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ASSESSMENT OF KINETIC AND METABOLIC FEATURES OF TWO HYBRIDOMAS IN SUSPENSION CULTURE FOR PRODUCTION OF TWO MONOCLONAL ANTIBODIES FOR BLOOD TYPING

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Abstract - The aim of this study was to analyze kinetic and metabolic features of two hybridomas for monoclonal antibodies (MAbs) production for blood typing. Two lines of hybridomas, ED7 and ED9, were evaluated to produce anti-A and anti-AB, respectively. Experiments with ED7 and ED9 lines were conducted in a 500mL spinner flask and 2L bioreactor, using two different basal culture media, both with 10 % v/v addition of fetal bovine serum. The results revealed the necessity of additional supplementation of the culture media to meet the nutritional demands of the cells. Through nutritional balance and cultivation in better controlled conditions in the bioreactor, it was possible to raise the maximum specific growth rate μ_{max} in 8.2 % and the productivity of anti-A in 148 % with the line ED7. For the ED9 line the μ_{max} decreased 9.5% but the productivity of anti-AB increased 33.3%.

Keywords: hybridomas, cell culture, monoclonal antibody, blood typing.

INTRODUCTION

In the last two decades, monoclonal antibodies (MAbs), due to their high selectivity, have been a valuable tool as reagents for treatment of infectious diseases, cancer (Adams and Weiner, 2005; Ozturk, 2006), identification of diseases in biomedical and microbiological research (Saleem and Kamal, 2008), and ABO blood typing (Kretzmer, 2002). The forecast for the global market of monoclonal antibodies is that it will grow at a yearly rate of 8%, reaching worldwide sales of US$ 94 billion by 2017 (Ecker et al., 2015).

The diagnosis of blood compatibility through the ABO system is of great importance in modern medicine. The test is based on the use of anti-A and anti-AB antibodies which specifically agglutinate red blood cells that have the corresponding A and B antigens. The ABO system from the clinical point of view can be considered the most important worldwide, especially in transfusion practices and in the field of organ transplantation. In the late 1980s, the advent of cell fusion technology of lymphocytes with myeloma cells, discovered a decade earlier by Köhler and Milstein (1975) and today commonly known as

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hybridoma technology, enhanced the efficiency and credibility of the system. This technology allowed the large-scale production of monoclonal antibodies by culturing these cells in bioreactors (Lebherz et al., 1985; Arathoon and Birch, 1986; Birch et al. 1987). Furthermore, a new cycle of utilization of hybridoma technology is being predicted for the production of therapeutic human antibodies in their native form (Smith and Crowe, 2015; Glukhova et al., 2016).

Large scale production of monoclonal antibodies is one of the few fields within biotechnology that has undergone significant improvements and standardization during the past twenty years (Birch and Racher, 2006; Jain and Kumar, 2008; Li et al., 2010; Shukla and Thommes, 2010; Chon and Papastoitsis, 2011; Gaughan, 2016). The most important improvements include better cell lines, high-performing culture media, process optimization, and improved bioreactors and purification processes. The result of these improvements is reflected in a substantial increase in productivity of the industrial process and, consequently, in the reduction of the cost per gram of MAbs. The technology used in the processes of cultivation, separation and purification of MAb requires a high degree of sophistication, thus implying that the operating costs for the production process reach values of the order of US$ 10,300 per gram of MAb (Vermasvuori and Hurme, 2011). In this context, efforts should be made during the development of the process to minimize production costs.

The cultivation of mammalian cells *in vitro* leads to a differential metabolism due to high rates of glycolysis and glutaminolysis (Zhou et al., 1995), having as main products ammonia and lactate. The accumulation of these products reduces cell growth and affects quantitatively and qualitatively the production of MAbs from hybridoma cells (Ozturk, 1992; Gawlitzek, 1998). Therefore, the production of MAbs is mainly carried out in stirred tank bioreactors on an industrial and laboratory scale (Wurm, 2004; Ozturk, 2006; Rodrigues et al., 2009) and under strictly controlled and monitored conditions (Jain and Kumar, 2008). The industrial version consists of a stainless steel tank fitted with a mechanical agitator and automatic temperature, pH and oxygen controllers (Chu and Robinson, 2001; Ozturk, 2006; Mel et al., 2008); one of its laboratory versions, known as spinner flask, is much simpler and consists of a cylindrical glass flask with Teflon® stirrer or actuated magnetically. The spinner flask is operated at fairly constant temperature and pH within a CO_2 incubator.

In South America, especially in Brazil, although R&D centers and experts are able to generate lines of hybridomas which can produce MAbs, there is still not enough know-how to implement the technology for large scale production. The use of MAbs in the region involves a broad market whose demand is met entirely through imports, a situation that makes the prices of MAbs even less accessible due to customs taxes. The installation of a large scale industrial production unit for various MAbs in the region could represent significant savings for governmental agencies responsible for public health.

This study was carried out to evaluate the feasibility of hybridoma culture processes for the production of MAbs for use in blood typing utilizing lineages obtained in a Brazilian research center. For this purpose, cultivation was realized in two stages: firstly, in spinner flasks (200 mL) to meet the key metabolic characteristics of the selected clones in two different culture media; and secondly, in a bench scale (1L) stirred tank bioreactor in conditions similar to an industrial scale to identify and dimensionalize important cultivation parameters for scale up. The results of this study will allow the identification of the culture conditions of hybridoma cells to provide high yields of MAbs, and gathering of valuable data for subsequent technical-economic assessments of what might become an industrial scale bioprocess.

MATERIALS AND METHODS

Cells and Culture Media

Two murine hybridomas known as ED7 and ED9, which produce immunoglobulin (IgG-k) with specificity for blood group antigens A (anti-A) and AB (anti-AB), respectively, were used. These were provided by the Blood Center of the Medicine Faculty of Universidade Estadual Paulista (UNESP) in Botucatu, state of São Paulo, Brazil. The RPMIS and MQYS culture media were used for their cultivation.

RPMIS consisted of RPMI (Roswell Park Memorial Institute) 1640 (Gibco™, Life Technologies, Grand Island, NY, USA) supplemented with 240 mM glucose (Sigma-Aldrich, Saint Louis, MO, USA), 10 mM sodium pyruvate (Gibco™, Life Technologies, Grand Island, NY, USA), 40 mM Glutamax (Gibco™, Life Technologies, Grand Island, NY, USA), 10 mL antibiotic-antimycotic (Gibco™, Life Technologies, CA, USA), 1 mM oxaloacetic acid (Sigma-Aldrich, Saint Louis, MO, USA), 0.4 UI insulin (Gibco™, Life Technologies, Grand Island, NY, USA), 14.3

mM mercaptoethanol (Gibco™, Life Technologies, Grand Island, NY, USA), 10 mL essential amino acids (Gibco™, Life Technologies, Grand Island, NY, USA), 0.5 mM nonessential amino acids (Gibco™, Life Technologies, Grand Island, NY, USA), 23.8 mM sodium bicarbonate (Sigma-Aldrich, Saint Louis, MO, USA) and 10% v/v fetal bovine serum (FBS) (Gibco™, Life Technologies, Grand Island, NY, USA). Two additionally supplemented versions of RPMIS media were used in the experiments with ED7 and ED9 cell lines: the first version, hereinafter RPMIS1, involved the addition of 3.52 $mg.L^{-1}$ Serine (Ser), 2.25 $mg.L^{-1}$ Methionine (Met), 7.7 $mg.L^{-1}$ Cysteine (Cys), 3.81 $mg.L^{-1}$ Lysine (Lys) and 15.5 $mg.L^{-1}$ Glutamine (Gln). The second version, hereinafter RPMIS2, involved the addition of 2.57 $mg.L^{-1}$ Proline (Pro), 7.7 $mg.L^{-1}$ of Cys and 15 $mg.L^{-1}$ Gln. These amino acids were purchased from Gibco™ (Life Technologies, Grand Island, NY, USA) and identified as deficiencies in the RPMIS media in studies that preceded this work (Ferreira, 2007). The MQYS culture media is a basal chemically defined media Quantum Yield (BD Biosciences, São José, CA, USA) supplemented with 10% v/v FBS.

Hybridoma culture techniques

For experimentation, cells were defrosted in 25 cm^2 culture T-flasks (Corning, Corning, NY, USA) and maintained in an incubator (Forma 310, Thermo Fisher Scientific, Waltham, MA, USA) at 37 ºC with 5% CO_2. After two days of growth with viability greater than 90%, cells were inoculated into 75 cm^2 culture T-flasks (Corning) at $2x10^5$ $cells.mL^{-1}$. After another two days of growth with viability above 95%, cells were then transferred aseptically to a 500 mL spinner flask (Wheaton, Millville, NJ, USA) in 200 mL of culture media at a concentration of $2x10^5$ $cells.mL^{-1}$. After the initial 24 hours of culturing, the oxygenation was conducted through a tubular gas-permeable silicone membrane immersed in the culture media. Once a suitable cell concentration and viability above 95% was reached, the cells were inoculated in a Bioflo 110 stirred tank bioreactor of 2L capacity (New Brunswick Scientific Co., Edison, NJ, USA) with a working volume of 650 mL and inoculum concentration of $2x10^5$ $cells.mL^{-1}$. It was equipped with temperature, pH and dissolved oxygen control. To avoid damage by hydrodynamic forces, a bubble free oxygenation system was set up using a silicone tubular membrane with dimensions 2 mm internal diameter, 0.8 mm wall thickness and 7.8 m long curled in a grid of stainless steel around the marine propeller supplied with the bioreactor.

Three experiments were conducted with each cell line. The first two experiments (ES1 and ES2 for ED7; ES3 and ES4 for ED9) followed the procedure described above only until the step of cultivation in the spinner flask using the culture media RPMIS1 for ES1, RPMIS2 for ES3 and MQYS for ES2 and ES4 experiments, respectively. The last experiment (EB1 for ED7 using RPMIS1 culture media and EB2 for ED9 using the MQYS culture media) followed the entire procedure for cultivation in a bioreactor. The choice of media in experiments EB1 and EB2 is justified in the discussion. The cultures of the hybridomas in the bioreactor were performed at 37±0.2ºC, pH 7.2±0.1, 40±5% air saturation and agitation of 80-100 rpm. During the culture an average of a sample of 5 mL every 12 hours was withdrawn. A 0.5 mL aliquot was immediately separated for cell density and viability quantification and the remainder was centrifuged at 200g during 5 minutes to store the supernatant at -80ºC for analysis of glucose, amino acids, lactate, ammonia, and MAb.

Analytical Methods

The total and viable cell densities (C_{xt} and C_{xv}) were obtained by microscopic counting in an Improved Neubauer hemocytometer chamber (Sigma-Aldrich, Saint Louis, MO, USA), and cell viability was determined by the dye exclusion method of trypan blue (Sigma-Aldrich, Saint Louis, MO, USA). Considering as a counting event the quantified cells in one of the halves of the hemocytometer, each sample was counted four times and the calculated averages correspond to the values reported in the graphs of concentration and cell viability in Figures 1, 2, 3 and 4. The maximum specific growth rate μ_{max} (h^{-1}) was calculated in the logarithmic growth phase as the slope ± standard deviation of the LnC_{xv} values as a function of the culture time.

To analyze the concentration of glucose and lactate, a high performance liquid chromatograph-HPLC (Waters, Milford, MA, USA) with Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) was used with a mobile phase of 5 mM sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA), at 65 ºC and a 0.6 $mL.min^{-1}$ flow, with detection of carbohydrates and organic acids through refractive index and UV 210 nm, respectively. The concentration of amino acids in the supernatant was determined by the same HPLC using the Pico-Tag® system, and that of ammonia by an ion selective electrode method (Orion 710A, Thermo

Fisher Inc, Beverley, MA, USA). The total amount of IgG-k produced was quantified using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) according to the general protocol of SIGMA involving anti-mouse polyvalent IgG as a coating, anti-mouse IgG conjugated to alkaline phosphatase as a detection antibody and alkaline phosphatase yellow (pNPP) as a substrate. The assay was calibrated using an IgG1 murine myeloma-k as a standard, and each sample was analyzed in a Multiskan™ FC Microplate Photometer (Thermo Scientific, Waltham, MA, USA) with culture medium containing 10% v/v of FBS as blank. The result was expressed as concentration (C_{MAb}) in $mg.L^{-1}$. The biochemicals used in the ELISA analysis were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The productivity of Mab in the culture was calculated with the expression $(C_{MAb,max} - C_{MAbi})/t_{max}$, where $C_{MAb,max}$ and C_{MAbi} are the maximum and initial concentrations of monoclonal antibody, respectively; t_{max} is the time (h) taken to reach $C_{MAb,max}$. All analyses of amino acids, glucose, lactate and ammonia were done without replication. The analyses of MAbs were done in duplicate.

Experimental Strategy

Knowledge of cell growth kinetics and metabolism is crucial for a better understanding of the physiology and optimization of the *in vitro* cultivation process, when it is aimed at the production of a protein. The metabolic patterns of mammalian cells are substantially changed when moved from an environment in a tissue of a multi-cellular organism to an artificial device of *in vitro* mono-cellular cultivation (Godia and Cairo, 2006).

The ES1 - ES2 and ES3 - ES4 sets of experiments were performed with the ED7 and ED9 cell lines, respectively, in the spinner flasks to recognize, in a first approach, the kinetic and metabolic characteristics of the hybridomas cultivated in suspension. The media which was found to be most appropriate for each cell line was used for the cultivation in the bioreactor Bioflo 110.

Statistical analysis

For the comparative analysis of the measurements performed with replicates such as those of concentration and cell viability and the concentration of MAb the mean ± standard deviation was used. Statistical significance was assessed by analyzing the data with the student *t*-test; significance was determined at a *p* value less than 0.05.

RESULTS AND DISCUSSION

Spinner flask cultivation of the ED7 and ED9 cell lines

The results of the experiments ES1 and ES2 with the ED7 cell line in spinner flasks are presented in Figure 1. Similarly ES3 and ES4 with the ED9 cell line, are presented in Figure 2. Both figures illustrate the main results of cell growth, development of glucose, most consumed and produced amino acids, production of metabolites lactate and ammonia and production of monoclonal antibodies during the cultivation process.

It can be observed in Figure 1 that in both experiments the cell line ED7 grew successfully with near 100% viability and maximum specific growth rates of $\mu_{max} = 0.0304 \pm 0.0014\ h^{-1}$ and $\mu_{max} = 0.0294 \pm 0.0010\ h^{-1}$ in the ES1 and ES2 experiments, respectively. The viable cell concentration of $2.00 \pm 0.24 \times 10^6$ cells.mL^{-1} achieved in the ES1 experiment was significatively greater (p=0.0042) than that in ES2, in which it reached $1.76 \pm 0.14 \times 10^6$ cells.mL^{-1}. In the case of the ES1 and ES2 experiments, growth was interrupted after 75 hours of cultivation due to the depletion of the Gln amino acid. It probably occurred due to stoichiometric limitation at a concentration around 1 mM, a behavior similar to that observed and characterized by Jeong and Wang (1995). The Gln amino acid is an important precursor in the synthesis of nucleic acids, and its scarcity implies a strong limitation on biosynthesis. Even an increase in the rate of glucose consumption, which can be observed in Figure 1 at the start of Gln limitation, may not be sufficient to supply the nitrogen needs that were being met by this amino acid.

For the ED9 cell line in Figure 2, the maximum values of specific cell growth and viable cell concentrations obtained were $0.0272 \pm 0.0014\ h^{-1}$ and 0.98×10^6 cells.mL^{-1}, and $0.0412 \pm 0.0024\ h^{-1}$ and 1.50×10^6 cells.mL^{-1}, with close to 100% viability in the ES3 and ES4 experiments, respectively. In the ES3 experiment, growth was interrupted due to the lack of Cys at 60 hours of cultivation because the rate of conversion of Met to Cys was not enough to offset the high consumption of Cys, to perform its two main functions in the cell: synthesis of biomass and free protein. Whereas, for ES4, the exponential phase was interrupted around 55 hours of cultivation due to limitation of Gln that had very low levels, about 0.7 mM. With the hybridoma ED9, there was no increase in the rate of glucose consumption, which was observed with the ED7 cell line at the start of Gln limitation.

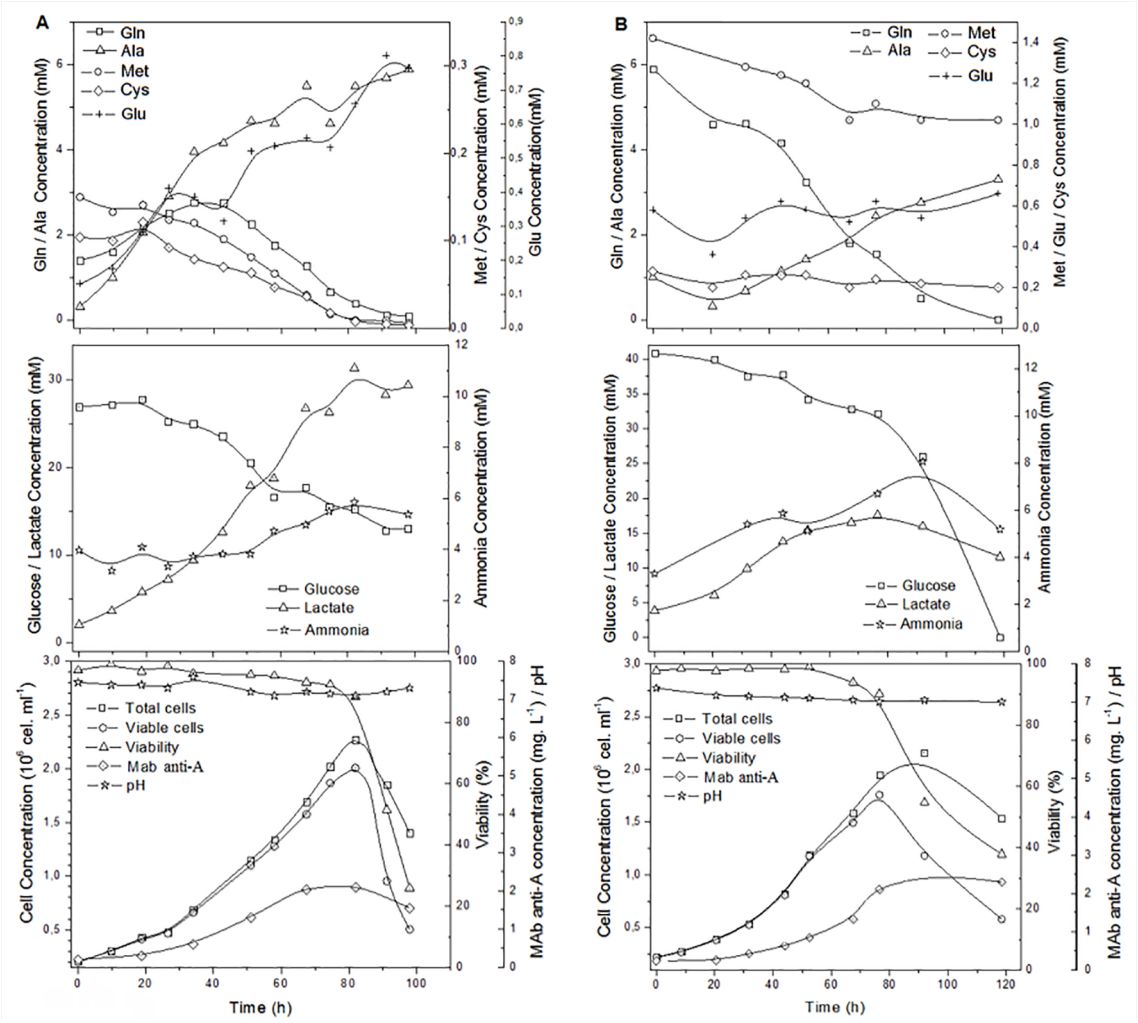

Figure 1. Results of total and viable ED7 cell concentration, cell viability, pH, monoclonal antibody (anti-A) concentration, and concentrations of glucose, lactate, ammonia and most consumed and produced amino acids, in 200 mL spinner flask cultures at 37°C, and 75 rpm. A) ES1 with RPMIS1 culture medium, and B) ES2 with MQYS culture medium.

The initial increase in the concentration of Gln in the ES1 experiment was probably due to the gradual release of glutamine by GlutaMAX™ (L-Alanine and L-Glutamine dipeptide) by the action of amino-peptidase originated from the cells themselves. This also explains the enhanced accumulation of Ala in the culture media. The addition of Ala produced by GlutaMAX™ to the Ala produced by the reaction of Glu with Pyr justifies the high concentration of Ala found in the ES1 experiment in comparison with ES2. The other amino acid produced sufficiently by the hybridoma ED7 was Glu; as a result, the production profile of these two amino acids resembles that of the KB-26.5 hybridoma cell line studied by Sanfeliu et al. (1996). Similar to the results of the ED7 cell line, the hybridoma ED9 also showed an initial increase in the concentration of Gln in the experiment ES3, attributed to the gradual release of Gln by GlutaMAX™. A marked accumulation of Ala was also observed in the culture media as a release product of the synthesis by the cell itself and by GlutaMAX™. Additionally, Glu was also significantly produced by the hybridoma ED9, thus also resembling the profile of amino acid production of the hybridoma-KB 26.5 (Sanfeliu et al. 1996).

In the experiments with both cell lines, ED7 and ED9, the highest concentrations of ammonia exceeded the 5mM value, which is considered as a cell growth inhibitor (Newland et al, 1994). This happened earlier and with higher concentrations in ES2 and ES4, which may have caused the inhibition of cell growth even before the Gln reached the value of 1 mM indicated as stoichiometrically limiting (Jeong and Wang, 1995). Concerning lactate, the maximum concentration achieved in ES1 (10 mM), in ES2 (5.2 mM), in ES3 (30 mM) and in ES4 (22 mM) should not have affected the cell growth since the value of 40 mM recognized as inhibitory when pH is reasonably controlled was not attained (Ozturk et al, 1992). In these cases pH was controlled by the CO_2 of the incubator in the range of 7.2 - 7.4.

The production of monoclonal antibody obtained in the four experiments in spinner flask is also presented in Figures 1 and 2. It can be noted in Figure 1 that the production of anti-A accompanies the cell growth in both experiments, with a maximum productivity of 0.023 $mg.L^{-1}.h^{-1}$ in ES1 and 0.028 $mg.L^{-1}.h^{-1}$ in ES2. In the case of Figure 2, the production of anti-AB accompanies the cell growth in both experiments, and its maximum productivity is 0.0066 $mg.L^{-1}.h^{-1}$ in ES3 and 0.0270 $mg.L^{-1}.h^{-1}$ in ES4, respectively.

Cultivation in spinner flask has the disadvantage of not having a precise control of culture parameters, such as pH and dissolved oxygen, which are necessary for a better understanding of the physiological state of the cell to optimize the production of antibodies (Kretzmer, 2002). Consequently, a culture environment was sought which is more homogeneous, controlled and similar to the industrial process. For this, a stirred tank bioreactor was used with the culture medium MQYS, which provided higher productivity in the spinner flask experiments for cell line ED9. For cell line ED7, which reached similar productivities with the two media, the RPMS1 medium was chosen because it enabled smaller inhibitory amounts of ammonia.

Bioreactor cultivation of the ED7 and ED9 cell lines

Figure 3 and Figure 4 present the results of experiments EB1 and EB2, respectively, including total and viable cell concentration, cell viability, and concentration of monoclonal antibody, glucose and amino acids obtained in the stirred tank bioreactor. In the experiment EB1 similar cellular growth in the stirred tank bioreactor can be observed in comparison with the spinner flask, with a μ_{max}=0.0329 ± 0.0011 h^{-1} and a maximum cell concentration of 1.8 x 10^6 viable cells.mL^{-1}. In the experiment EB2 a maximum

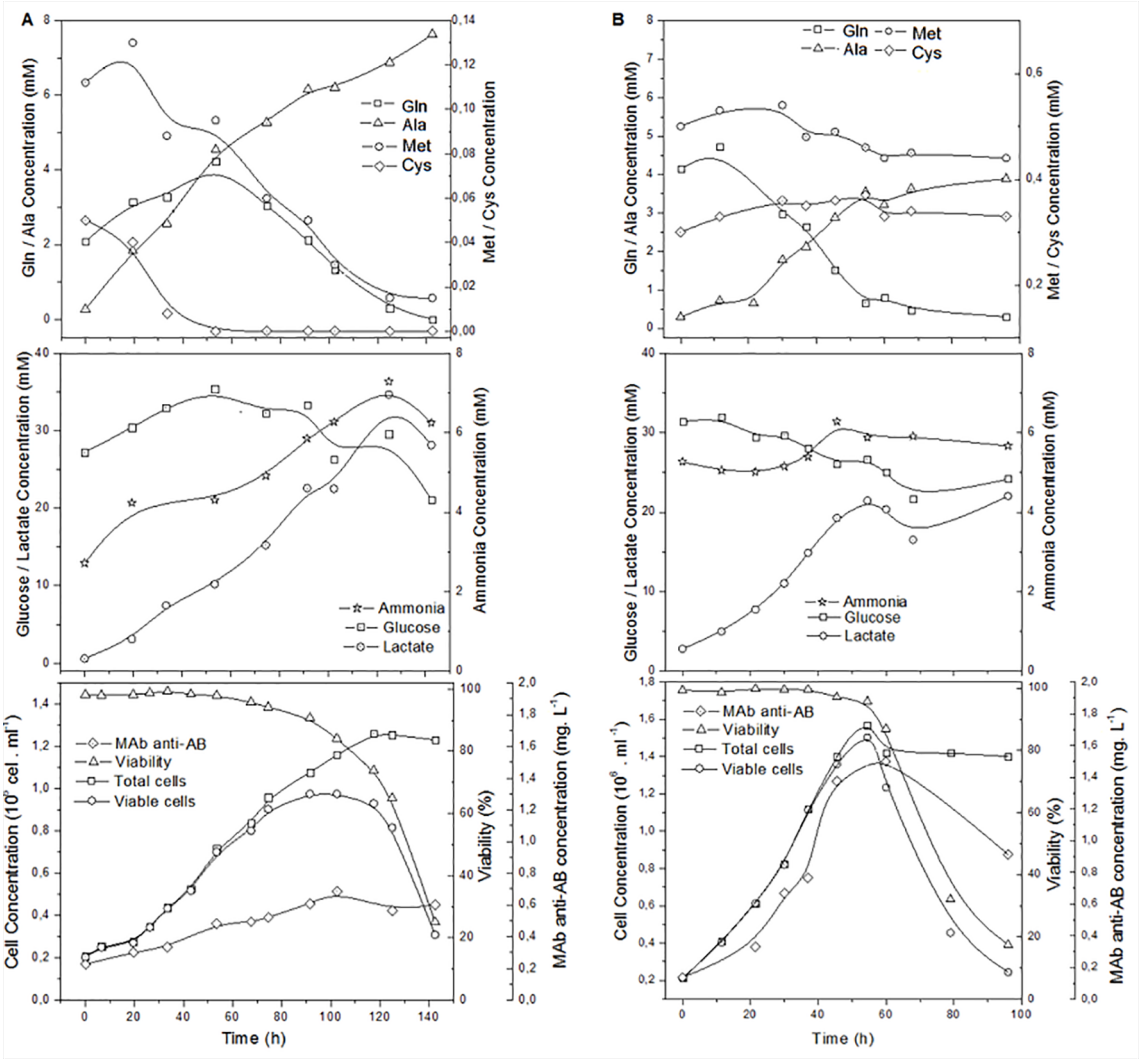

Figure 2. Results of ED9 cell growth, cell viability, monoclonal antibody (anti-AB) concentration, and concentrations of glucose, lactate, ammonia and most consumed and produced amino acids, and pH of the experiments performed in 200 mL cultures in spinner flasks at 37°C and 75 rpm. Experiments A) ES3 with RPMIS2 culture medium, and B) ES4 with MQYS culture medium.

cell growth of $2x10^6$ cells.mL^{-1} was obtained. When compared with the ES4 experiment (Figure 2) performed in the spinner flask ($1.5x10^6$ viable cells.mL^{-1}), 33% higher cell growth was reached. The value of μ_{max} = 0.0373 ± 0.0019 h^{-1} is slightly lower than the 0.0412 ± 0.0024 h^{-1} obtained in the spinner flask. The instant where the viable cell concentration begins to decay coincides with the concentration of Gln lower than 1 mM value, indicated by Jeong and Wang (1995) as a cause for cell death.

It can be noted in Figure 3 that the cell viability in experiment EB1 dropped dramatically after 70 hours of culture. This point coincides with the depletion of Gln, an important amino acid for growth. Other amino acids such as Cys and Met were also significantly consumed, which implies that the low concentration of these amino acids must have also contributed to the decrease in viability, as it has been demonstrated earlier that the deprivation of any of these results in the induction of cell death by apoptosis (Singh, 1997). The glucose consumption remained low, as shown by the presence of an appreciable amount of this substrate at the end of the experiment. Similar to the spinner flask experiments, the lactate production by the ED7 cell line in the RPMIS1 culture medium did

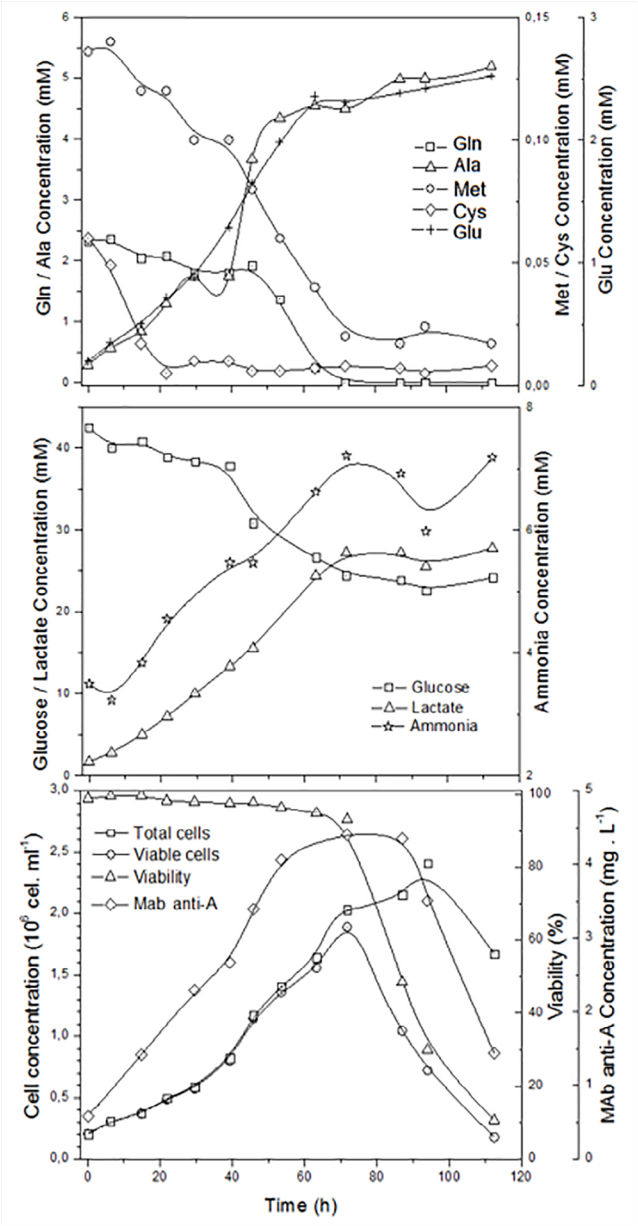

Figure 3. Results of total and viable ED7 cell concentration, cell viability, concentration of glucose, lactate, ammonia, amino acids and monoclonal antibody (anti-A) in EB1 experiment. The experiment was carried out in a stirred tank bioreactor with a working volume of 650 mL at 37°C, 100 rpm, dissolved oxygen of 40% air saturation, and RPMIS1culture medium controlled at pH 7.2.

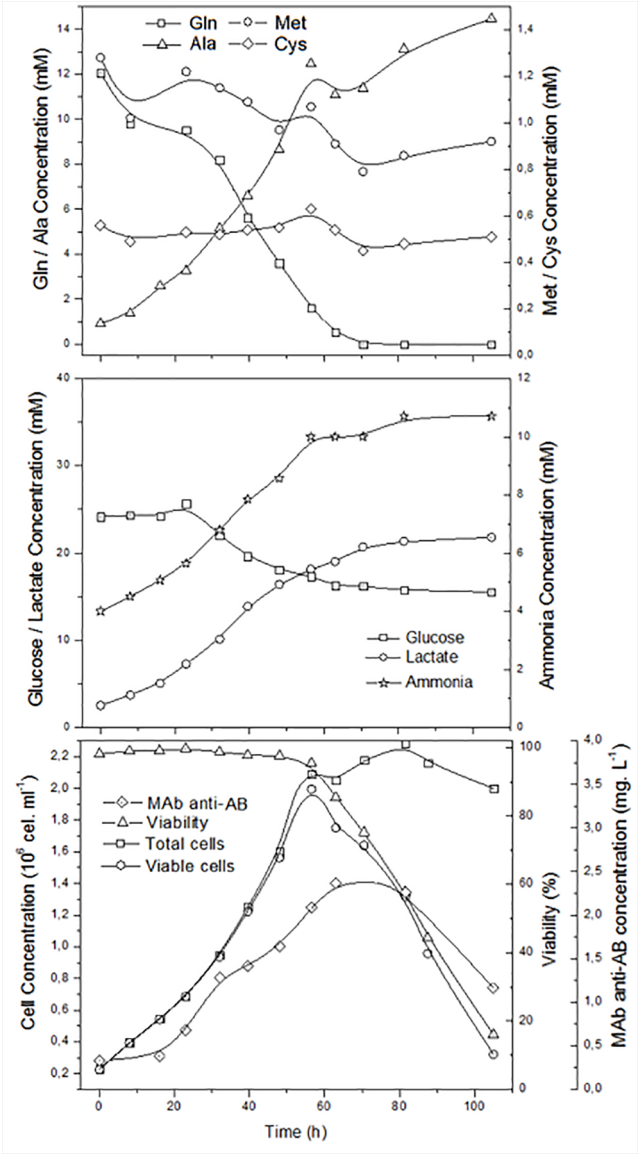

Figure 4. Results of total and viable ED9 cell concentration, cell viability, concentration of glucose, lactate, ammonia, amino acids and monoclonal antibody (anti-AB) in EB2 experiment. The experiment was carried out in a stirred tank bioreactor with a working volume of 650 mL at 37°C, 80 rpm, dissolved oxygen of 40% air saturation, and MQYS culture medium controlled at pH 7.2.

not seem to affect cell growth, since the concentration did not reach values considered to be inhibitory in cultures with controlled pH (Ozturk, 1992). On the other hand, the concentration of ammonia attained values around 7mM, which is considered inhibitory (Newland et al, 1994).

In the case of experiment EB2 it can be observed that, after 57 hours of cultivation, the viability dropped dramatically. This coincides with the depletion of Gln, as already pointed out, an amino acid crucial for cellular growth. Glucose consumption remained low, with a substantial amount of this substrate still available at the end of the experiment. Lactate production by the ED9 cell line in the MQYS culture medium, as in the case of spinner flask cultivation, should not have affected the cell growth, as the concentration did not reach the value of 40 mM considered inhibitory. Referring to production of ammonia, the ED9 produced even more inhibitory quantities than ED7, attaining values around 11 mM.

The production of anti-A in experiment EB1 accompanied the cell growth and, in the late exponential phase, reached its maximum yield of 0.062 $mg.L^{-1}.h^{-1}$, which is almost double that obtained in the spinner flask. Thereafter, the antibody concentration began to decline, possibly by the action of proteases released by cell lysis in the culture media. Despite the supplementation of the Gln amino acid, it was depleted after 70 hours of cultivation, a fact which led to the interruption of cell growth and monoclonal antibody production. The Cys was also consumed considerably, almost to exhaustion within 20 hours of cultivation. From this moment on, Cys, being a nonessential amino acid, started to be supplied by Met through a reaction which is also found in the metabolism of other hybridomas (Jo et al., 1990; Sanfeliu, et al., 1996).

The production of anti-AB in experiment EB2 accompanied the cell growth, having reached its maximum yield of 0.040 $mg.L^{-1}.h^{-1}$ at the end of the exponential growth phase. Thereafter, the antibody concentration began to decline, possibly as a consequence of the action of proteases released by cell lysis. The maximum yield achieved in the bioreactor Bioflo 110 was almost double that obtained in the spinner flask cultures. The amino acid Gln was the main limiting nutrient; its depletion started around 70 hours of cultivation, which led to the interruption of cell growth and of production of monoclonal antibody anti-AB. The other amino acids did not reach limiting values, thus showing a better balanced culture medium than the supplemented RPMIS.

Even though an increment in ED7 cell growth and antibody production was observed in batch cultures with RPMIS1 in both spinner flask (ES1) and bioreactor (EB1), it appears that the amount of glutamine supplemented in this medium was not yet sufficient to compensate for the deficit of that amino acid in the RPMI medium. The increase in glutamine consumption has been detected in several hybridomas when higher concentrations of glutamine are supplemented (Butler and Spier, 1984; Glacken et al., 1986; Miller et al., 1988; Jeong and Wang, 1995), accompanied by high production of ammonia. The recommendation for more efficient utilization of that amino acid to achieve high concentrations of pharmaceuticals is the use of the fed batch cultivation strategy (Xie and Zhou, 2006). When comparing the experiment conducted with MQYS culture medium in the spinner flask (ES4) and bioreactor (EB2), an increase in ED9 cell growth and antibody production is also evident, however, at the cost of a more harmful concentration of ammonia.

Summarizing the results of growth kinetics and monoclonal antibody production obtained in the six experiments, it can be noted that the two experiments conducted in the stirred tank bioreactor achieved higher values of productivity of the anti-A and anti-AB by ED7 and ED9 hybridomas, respectively. A 2-fold increase in the productivity for anti-A and 1.4-fold for anti-AB were observed in the bioreactor with respect to the spinner flask. The most decisive factors in achieving these results are probably the nutritional supplementation in the case of RPMIS culture media and better controlled processing conditions, especially pH and dissolved oxygen, accomplished in the bioreactor.

A general analysis of these results, conducted during the course of nearly six years, shows that the hybridomas used in this study and a previous study (Ferreira, 2007) proved to be quite stable, with little variation in growth and metabolism in the supplemented RPMIS culture media. Isotyping and agglutination tests realized in the Blood Center of the Faculty of Medicine of UNESP in Botucatu showed that both monoclonal antibodies were authentic and functional.

The results obtained in this work with the hybridomas ED7 and ED9 give an insight into the strategy of balancing nutrition of the culture media, which can lead to an improvement in batch cultures in terms of the ability of growing cells and synthesizing anti-A and anti-AB antibodies. However, it is important to remember that the concentration of Gln in the presence of high glucose

concentrations, often causes deregulation of the metabolism of mammalian cells (Godia and Cairó, 2006), as shown by several investigators (Miller et al, 1988; Glacken et al, 1986; Butler and Spier, 1984; Jeong and Wang, 1995). This deregulation is characterized by an excessive production of lactate and ammonium salts, in addition to an inefficient use of these sources of carbon and energy.

A convenient solution, to be adopted to alleviate this problem with ED7 and ED9 cell lines, should be the use of the cultivation in a fed batch bioreactor. In this system, the limiting nutrients such as Gln and glucose are added in a modulating manner during the course of cultivation to maintain their low concentrations and, consequently, an efficient metabolism with high cell yield, low production of toxic metabolites (lactate and ammonia) and higher concentration of monoclonal antibody.

CONCLUSIONS

Both cell lines of hybridomas proved to be very stable with regards to metabolic characteristics and cellular growth during the course of this research. Overall the results show that the basic culture media RPMIS and MQYS, both containing 10% v/v fetal bovine serum, need supplementation of some amino acids to meet the nutritional demands of the two hybridomas in a more balanced way. The MQYS culture medium requires less supplementation, possibly because it has been designed as a medium with a formulation more specific for hybridomas.

For the ED7 cell line, cultivation in spinner flask showed a 12% higher yield of anti-A using the MQYS culture medium in comparison with the RPMIS1, though it achieved 14% lower viable cell concentration. On the other hand, when cultivated on larger scale in a stirred tank bioreactor, with better controlled culture conditions, productivity was 2.7 times higher than the cultivation in spinner flask using the RPMIS1 medium. As for the ED9 cell line, when cultivated in spinner flask, the use of MQYS culture medium presented a possibility of having 50% higher viable cells concentration and an anti-AB productivity four times greater than the RPMIS2 culture medium. When cultivated in a stirred tank bioreactor using the MQYS culture medium, 33% more viable cells and 1.5 times higher anti-AB productivity were obtained in comparison with the spinner flask cultivation.

The supplementation with amino acids showed improvements in cell growth and monoclonal antibody production in the batch cultures in this study. However, they highlight a limitation of this cultivation system, which has been widely debated in the specialized literature concerning the technology of hybridoma culture. The problem of disrupting the metabolism of hybridomas with the presence of high concentrations of glucose and glutamine, reflecting in poor cell growth and low synthesis of monoclonal antibody, should be overcome in a fed-batch cultivation system.

NOMENCLATURE

C_{MAb}	Monoclonal antibody concentration, $mg.L^{-1}$
C_{MAbi}	Monoclonal antibody concentration at the start of the culture, $mg.L^{-1}$
$C_{Mab,max}$	Maximum monoclonal antibody concentration, $mg.L^{-1}$
C^{xt}	Total cell concentration, $cells.mL^{-1}$
C^{xv}	Viable cell concentration, $cells.mL^{-1}$
t_{max}	Time to reach maximum monoclonal antibody concentration, h.

Greek letters:

μ_{max}	Maximum specific growth rate, h^{-1}

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