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ORIGINAL RESEARCH

Effects of passage number on growth and productivity of hybridoma secreting MRSA anti-PBP2a monoclonal antibodies

Arthur Luiz Corrêa · José Procópio Moreno Senna · Álvaro Paiva Braga de Sousa

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Abstract Monoclonal antibodies (mAb) are high added value glycoproteins recommended for immunotherapy, diagnosis, and also for the treatment of bacterial infections resistant to multiple drugs such as Methicillin Resistant *Staphylococcus aureus* (MRSA). In addition to environmental conditions related to cell cultures, the intrinsic characteristics of hybridoma cells, like the secretion stability of monoclonal antibodies by the cells through successive subcultures, are relevant for the characterization of cell lines related to the productivity of mAb. The rate of mAb production differs significantly between different cell lines and different passage numbers, and it is an important variable in characterization of cell lines. In order to find a more robust, faster-growing, and higher-productivity cell line of hybridoma, cultivations in 24-well plates were performed in different subculture periods, or cell passages (P), of hybridoma cells producing MRSA anti-PBP2a monoclonal antibodies [MRSA-antiPBP2a (mAb)]. The objective of this study was to study the effects of cell growth and production of MRSA-antiPBP2a mAb secreted by murine hybridoma cells grown in different passages as well as determine the which passages the hybridomas can be cultivated without harming their growth and productivity. So, cell growth profiles of hybridomas secreting MRSA-antiPBP2a (mAb) and the production of MRSA-antiPBP2a mAb in different subculture periods or cell passages (P) were studied. Cell growth tests, monoclonal antibody productivity, and metabolite characteristics revealed substantial differences in those cells kept between P10 and P50. Similarities in the secretion of monoclonal antibody, growth, and metabolic profiles, were noted in the MRSA-antiPBP2a mAb producing hybridoma cells kept between P10 and P20. Also, glucose consumption (g/L) and lactate production (g/L) in the latter cell cultures were monitored daily through biochemical analyzer. As of P30, it was observed a 4.4 times reduction in productivity, a 13 % reduction in metabolic yield, and a significant change in cell growth. Secretion of MRSA-antiPBP2a mAb should be obtained through the culture of hybridomas up to P20 in order to keep its stability.

Keywords Hybridoma · Antibody anti-PBP2a · *Staphylococcus aureus* · Methicilin

A. L. Corrêa
Laboratório de Tecnologia de Produtos Naturais, Universidade Federal Fluminense, UFF, Niterói, RJ 24241-000, Brazil

J. P. M. Senna
Laboratório de Tecnologia Recombinante, Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil

Á. P. B. de Sousa (✉)
Centro Integrado de Protótipos, Biofármacos e Reativos para Diagnóstico, Instituto de Tecnologia em Imunobiológicos, Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ 21040-360, Brazil
e-mail: alvaro.paiva@bio.fiocruz.br

Introduction

Monoclonal antibodies (mAb) are a class of high specificity antibodies produced in laboratory by the fusion of B lymphocytes removed from animal spleens and immunized with a specific antigen target, with a myeloma cell, generating a so-called hybrid hybridoma cell line (Köhler and Milstein 1975; Kaur et al. 2007).

Growth profile and physiological behavior are required investigations when it comes to bioprocesses of mammalian cells, as well as knowledge of their productivity rate so as to avoid harm to the quality of the purified biomolecule and the active pharmaceutical ingredient (IFA) which shall be used in the final formulation of the biopharmaceutical biomedical interest, e.g. monoclonal antibodies (mAb).

The growing use of mAb has been due to its high specificity and selectivity enabling its use as a therapeutic agent for diagnosis, chronic inflammatory conditions and immunobiological disorders, proving to be a promising alternative in fighting multi-drug resistant organisms (Bebbington and Yarranton 2008; Jang and Barford 2000).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen causing severe nosocomial infections worldwide. Such an opportunistic bacterium is resistant to all beta-lactam antibiotics due to its ability to produce an additional penicillin binding protein (PBP) called PBP2a which has a low affinity for those antibiotics (Lim and Strynadka 2002; Selvey et al. 2000; Stapleton and Taylor 2002).

A specific mAb against MRSA anti-PBP2a was obtained by Bio-Manguinhos using the hybridoma technology, patent application number WO/2011/017791. This mAb is able to recognize PBP2a from MRSA strains and can be applied in the manufacture of diagnostic kits. Besides, once properly humanized, it can be applied against MRSA infections.

Usually, production cells undergo prolonged cultivations due to selection phase and up scaling procedures. An issue to consider in this process is the cell stability in respect to productivity. Decrease or loss of cell-specific productivity are unpredictable events reported for various cell lines, e.g., hybridoma, Chinese hamster ovary (CHO), and no-secreting (NS0) myeloma cell lines (Barnes et al. 2003). The underlying causes for such instability vary and include loss of genes, chromosome rearrangements, mutations, methylation of promoter, and silencing processes (Beckmann et al. 2012). Among the major concerns in the production of mAb and recombinant proteins in animal cells cultivation are the conditions in which the stability, safety, and quality of the product of interest is maintained. Besides the environmental conditions involved in cell cultures that affect the quality and concentration of the product of interest, there are still the concentration of dissolved oxygen in the culture medium, the temperature, pH levels, and nutrient supply. Such conditions are important in characterizing cell cultures and consequently for the understanding of cell stability as regards to quality and concentration necessary to obtain the cell growth profile and mainly their productivity (Li et al. 2006). Thus avoiding potential losses related to factors influencing cell cultures and the time of subcultures that might impact the required quality of the product of interest.

Literature reports that a population of hybridoma cells can present productive and unproductive subpopulations. Unproductive populations, i.e. non-producer of mAb, may present different cell growth profiles and even higher growth rate compared to cell populations of hybridomas producers of mAb (Roshni et al. 1999).

The increase in the number of cell subcultures performed over a time, or cell passages over a time (number of cell passages), could favor the growth of unproductive cell cultures, reduce productivity and reduce the mAb protective potential against infections (Xin and Cutler 2006; Zhu and Yang 2004).

Longer-term cultures might lead to an instability in the antibody production between different hybridoma cell lines (Barnes et al. 2003; Schmid et al. 1990). For instance, it has been reported that murine hybridoma strain AB-1432 lost 50 % of mAb productivity when cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum (FBS) as of P20 (Schmid et al. 1990). Whereas hybridoma B6.1 cells maintained at high passages reduced the protective capacity of their secreted mAb (Xin and Cutler 2006). The production instability in recombinant mAb in CHO cells can occur through gene silencing mechanism, or else the progressive loss in the number of copies of the recombinant gene, reducing mAb yield as of P14 or P95 (Beckmann et al. 2012; Kim et al. 2011).

Long-term instability can impact the productivity of proteins of interest and yet promote doubling of cell

populations which are non-mAb secretive. Such loss of production between initial and end-of-production cells might compromise regulatory approval and, in the worst-case scenario, could result in rejection of a particular cell line after months of wasted development effort (Barnes et al. 2003). In addition to cell line stability, growth and metabolite characteristics affecting process robustness and scalability also need to be assessed (Li et al. 2010). Despite the mAb tendency to lose productivity over a time, the limit in the number of passages that a given cell can be subjected to without impinging on its stability is singular to each cell line (Lee et al. 1991).

Regardless of cell line origins, studies in cell cultures and subcultures (passages) can alter cellular biochemical metabolism and the production of proteins. Considering cost-effectiveness, it is important to have a cell line secreting proteins or antibodies of interest for development of diagnostics kits (Barnes et al. 2003; Schmid et al. 1990; Xin and Cutler 2006; Beckmann et al. 2012; Kim et al. 2011; Li et al. 2010; Lee et al. 1991).

Materials and methods

Cell line and culture medium

Cultures employed a kind of hybridoma murine cells secreting mAbs, targeting the Penicillin binding protein PBP2a that is a transpeptidase enzyme located only on the surface of Methicillin-Resistant *Staphylococcus aureus*. These cells were developed at the Institute for Immunobiology Technology, Bio-Manguinhos, Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro/RJ; patent application number WO/2011/017791). Hybridoma cells were preserved in liquid nitrogen at −196 °C. After thawing, they were grown in T_{25} cm^2 flasks containing Dulbecco's Modified Eagle's Medium, working volume 5 mL (DMEM, Invitrogen®, Carlsbad, CA, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS, Sigma®, St. Louis, MO, USA), 3 mM glutamine (Merck®), ITS: 5 mg/L insulin, 5 mg/L transferrin, 5 µg/L selenium (Sigma®), 15 mM Hepes (Sigma®), sodium bicarbonate (Merck®, Darmstadt, Germany), 0.003 % (v/v) β-Mercaptoethanol (Sigma®), and 1 % (v/v) antibiotic solution: Penicillin—10,000 U, Streptomycin—10 mg, and Amphotericin—25 g (Sigma®).

Cell culture in static systems

Hybridomas MRSA anti-PBP2a mAb were cultured and subcultured in polystyrene flasks type T_{25} and T_{75} cm^2 containing DMEM medium supplemented with 10 % (v/v) FBS, with working volume 5 mL (T_{25} cm^2 flask) and working volume 20 mL (T_{75} cm^2 flask), respectively. They were kept at 37 °C with a moisty 5 % CO_2 atmosphere. An exchange of culture-medium was carried out every 2 days to maintain some supply of nutrients and pH control. The subcultures were performed every 3 days after mechanical detachment of hybridoma cells from culture flasks surfaces.

In the mid exponential phase of cell growth, the hybridoma cells in the passages 10, 15, 20, 30, 40, and 50 were transferred in sequence to 24-well polystyrene plates (Falcon®) in the concentration of 0.5×10^5 viable cells/mL with 1 mL per well, and taken to an incubator at 37 °C and a 5 % CO_2 atmosphere for 8 days. For each passage, cell cultures were carried out in biological duplicate, and the analytical determinations in quadruplicate, Student's T-test (Harris 2001) following suit. The average standard of analytical determinations and deviation values were compared with P10 (control passage) using the Student's T-test statistical reliability test with a margin of 95 % so as to verify the difference in the pattern of MRSA-antiPBP2a mAb secretion during cell subcultures.

Analytical methodology

Samples of 1 mL/well were collected daily and 200 µL were used for cell count in hemocytometer (Neubauer Chamber), through Trypan Blue exclusion at 0.4 % (w/v) (Freshney 1994).Then, 800 µL were centrifuged the remaining supernatant was frozen at −70 °C for later analysis of glucose (g/L) and lactate (g/L) in a biochemical analyzer type YSI 2700 (Yellow Spring Instruments), and quantification of MRSA-antiPBP2a mAb by direct enzyme immunoassay (ELISA; Senna et al. 2003).

The hybridoma cell cultures were performed in biological duplicate and the average standard of analytical determinations and deviation values were compared with the available younger cells maintained at P10 (control passage) using the statistical reliability Student's T-test (Harris 2001) with a margin of 95 % so as to verify the difference in the pattern of MRSA-antiPBP2a mAb secretion during cell subcultures.

Determination of kinetic variables and cellular metabolism

The cell specific growth rate, constant during exponential growth phase (μ_{exp}), was determined by the slope of the line adjusted to Natural Logarithm variations of cell concentration in relation to time, during the exponential growth phase of the cell culture, according to Eq. 1.

$$\mu_{exp} = \frac{1}{X}\frac{dX}{dT} \quad (1)$$

Specific production rates of monoclonal antibody (q_{mAb}), of glucose consumption (q_{Gluc}) and of lactate formation (q_{Lac}) were determined by the relationship between their average values and the integral of viable cell concentration over time (ICV) as per Eqs. 2, 3, and 4, respectively (Chen et al. 2012).

$$q_{mab} = \frac{1}{X_v}\frac{d[mab]}{dt} \quad (2)$$

$$q_{Gluc} = \frac{1}{X_v}\frac{d[Gluc]}{dt} \quad (3)$$

$$q_{Lac} = \frac{1}{X_v}\frac{d[Lac]}{dt} \quad (4)$$

Results and discussion

Growth kinetics

The cell cultures performed between passages P10 and P20 had similar growth profiles and cell death (Fig. 1). At the end of the second day of cultivation, P10, P15, and P20 reached maximum concentrations of $3.2 \pm 0.5 \times 10^5$ viable cells/mL, approximately. The kinetics at P30 showed a lower concentration of viable cells/mL of $2.6 \pm 0.6 \times 10^5$ at the second day (Fig. 1) showing a 1.23 times reduction compared to former P10 and P20 at the same day.

1.23 times corresponds to a reduction of 18.75 % of viable cells, showing relevant alteration in the cell cycle for the synthesis of new cells (Fig. 1). Last but not least, it showed a significant reduction in the production of mAbs once the maximum concentration of MRSA-antiPBP2a mAb produced at P30 reached 2 μg/mL, approximately, and the maximum concentration of MRSA-antiPBP2a mAb produced in P15 e P20 was of 7 μg/mL, approximately, both at the fourth day. In P15 and P20, the concentration of MRSA-antiPBP2a mAb was 3.5 times bigger; corresponding to an increase of 28.57 % in the concentration of mAb in P30 also at the fourth day.

Significant differences in cell growth and production of MRSA-antiPBP2a mAb profiles occurred as of P20. In P40 there was an extension of the exponential phase of cell growth until the fifth day of culture, when the viable cells/mL maximum concentration reached $2.3 \pm 0.7 \times 10^5$, as shown in Fig. 1E.

No change was observed in the cell growth specific rate in the cultivation of hybridoma cells in passage P40 which was of $\mu = 0.8 \pm 0.1$ day^{-1}. These values are quite different from the values of passages P10–P20 at the second day of culture, showing that cell metabolism was altered (Fig. 1).

There were two significant reductions in P50, namely in the maximum concentration of $1.1 \pm 0.1 \times 10^5$ viable cells/mL, and cell specific growth rate of $\mu = 0.3 \pm 0.1$ days^{-1}, when compared to other cultures performed at different passages, as shown in Fig. 2A, B. It demonstrated that, as time and passages passed, cultures of hybridoma secreting MRSA-antiPBP2a mAb showed alterations in cell concentrations (Fig. 2A) and specific growth rate (Fig. 2B).

P30 showed a higher specific growth rate of 1.03 ± 0.27 day^{-1} (Fig. 2B), but mAb (μg/mL) total concentration dropped 28.9 %. Besides, total concentration of mAb of 41.2 μg/9 mL at P20 dropped to 11.9 μg/9 mL at P30 (Fig. 2C).

The initial cell viability used to perform the initial inoculum of all hybridoma cell cultures performed in all passages was >80 % (data not shown).

"T-Student" reliability test (Harris 2001) was applied to the values of viable cells/mL, to the specific concentration of mAb/mL, to the cell growth specific rate, and finally to the specific production rate of mAb (Table 1). "T-Student" values greater than "tabulated" T-values (4.303), applied for data in Fig. 2, show significant differences when compared with the control sample (P10), with statistical reliability of 95 % (lines).

Schmid et al. (1990) noted that in murine hybridoma AB2-143.2 strain grown in 70 mL working volume in a shaker system increased in the maximum number of viable cells/mL between passages P5 and P57, there was an increase in the maximum number of viable cells/mL. Characterization of every different cell lineage is paramount because upon studying the

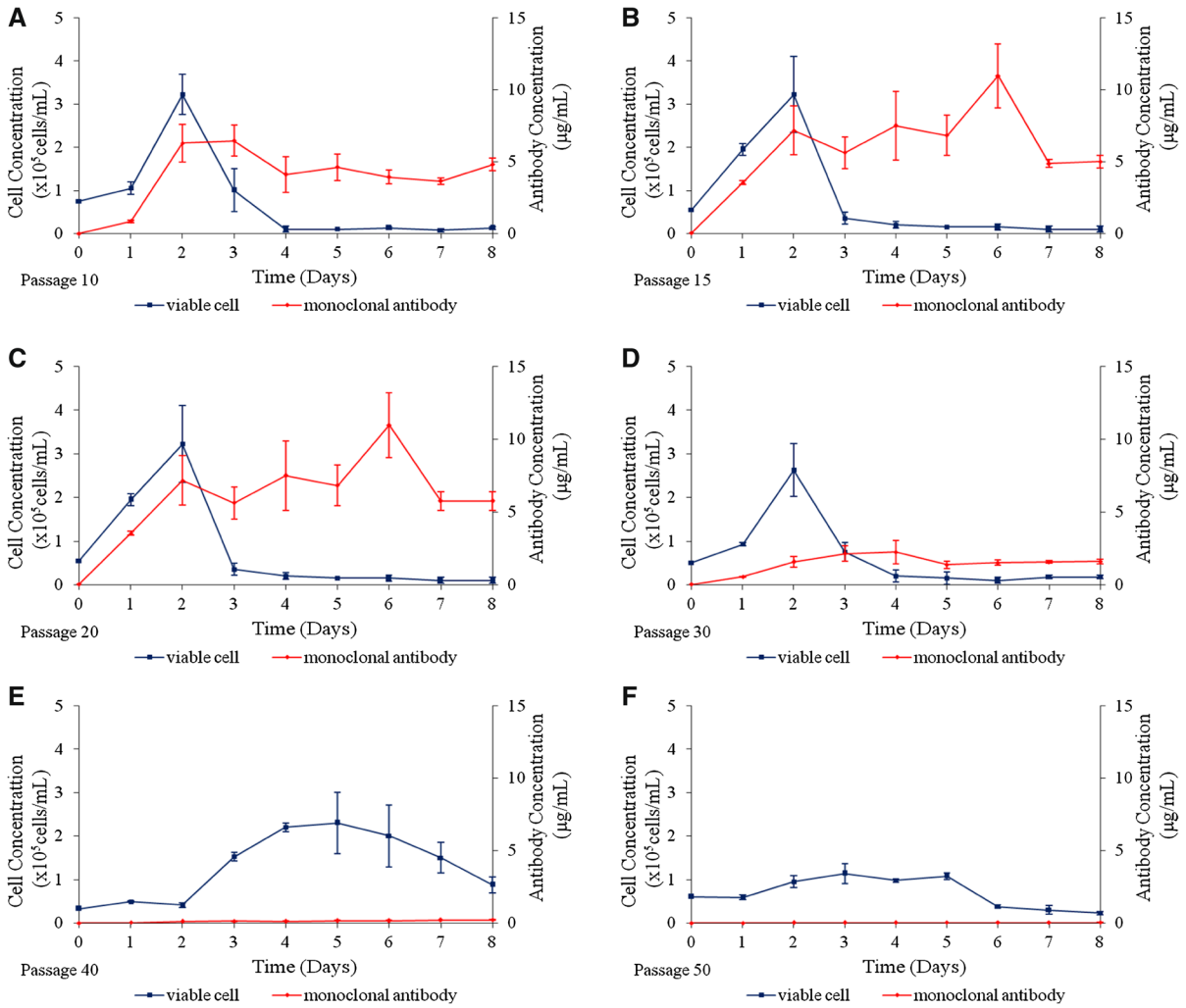

Fig. 1 Concentration of viable cells/mL and the concentration of MRSA-antiPBP2a mAb µg/mL obtained in cell cultures carried out in polystyrene plates at different passages: **A** P10, **B** P15, **C** P20 **D** P30, **E** P40, and **F** P50

line of hybridoma secreting MRSA-antiPBP2a mAb, the opposite outcome was observed, i.e., a decrease in the number of viable cells/mL. Such decrease until P20 is relevant for future optimization of cell bioprocesses.

A reduction of specific growth rate in the cultures of hybridoma MRSA-antiPBP2a mAb was not reported for other cell lines (Li et al. 2006).

Characterization of different cell lines need to be obtained, as per present work, with hybridoma secreting MRSA-antiPBP2a mAb. Thus, knowledge about cell growth patterns and secretion of the antibodies of interest are relevant data. Once identified, the data shall serve future steps in antibody production, namely, the optimization of bioprocesses and establishment of cell banks.

Monoclonal antibody production

The specific productivity of MRSA-antiPBP2a mAb showed an inverse increase to the passage number, being the highest antibody production specific rate observed in P10, 2.2 ± 0.5 pg/cell*day (Fig. 2D).

The specific productivity of MRSA-antiPBP2a mAb in cultures performed between passages P10 and P50 ranged from 2.2 ± 0.5 pg/cell/day (P10) to

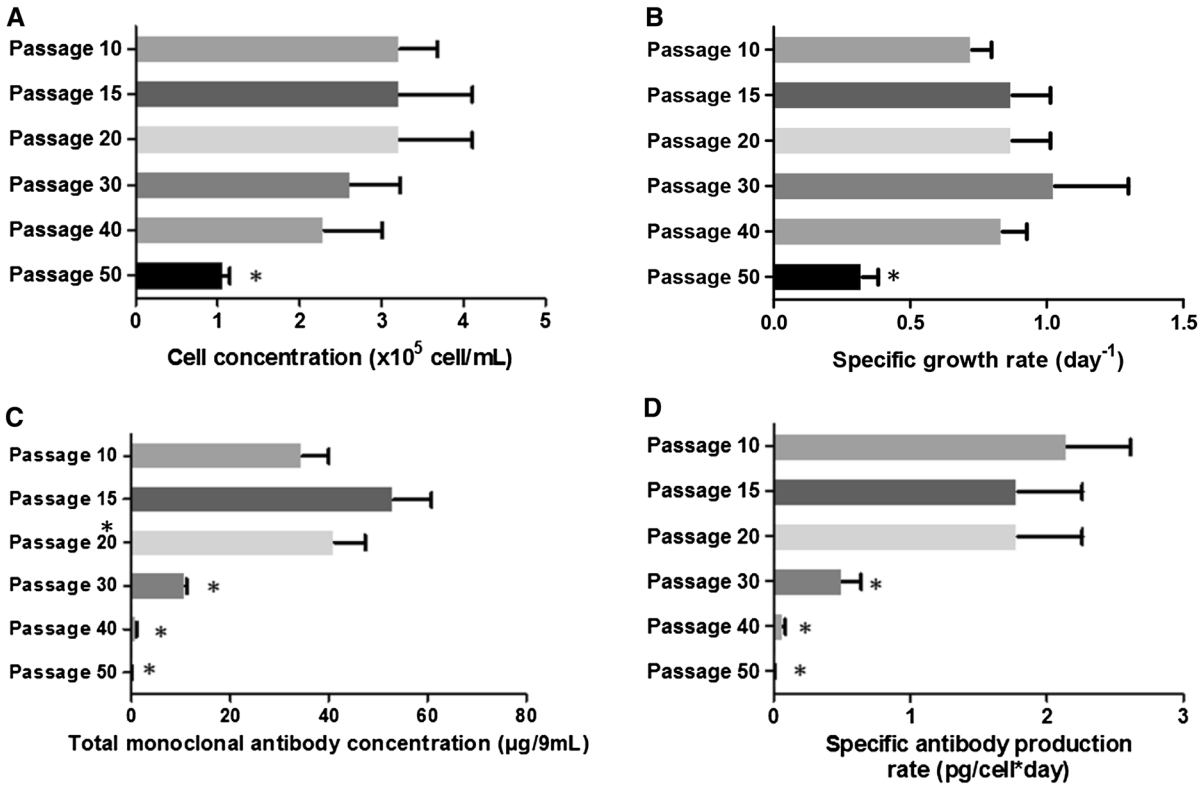

Fig. 2 **A** Maximum cell concentration ($\times 10^5$ viable cells/mL). **B** Specific growth rate (day^{-1}), **C** total monoclonal antibody concentration (µg/9 mL), and **D** Specific antibody production rate (pg/cell*day)

Table 1 Specific glucose consumption rate (q_{Gluc}), specific lactate formation rate (q_{Lac}), cell yield coefficient with respect to glucose consumption ($Y_{XV/gluc}$), mAb yield coefficient of monoclonal antibody in relation to glucose consumption ($Y_{mAb/gluc}$), and yield coefficient of lactate formation relative to glucose consumption ($Y_{Lac/Gluc}$) of hybridoma cells cultured in polystyrene plates in the passages: P10, P15, P20, P30, P40 and P50

	Q_{Gluc} (pg/cell/day)	q_{Lac} (pg/cell/day)	$Y_{Xv/gluc}$ (10^5 cell/g)	$Y_{mAb/gluc}$ (µg/g)	$Y_{Lac/gluc}$ (g/g)
Passage 10	0.182 ± 0.004^a	0.145 ± 0.002^a	3.4 ± 0.6^a	9 ± 2^a	0.82 ± 0.01^a
Passage 15	0.171 ± 0.006^a	0.179 ± 0.001^b	3.3 ± 0.9^a	7 ± 1^a	0.83 ± 0.02^a
Passage 20	0.393 ± 0.006^b	0.179 ± 0.001^b	3.6 ± 0.9^a	7 ± 2^a	0.88 ± 0.03^a
Passage 30	0.230 ± 0.001^c	0.144 ± 0.007^a	3.8 ± 0.6^a	3 ± 1^b	0.72 ± 0.02^b
Passage 40	0.279 ± 0.009^d	0.110 ± 0.001^c	2.5 ± 0.9^a	0.25 ± 0.01^c	0.52 ± 0.01^c
Passage 50	0.219 ± 0.005^e	0.203 ± 0.002^d	0.6 ± 0.1^b	0.024 ± 0.001^d	0.36 ± 0.03^d

Averages in the same column with different superscripts showing significant differences among the different passages of cell culture ($p < 0.05$)

0.007 ± 0.001 pg/cell/day (P50). The specific production rate of anti-PBP2a mAb was constant from the beginning of culture until 1 day after the end of the exponential phase of cell growth when the maximum concentration of MRSA-antiPBP2a mAb reached 0.02 ± 0.01 µg/mL (P50), and 6.5 ± 1.1 µg/mL (P10). These levels remained unmodified until the last day of culture (Fig. 1).

Both the total MRSA-antiPBP2a mAb concentration (40 µg/9 mL), and specific antibody production rate (2.0 ± 0.5 pg/cell/day, approximately), achieved at P20 did not differ significantly from the values

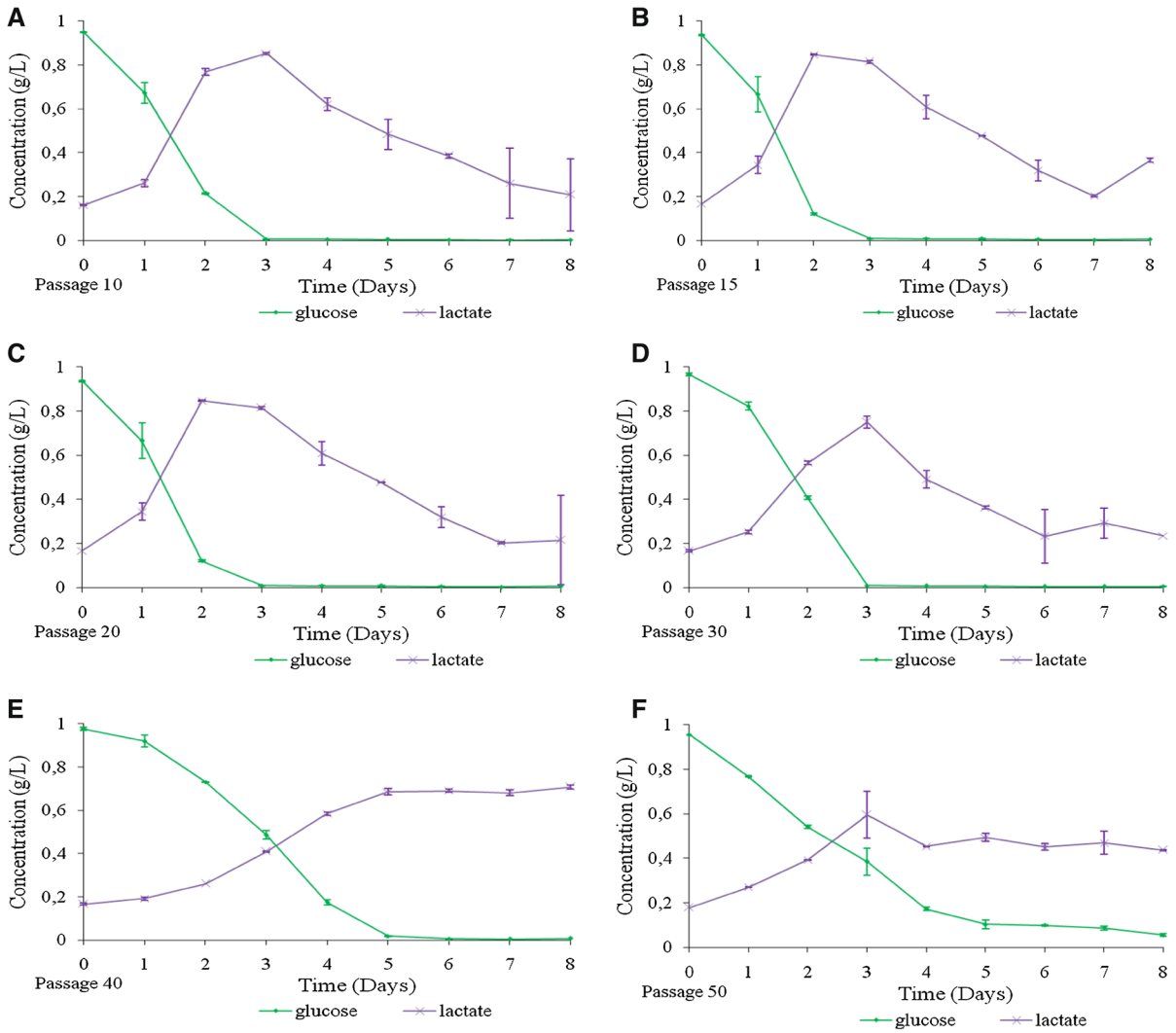

Fig. 3 Glucose and Lactate concentration in cultures of MRSA's hybridomas anti-PBP2a mAb performed in polystyrene plates at different passages: **A** P10, **B** P15, **C** P20, **D** P30 **E** P40 and **F** P50

obtained for the same variables studied in cell cultures performed at P10 and P15, as shown in Fig. 2C, D.

There was a reduction of about 3.6 times in the concentration of MRSA-antiPBP2a mAb observed in the cultures as of P30 (Fig. 2C). Hybridoma cells producing MRSA-antiPBP2a mAb showed a too early decline in productivity. This is similar to what Lee et al. (1991) reported upon studying other hybridoma cell lines, different from the MRSA-antiPBP2a mAb producing hybridomas, which had also lost production ability of around 10^{-2} to 10^{-5} per cell generation. HAb18 hybridoma cells reduced the antibody productivity at 15 % after 3 months of subculture (Li et al. 2006). These results show that the characterization of cell cultures at different passages is paramount and must be considered when large-scale production is needed.

Hybridomas secreting murine monoclonal antibodies against *Neisseria meningitidis* capsular polysaccharide (MCPS) showed a reduction in the oxygen consumption rate and an increase in the specific lactate formation rate as of passage P50. This fact led to a decrease in the energy supply needed for cell development, consequently leading to a drop in the cell growth specific rate, as reported by Vallejos et al. (2009).

Maximum concentration of viable cells/mL at the second day of culture (Fig. 1A–C) and mAb (μg/mL) at the eighth day (Fig. 2D) were similar at P10, P15 and P20.

As mentioned before, reductions in the mAb productivity (Li et al. 2006) as well in cell growth (Vallejos et al. 2009) show that different cell lines can behave differently. Data obtained for MRSA-antiPBP2a mAb producing hybridoma cells which showed high specific mAb antibody production rate until P10 (Fig. 2D) but a variable rate between P10 through P20 (Fig. 2D), within the error, demonstrated that different passages (P10 through P20) did not compromise cell growth (Fig. 2A) neither the specific mAb production rate (Fig. 2D).

Metabolism

Difference in primary metabolism was assessed through analysis of glucose consumption and lactate production, as shown in Fig. 3.

Nutrient shortage as well as accumulation of toxic metabolites are important factors that can influence cell growth (line). Lactate concentrations greater than 3 g/L can inhibit cell growth (Legazpi et al. 2005). High-values for the yield of lactate formation ($Y_{Lac/Gluc}$) are usually an indication that the cells may be in a state of less energetic efficiency, and can adversely affect productivity and quality of the product of interest (Vallejos et al. 2009; Acosta et al. 2007). The maximum concentration of lactate obtained did not exceed 0.9015 g/L during cultures performed at passage P20, for example. The inhibition of cell growth may be caused by shortage of nutrients such as glucose, whose exhaustion occurred as of the third day of culture. Despite the culture medium had been supplemented with glutamine, the lack of this amino acid can also be limiting as verified by Schmid et al. (1990) who cultured hybridoma cells AB2-143.2 in culture medium supplemented with 13.5 mM/L glucose and 4.9 mM/L glutamine.

The efficiency of glucose consumption, however, is not reflected in the increase in maximum concentration and specific productivity of MRSA-antiPBP2a mAb. Table 1 shows the Metabolic parameters determined during the exponential growth phase of hybridomas secreting MRSA-anti-PBP2a mAb grown in microtiter plate at different passages.

The conversion rate of glucose in cells, the yield per cell, and $Y_{XV/Gluc}$, were kept constant in the cultures performed between P10 and P40. However, glucose consumption rate used in the production of mAb (yield—$Y_{mAb/Gluc}$) was similar at P10, P15 and P20 (Table 1), showing higher monoclonal antibody concentration (μg/9mL) until P20 (Fig. 2C). There were still reductions in mAb yield coefficient in relation to glucose consumption ($Y_{mAb/Gluc}$) as of P30, and in the cell yield coefficient with respect to glucose consumption ($Y_{xv/Gluc}$) as of P40 (Table 1).

Reductions in rates and yields above show that cells can suffer harmful interferences, as passages run, for the production of mAb as, already in P30, its concentration did not reach the maximum of 2 μg/mL at the fourth day of culture (Fig. 1D), total mAb production was 10 μg/9 mL (Fig. 2C), and specific antibody production rate was 0.5(pg/cell/day; Fig. 2D).

Conclusions

Patterns of secretion of murine MRSA anti-PBP2a mAb were found in hybridoma cells sub-cultured at different passages. The concentrations of viable cells/mL were similar in hybridoma cells in P10, P15 and P20. The highest total MRSA anti-PBP2a mAb concentrations were obtained in passages P15 and P20. These results indicate that the MRSA anti-PBP2a mAb producing hybridoma cells must be cultivated until P20 so as not to impair cell growth and total production of mAb.

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