TECHNIQUE

Semicontinuous cultures as a tool for characterization of murine hybridoma 1E10 cells

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ABSTRACT

Semicontinuous cultures are a powerful tool for cell characterization. However, it is hard to be established at small scale due to the far more complex experimental configuration required. In this work, the use of semicontinuous culture in spinner flasks was explored with daily feeds and extractions, considering that metabolic and growth rates are lower in animal cells under continuous culture regimes. Relatively stabilized conditions were established, characterized by small fluctuations in the concentration of the different metabolic species (glucose, lactate, *etc.*) at the dilution rates evaluated, and the kinetic parameters (μ, q_P, qGlu) were calculated as a function of the dilution rate for each phase. Results obtained were equivalent to those expected for a continuous culture, the semicontinuous operation mode being a much simpler variant. Up to now there are only scarce reports on using semicontinuous cultures as a feasible alternative to simulate the kinetic behavior at stationary states. Finally, the antibody production pattern was confirmed as growth independent, with glutamine as the limiting substrate in the medium. The results were validated by an statistical homogeneity of variance test (ANOVA) and with normally distributed data as evidenced by a Student´s t test.

Keywords: animal cells, semicontinuous culture, cell characterization

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RESUMEN

Cultivos semicontinuos como una herramienta para la caracterización celular del hibridoma murino 1E10. Los cultivos continuos constituyen una herramienta poderosa para la caracterización celular. Sin embargo, la configuración experimental necesaria en este modo de operación es más compleja, por lo que se dificulta su ejecución principalmente a pequeñas escalas. Considerando que en cultivos continuos de células de origen animal, las velocidades metabólicas y de crecimiento son menores, en esta investigación se explora el empleo de cultivos semicontinuos en frascos agitados con adiciones y extracciones puntuales diarias. Durante el estudio se crearon ambientes relativamente estables, caracterizados por pequeñas fluctuaciones en las concentraciones de las diferentes especies (glucosa, lactato, etc.), para las velocidades de dilución evaluadas, y se calcularon los parámetros cinéticos (μ, qP, qGlc) en cada fase estable en función de la velocidad de dilución. Los resultados fueron equivalentes a los de los cultivos continuos, por lo que el modo de operación se distinguió como una variante mucho más simple. Hasta el momento existen muy pocos reportes que describan el empleo de cultivos semicontinuos como una alternativa viable para simular el comportamiento cinético una vez alcanzados los estados estacionarios. Finalmente se confirmó el patrón de producción de anticuerpo no asociado al crecimiento, y se detectó la glutamina como el sustrato limitante en la formulación del medio usado. Los resultados se validaron mediante un análisis estadístico por una homogeneidad de varianza (ANOVA) y una distribución normal t-*Student*.

Palabras clave: células animales, cultivo semicontinuo, caracterización celular

*I*ntroduction

Animal cell culture has been extensively used for the expression of complex human proteins at industrial scale [1]. Among these proteins are monoclonal antibodies (MAbs) for therapeutic applications, with nineteen of them already registered [2]. The Center of Molecular Immunology (CIM) has several MAbs being tested in clinical trials, one of them registered for head and neck cancers and with potential sales of hundreds of million USD [3]. To achieve such goals, cost reduction in the production of recombinant proteins is of outstanding relevance [4], essentially based on process development and cell characterization. Continues cell culture is ideal for cell characterization studies, providing stabilized culture conditions and then, reliable determination of cellular parameters [5]. However, the evaluation of a wide range of dilution rates is extremely expensive, with dispersion of data reaching the same magnitude than the data itself, arguing against the usefulness of the method under these conditions [6, 7].

Cellular characterization studies are also carried out in batch cultures, due to the simplicity of the configuration. Nevertheless, the data generated are only reproducible and reliable at the exponential growth phase, when nutrients are non-limiting and inhibitory substances almost inexistent. The metabolic burden is low in batch and continuous cultures, at cell concentrations that never surpass 3×10^6 cells/mL. Thus, we studied the use of batch cultures with daily feeds at constant dilution rates for cell characterization (semicontinuous). The intermittent fresh media supply and exhausted media extractions, at constant intervals and amounts, would resemble a continuous culture behavior if these amounts and intervals would be near zero, or if fluc-

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tuations of the concentrations of different substances would remain steady.

Materials and methods

Cell line

The murine hybridoma 1E10 cell line, obtained at CIM [10], was employed. It secretes an anti-idiotypic IgG_1 antibody that specifically recognizes the MAb P3, which subsequently recognizes the NGcGM3 ganglioside.

Culture media and supplements

Control medium: Protein-free hybridoma medium II (PFHM-II; Gibco, USA), supplemented with 2 mM of L-glutamine (Sigma, USA), 13 mM of glucose (Sigma, USA), 0.1% of Pluronic F68 (Gibco, USA) and 0.35 g/L of sodium hydrogen carbonate (Sigma, USA).

Medium 1: Control medium supplemented with 6 mM of L-glutamine (Sigma, USA).

Medium 2: Medium 1 further supplemented with an aminoacid concentrate (asparagine, methionine, proline, leucine and cystein).

Medium 3: Medium 2 further supplemented with 28 mM of glucose (Sigma, USA).

All the media were sterilized by filtration through Sartobran P (Sartorius) filters, with a pore size of 0.22 mm.

Experimental procedures

Batch culture

The initial concentration of all the inoculums was of 0.3×10^6 cells/mL. One-hundred milliliters spinner flasks (Integra Biosciences) were used into a CO_2 incubator (Revco). Operational conditions were: temperature 37 ºC, 5% CO_2 and 100 rpm agitation speed.

Semicontinuous culture

Operational conditions were similar to those employed in batch cultures. Cultures were started as batch cultures until reaching a cell concentration of 1×10^6 cells/mL, when point dilutions were initiated at daily intervals. The volume exchanged (Vi; volumes extracted and fed) were calculated for the dilution required according to the equation:

$$Vi = D \cdot V_{Spinner} \cdot \Delta t \quad (1)$$

Where:

D: dilution rate (h^{-1})

$V_{Spinner}$: Volume of the spinner flask (mL)

Δt: Interval between medium addition

The regimen of fluid exchange was kept between 3 and 4 residence times, and was also calculated by the equation:

$$\tau = \frac{1}{D} \quad (2)$$

After this time with stabilized variables, the culture was maintained for five days to carry out 3 or 4 experimental determinations.

Four dilution rates were used: 0.12, 0.24, 0.42, 0.59 d^{-1}.

Analytical determinations

Glucose and lactate concentrations were determined by using a DT 60clinical analyzer (Johnson & Johnson, Rochester, New York, USA).

The IgG1 titer was determined by an immunoenzymatic ELISA assay, in a Maxisorp plate (Nunc, USA), coated with an anti-murine-IgG antibody. Samples were further applied, followed by an anti-murine-IgG antibody-alkaline phosphatase conjugate (Sigma, USA). Afterwards, the reaction was started by adding the para-nitrophenyl-phosphate substrate, and it was finally read in an ELISA plate reader (Organotechnica, Germany).

Cell concentration and viability of samples were estimated by the dye exclusion method, with tripan blue in a Neubauer chamber (PolyLabo™).

Calculations

Kinetic parameters

The specific production and consumption rates for any generic specie in the exponential growth curve were determined for the batch culture mode by the integrative method. The v_i (slope) was calculated by the best linear adjustment of equation 3:

$$I = v_i \int X_v dt \quad (3)$$

Where:

I: concentration of a generic specie (cells, products or nutrients).

v_i: production or consumption rate of the given generic specie.

Xv: concentration of viable cells.

t: time.

The differential method was used for semicontinuous culture determinations, applied to each interval between additions, by the following equation:

$$v_i = \frac{1}{X_v}\frac{dI}{dt} \quad (4)$$

Yields

The biomass-glucose yields were calculated for batch cultures in the exponential phase by the following equation:

$$Y_{X/Glc} = \frac{\Delta X}{\Delta Glc} \quad (5)$$

Considering that the semicontinuous culture behaves as a batch culture between one addition and the following extraction, the same methodology was followed and the calculated values averaged for the phase where they were stable.

Statistical analysis

The values averaged for calculations of the kinetics parameters were subjected to the following statistical tests with the MINITAB program: randomness (Kolmogorov-Smirnov´s test), and normality (variance homogeneity by ANOVA and normal distribution by a Student´s t test, respectively). The confidence interval was of 95% for all the statistical analyses.

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Results

Behavior of the semicontinuous culture

The concentration profile of viable cells and antibody secreted are shown in figure 1, in three replicates from two dilutions studied at stable culture conditions. Values shown correspond to samples taken before media exchange for each given dilution. The antibody concentration (IgG) and Xv values are relatively constant, with a profile similar to that expected for a pure continuous culture and small variations in the inter-experiment deviations. The exchange event introduced the highest point variations in the concentration of the given species. For example, the range of residual glucose concentrations reached for the different dilutions before and after adding media remained between 3.5 and 1.3 mmol/L. These values can be considered small, compared to the 13 mmol/L of initial glucose concentration in the medium. Fluctuations were lower for the rest of nutrients, since glucose is the preferentially consumed nutrient, with the highest consumption rate [11]. Noteworthy, all these fluctuations in the concentrations are small in spite of relatively long intervals between media exchange (24 h). This confirms the initial hypothesis of the present work, of keeping relatively constant conditions with semicontinuous cultures of daily exchange of media, due to the lower metabolic burden existing in animal cell cultures.

The mean specific growth rates, calculated after stabilizing culture conditions, are shown in figure 2. Standard deviations are shown, with a sample size of at least five points. The deviation calculated is relatively small compared to the absolute mean value. This demonstrates the reproducibility of the cell behavior under these conditions, and its steadiness and adequacy for its characterization. The growth rate value almost reaches in magnitude the dilution rate at low dilution rates (0.005 y 0.01 h^{-1}), as expected for a continuous culture. However, growth rate values at higher dilutions rates are remarkably higher than the dilution rates.

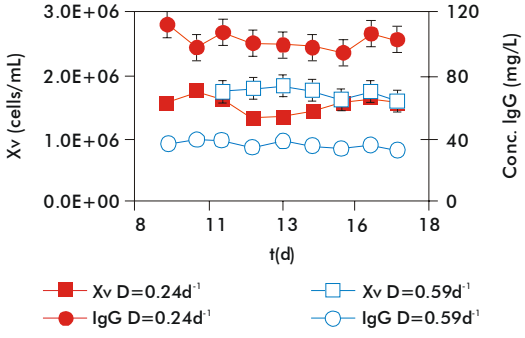

Figure 1. Concentration profiles of viable cells and antibody over time, corresponding to both evaluated dilutions of fed-batch cultures.

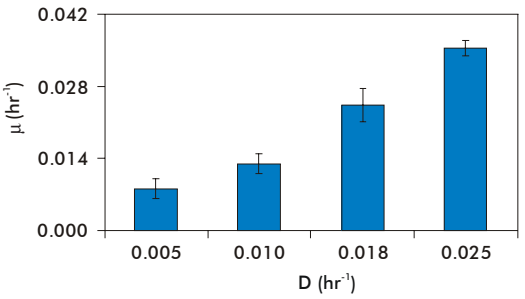

Figure 2. Relation between growth and dilution rates.

Cell characterization

Production profile

Diagram on figure 3 represents the IgG and Xv profiles against the integral of viable cells from three replicates of a batch culture. The best linear tendency adjustments are also included, with the respective parameters and linear regression coefficients by segments.

Considering the regression coefficients, some groups of experimental data can be linearly adjusted, indicating the steadiness of the kinetic variables for the interval. During the adaptation phase, cells produce the protein of interest at a specific rate (q_p) of 3 x 10^{-7} μg/cell*h, while decreasing to 2 x 10^{-7} μg/cell*h at the beginning of the exponential growth phase.

Figure 4 represents the relation between the specific growth rate (μ) and diltution rate (D) in semicontinuous cultures, additionally including the exponential growth phase of the batch culture corresponding to μ = 0.054 h^{-1}.

The q_p values obtained are of he same order of mag-nitude as previously reported [6], either for batch or semicontinuous cultures. The specific growth rate peaked at a growth rate of 0.012 h^{-1}. A proportional inverse correlation is observed between q_p and μ, when the specific growth rates is considered. This profile is identified as the most common between cell growth and antibody production in hybridoma cells [6, 12, 13]. According to those studies, is more probable that the antibody secretion from a given cell population depend on retention of the cell cycle progression in the culture. At low dilution rates, limiting concentra-tions for any nutrient can be

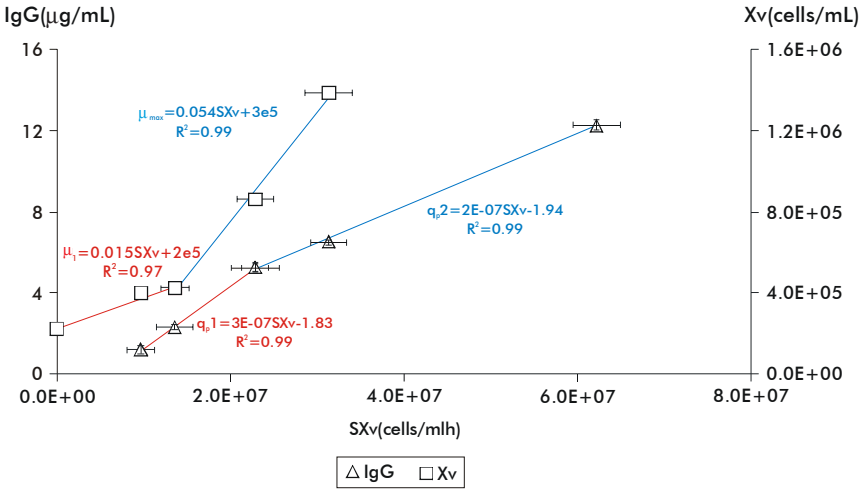

Figure 3. Viable cells (Xv) and product (IgG) plot of concentration profiles against the integral of viable cells in batch culture.

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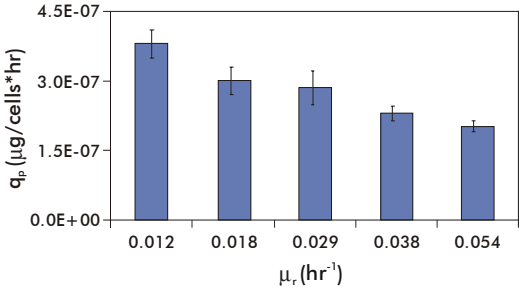

Figure 4. Profiles of μ_r with D in semicontinuous cultures and the exponential phase of batch culture.

achieved, leading to a lower than normal cell duplication rate. In this si-tuation, significant number of cell population can be retained in a phase of the cell cycle, with increased productivity for product formation. It has been de-monstrated in several cell lines that genes responsible for antibody production are transcribed more rapidly when cells are in phase Go/G1 [14, 15]. Otherwise, increased expression of recombinant proteins was evidenced in CHO cells during the S phase, indicating that more than one phase can contribute to the effect shown [16].

The specific rates of glucose consumption (q_{Glu}) and biomass/glucose ($Y_{Xv/Gluc}$) yields for each semicontinuous growth rate are shown in figure 5. Besides, results on the exponential phase of batch culture are shown ($\mu = 0.054\ h^{-1}$).

The q_{Glu} values in semicontinuous cultures are comparable in magnitude to those from batch cultures, in agreement with previous reports of q_{Glu} in animal cells [17-21]. Glucose consumption was higher in the batch culture, with cells during the exponential phase growing at its maximal rate and with fastest consumption of nutrients. Otherwise, growth rate, cell metabolism and consumption of critical metabolites are controlled in semicontinuous cultures. All these were confirmed with the highest q_{Glu} being obtained at $\mu = 0.012\ h^{-1}$).

A low yield correlated with a low m, when analyzing the profiles of biomass/glucose yields ($Y_{Xv/Gluc}$). This indicated a low glucose consumption rate under these conditions, and a less efficient metabolism at this dilution. For the rest of dilutions (between 0.018 and 0.038), $Y_{Xv/Gluc}$ values were insensitive in order of magnitude in a range between 1×10^8 and 3×10^8 cells/mL, that in agreement with that reported in the literature. In general, reports in the literature are contradictory, depending on the growth rate of the cell line and the glucose concentration in the culture media.

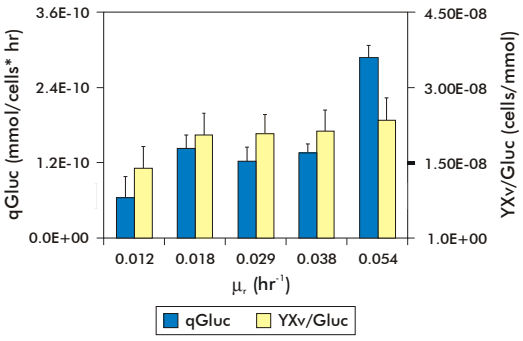

Figure 5. Specific glucose consumption rates (qGluc) and $Y_{Xv/Glu}$ yields in fed-batch cultures and the exponential phase of batch culture ($m = 0.054\ h^{-1}$).

Use of semicontinuous cultures to seek for nutritional limitations

In general, the dependence of the growth rate on the concentration of the limiting substrate in culture can be described by the Monod´s equation. The dependence of µ on residual glucose concentrations for every dilution rates is shown in figure 6.

A saturation model such Monod´s cannot describe the experimental points obtained, as evidenced in the data. This could be interpreted as glucose not being the limiting growth substrate in conditions tested. Figure 7 shows q_p and µ values of semicontinuous cultures with fortified media. The use of these fortified media increased growth rates with respect to control media. The common feature among media from 1 to 3 was the three-fold increase in glutamine concentration. A given substrate is established as kinetically limiting when its decreased concentration decreases growth. Data were statistically analyzed for possible indicators of nutritional limitations, to prove whether there were or not significant differences for µ values between the control medium and medium 1.

First of all, glutamine was checked as kinetically limiting substrate. The hypothesis test for the µ values was:

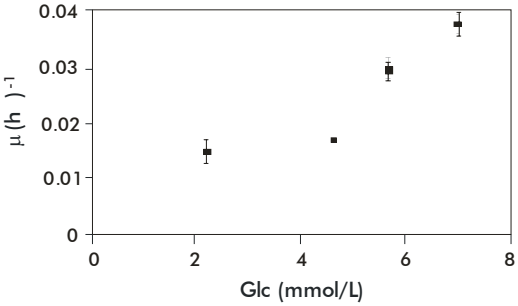

Figure 6. Plot of specific growth rates against residual glucose concentrations for all the dilution rates tested.

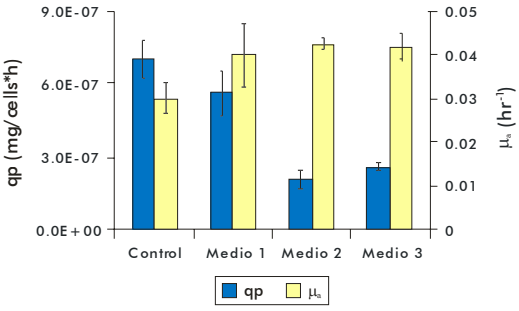

Figure 7. Specific growth and antibody production rates in cultures with different media formulations, at a dilution rate of $D = 0.65\ h^{-1}$.

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H_0: $\mu_{Control} = \mu_{Medium1}$

H_1: $\mu_{Control} \neq \mu_{Medium1}$

Where H_0 and H_1 were the null and alternative hypotheses, respectively.

According to the hypothesis test, the null hypothesis was rejected with a confidence interval of 95%, demonstrating significant differences between the μ values obtained. Thus, the addition of glutamine increased growth rate, suggesting that glutamine could be the kinetic limiting substrate under these conditions.

Additionally, samples from media 1 to 3 were compared to further explore if glucose or amino acid concentrations could also be limiting culture growth. It was evaluated by an ANOVA test, with the similarity of all the means as the null hypothesis accepted. Therefore, no significant differences were detected for μ values, with a 95% of confidence, evidencing that increased concentrations of glucose or the amino acids tested did not influence μ. Considering that the previously mentioned nutrients were consumed faster than glutamine, the L-glutamine was confirmed as the limiting substrate in the media formulation used as control medium for this cell line.

Results shown in figure 7 also demonstrated that q_p decreased by supplementing the medium with glutamine and aminoacids. Our previous result in this work, of μ inversely correlating with q_p, was not reproduced in media from 1 to 3, in spite of showing similar q_p values.

Discussion

In continuous culture, the apparent μ is equal to D, a tendency observed in semicontinuous cultures and mainly at low dilution rates (Figure 2). At lower dilutions, fluctuations in the concentrations were consequently lower, evidencing relatively steady conditions. Steadiness in semicontinuous cultures make them equivalent to continuous cultures, where dI/dt = 0. On the contrary, at high D, μ values are far from these tendency with higher variations associated to media exchange.

Continuous cultures are not reliable at low D. Therefore, our results seem to indicate that semicontinuous cultures can be equivalent to continuous cultures for cell line characterizations, specially at low dilution rates.

A tendency arising from figure 3 is observe of lower protein production at the maximum growth rate. A similar situation is evidenced in figure 4, with a tendency of q_p to increase with a decreased μ.

The possible existence of an μ value correlating with a maximal q_p could have implications in process development. Therefore, the strategy of feeding specific nutrients in a semicontinuous operational mode, or in a continuous culture with biomass retention, should be designed to keep the μ value maximal during the production phase.

$$q_S = \frac{1}{Y_{X/S}}\mu + m \qquad (6)$$

The Maintenance energy model [22] has been used to try to explain the dependence of q_{Glu} as function of μ.

It can be observed that q_{Glu} reaches a value of 6×10^{-11} mmol/cell*h for a dilution rate 0.005 h^{-1}. Such a decrease can be interpreted as cells consuming only glucose for maintenance while decreasing D. However, it was recognized in a review paper about modeling of continuous cultures that q_{Glu} values as function of μ reported by many research groups cannot be described by using the linear model proposed by Pirt for the whole range of values [6]. This questions how valid is equation 6 for animal cell culture.

The experimental approach used to fortify the culture media allowed the identification of L-glutamine as the kinetically limiting substrate. It would be interesting to use a similar approach in future work to try to determine possible simultaneous limitations or inhibitions in animal cell growth and/or antibody production.

In summary, a new methodology was established for animal cell culture at low costs, able to generate results at small scale that allowed characterization of the 1E10 hybridoma cell line. Furthermore, these results will allow us to predict its behavior when cultured in bioreactors, to face strategies for future process development. Our group is currently implementing continuous cultures, to ultimately validate our results.

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