inhibited by ammonium and especially by lactate that had accumulated in the medium.

List of symbols and acronyms

A – total fiber area, cm^2
AM – ammonium
C_e – concentration in cartridge ECS, mg mL^{-1}
$(C_e)_{initial}$ – initial concentration in cartridge ECS, mg mL^{-1}
$C_{e,AM}$ – ammonium concentration in the ECS, mg mL^{-1}
$C_{e,GLC}$ – glucose concentration in the ECS, mg mL^{-1}
$C_{e,GLN}$ – glutamine concentration in the ECS, mg mL^{-1}
$C_{e,LAC}$ – lactate concentration in the ECS, mg mL^{-1}
$C_{e,MAb}$ – antibody concentration in the ECS, mg mL^{-1}
$(C_{e,LAC})^*$ – maximum lactate concentration, mg mL^{-1}
$(C_{e,AM})^*$ – maximum ammonium concentration, mg mL^{-1}
C_i – concentration in the bottle or ICS, mg mL^{-1}
$(C_i)_{initial}$ – initial concentration in the bottle or ICS, mg mL^{-1}
$C_{i,inlet}$ – bundle inflow concentration, mg mL^{-1}
$C_{i,outlet}$ – bundle outflow concentration, mg mL^{-1}
$C_{i,AM}$ – ammonium concentration in the ICS, mg mL^{-1}
$C_{i,GLC}$ – glucose concentration in the ICS, mg mL^{-1}
$C_{i,GLN}$ – glutamine concentration in the ICS, mg mL^{-1}
$C_{i,LAC}$ – lactate concentration in the ICS, mg mL^{-1}
ECS – extra-capillary space
GLC – glucose
GLN – glutamine
$HFBR$ – hollow fiber bioreactor
ICS – intra-capillary space
$K'_{L,AM}$ – mass transfer coefficient for ammonium, cm h^{-1}
$K'_{L,GLC}$ – mass transfer coefficient for glucose, cm h^{-1}
$K'_{L,GLN}$ – mass transfer coefficient for glutamine, cm h^{-1}
$K'_{L,LAC}$ – mass transfer coefficient for lactate, cm h^{-1}
LAC – lactate
MAb – monoclonal antibody
p_{AM} – maximum specific production rate for ammonium, h^{-1}
p_{LAC} – maximum specific production rate for lactate, h^{-1}
q_{GLC} – maximum specific consumption rate for glucose, h^{-1}
q_{GLN} – maximum specific consumption rate for glutamine, h^{-1}
q_{MAb} – maximum specific production rate for antibody, h^{-1}
Q – flow, mL h^{-1}
n_{AM} – exponent for inhibitory term due to ammonium in eq. 19–20
n_{LAC} – exponent for inhibitory term due to lactate in eq. 19–20
n'_{AM} – exponent for inhibitory term due to ammonium in eq. 23
n'_{LAC} – exponent for inhibitory term due to lactate in eq. 23
r_{AM} – ammonium production rates, mg mL^{-1}
r_{GLC} – glucose consumption rates, mg mL^{-1}
r_{GLN} – glutamine consumption rates, mg mL^{-1}
r_{LAC} – lactate production rates, mg mL^{-1}
SD – standard deviation
t – time, h
V_e – cartridge extra-capillary volume, mL
V_i – volume of medium in the bottle, mL
X_v – cell concentration, cell mL^{-1}
τ – retention time in the bottle, h

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