Approach toward an efficient inoculum preparation stage for suspension BHK-21 cell culture

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Abstract Mammalian cells are the most frequently used hosts for biopharmaceutical proteins manufacturing. Inoculum quality is a key element for establishing an efficient bioconversion process. The main objective in inoculation expansion process is to generate large volume of viable cells in the shortest time. The aim of this paper was to optimize the inoculum preparation stage of baby hamster kidney (BHK)-21 cells for suspension cultures in benchtop bioreactors, by means of a combination of static and agitated culture systems. Critical parameters for static (liquid column height: 5, 10, 15 mm) and agitated (working volume: 35, 50, 65 mL, inoculum volume percentage: 10, 30 % and agitation speed: 25, 60 rpm) cultures were study in T-flask and spinner flask,

respectively. The optimal liquid column height was 5 mm for static culture. The maximum viable cell concentration in spinner flask cultures was reached with 50 mL working volume and the inoculum volume percentage was not significant in the range under study (10–30 %) at 25 rpm agitation. Agitation speed at 60 rpm did not change the main kinetic parameters with respect to those observed for 25 rpm. These results allowed for a schedule to produce more than 4×10^9 BHK-21 cells from 4×10^6 cells in 13 day with 1,051 mL culture medium.

Keywords Inoculum preparation · Mammalian cells · BHK-21 · Static cell culture · Suspension cell culture · Spinner flask · Bioreactor

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Introduction

Mammalian cells are the most frequently used hosts for biopharmaceutical proteins manufacturing, 66 % of the revenue being from those produced in different mammalian cell lines. The majority of approved proteins have utilized rodent cell lines like: Chinese hamster ovary (CHO), baby hamster kidney (BHK) and mouse myeloma (NS0) cells. All of them can be grown in suspension culture (Berlec and Štrukelj 2013). Besides, mammalian cell culture is used widely in lab scale for performing researches about several diseases as well as new drug screening and safety testing (Griffiths and Wurm 2003). Specially, BHK-21 has also been employed extensively for veterinarian viral vaccines (foot-and-mouth disease virus and rabies virus) since 1960s (Auninš 2010).

The development of suspension culture systems, in most of the cases, allows high cell concentrations and volumetric yields, as well as facilitates the scale-up. Inoculum quality, culture medium composition, proper definition and control of bioreactor operational parameters are key elements for establishing an efficient bioconversion process (Griffiths 2010).

Inoculum preparation passes through several stages, starting from frozen vial with few milliliters of the cell line and moving to the production fermentation volume (Sood et al. 2011; Junker 2007). Cell cultures in mid-exponential growth are preferred for transfer to the next stage in order to minimize the lag phase (Junker 2007). The main objective in inoculation expansion process is to generate large volume of viable cells in the shortest time (Kloth et al. 2010; Seth et al. 2012). In that sense, many factors must be considered such as: media and supplement storage, media preparation, transfer parameters for the flask expansion (container usage, volume range, splitting schedule as well as range for seeding and transfer cell densities) and equipment setting (Kloth et al. 2010). For animal cells, the inoculum transfer time and volume are in the 5–10 days and 5–10 % ranges, respectively (Junker 2007).

Mammalian cells in a freezing mixture are usually stored in liquid nitrogen vessels. In general, freezing mixtures are composed of heat inactivated serum, like fetal bovine serum (10–95 % v/v final concentration) in conjunction with cryoprotectants like dimethyl sulfoxide (DMSO) and conditioned culture medium.

In order to get highest viable cell concentration, a rapid thaw above 20 °C/min is recommended (Kloth et al. 2010).

After thawing, the suspension cells are maintained in static and agitated cultures or a combination of both. The free shear-stress environment in static cultures (T-flask or Nunc cell factories) may be advantageous immediately after thaw. Nevertheless, the cell attachment to the plastic surface, cell aggregation and the ineffective gas diffusion are limiting factors of these culture systems (Kloth et al. 2010). Agitated cultures are superior to static flask cultures because of maintaining homogenous environment for the cells, like pH, nutrients concentration and temperature, as well as improving better gas diffusion (Kloth et al. 2010).

The aim of this paper was to optimize the inoculum preparation stage of BHK-21 cells for suspension cultures in benchtop bioreactors, by means of a combination of static and agitated culture systems.

Materials and methods

Cell line, culture medium, freezing mixture and procedure

BHK-21 (C-13) cells (Sigma-Aldrich ECACC Cell Lines, Lyon, France) adapted to single cell suspension culture were kindly supplied by Dr. Renaud Wagner from Ecole Supérieure Biotechnologie de Strasbourg (France). The culture medium used had the following composition by volume: Iscove's Modified Dulbecco Medium with glutamine and phenol red (IMDM, catalog number 12200-036, Gibco, Grand Island, NY, USA) 45,5 %, High glucose Dulbecco's Modified Eagle Medium (DMEM, catalog number 12100-046, Gibco) 45,5 %, heat inactivated fetal bovine serum (Catalog number 10082-147, Gibco) 5 %, 10 % m/v Pluronic F-68 (Sigma-Aldrich, St. Louis, MO, USA) aqueous solution 2 %, and 4 mM glutamine (Sigma-Aldrich) aqueous solution 2 % (Fernández Núñez et al. 2013).

The freezing mixture was formulated with fresh culture medium as described above (45 % v/v), cells in conditioned medium (at the end of growth phase in spinner flask) (45 %) and dimethyl sulfoxide (Catalog number D 2650, Sigma-Aldrich) (10 %). The cell concentration in the freezing mixture was 2×10^6 - cell mL⁻¹. The mixture was stored in 2 mL

cryopreservation tubes (Corning Incorporated, Monterrey, Mexico). The rate freezing protocol was: after filling cryopreservation tubes, samples were kept at $-20\text{ }^{\circ}\text{C}$ (349-FV Plasma freezer, Fanem, Brazil) for 12 h, then they were transferred to $-80\text{ }^{\circ}\text{C}$ (Revco ULT 1386-3-D39, Kendro Laboratory Products, Newtown, CT, USA) for 12 h and finally they were stored in nitrogen liquid tank ($-196\text{ }^{\circ}\text{C}$).

Culture in 25 and 75 cm² T-flask

Two milliliter of cryopreserved BHK-21 cell line was thawed ($22\text{ }^{\circ}\text{C}/\text{min}$) and added to 30 mL of culture medium in 75 cm² T-flask (vertical position, in order to reduce cell adherence to treated surface of the flask, and as a consequence, to avoid enzymatic cell detachment) (TPP, Trasadingen, Switzerland). After 96 h in culture ($0.8 \times 10^6\text{ cell mL}^{-1}$), this cell suspension was used as inoculum ($2.5 \times 10^5\text{ cell mL}^{-1}$) in 25 cm² T-flask (TPP) (vertical position) for studying the influence of liquid column height (5, 10 and 15 mm) on maximum viable cell concentration (X_{max}), maximum specific growth rate (μ_{max}) and cell viability for static culture. Each condition was performed in triplicate and monitored during 120 h.

Another experiment was carried out to corroborate the significance of liquid column height (5 and 10 mm) in 75 cm² T-flask in vertical position. The post-inoculation time under study was 72 h. The inoculum was provided from the previous 25 cm² T-flasks, it was taken at the end of the exponential phase.

Culture in 100 mL spinner flask

In the spinner flasks culture stage (100 mL, Bellco Glass Inc., Vineland, NJ, USA), a multilevel factorial experimental design was performed to assess the significance of working volume (35, 50, 65 mL) and inoculum volume percentage with respect to the working volume (10 and 30 %) on the same kinetic parameters studied in the previous stage. Initial concentration was $2.5 \times 10^5\text{ cell mL}^{-1}$. For 10 % inoculum volume percentage was necessary inoculum centrifugation (750g for 4 min) (Sorvall Biofuge Primo R Centrifuge, Thermo Electron Corp., Germany) in order to concentrate cells and decrease toxic metabolites, the remaining volume was discharged. The inoculum for spinner flasks was generated in

75 cm² T-flask. The agitation speed was controlled at 25 rpm (Four position magnetic stirrer, Sci Era, Quad drive system, Bellco Biotechnology, Vineland, NJ, USA). All experiments were performed at $37\text{ }^{\circ}\text{C}$ and 5 % CO₂ atmosphere (Thermoforma 3110 incubator, Marietta, OH, USA).

The influence of agitation in spinner flasks was also studied at 60 rpm with 50 mL working volume and 30 % of inoculum volume.

Culture in benchtop bioreactor

Batch experiments in bioreactors were carried out in a 2 L Bioflo 110 reactor (New Brunswick Scientific, Edison, NJ, USA) with a working volume of 1 L, at $37\text{ }^{\circ}\text{C}$, 80 rpm. The initial cell concentration was $0.25 \times 10^6\text{ cell mL}^{-1}$ and dissolved oxygen and pH was controlled at 50 % air saturation and 7.2, respectively, over the course of the batch culture (103 h). The inoculum for this benchtop bioreactor was prepared from spinner flask cultures at the end of the growth phase ($4 \times 10^6\text{ cell mL}^{-1}$). Retrospectively, a cryopreservation vial with 2 mL cell suspension was thawed and added to 30 mL culture medium for 96 h. Then, a 75 cm² T-flask was inoculated with $2.5 \times 10^5\text{ cell mL}^{-1}$ and cells were cultured for 72 h. This cell suspension was used to inoculate a spinner flask with 50 mL working volume at 25 rpm. The inoculum for bioreactor was taken at 72 h post-inoculation.

Cell counting, maximum specific growth rate, glutamine and glucose quantification

The cells were counted using an improved Neubauer counting chamber (Precicolor HBG, Giessen-Lützelinden, Germany) with proper sample dilution with Phosphate Buffer Saline (0.13 M NaCl, 2 mM KCl, 8 mM Na₂HPO₄, 15 mM KH₂PO₄). Viable cells were quantified in parallel using the trypan blue exclusion method (Augusto et al. 2010).

Maximum specific growth rate, μ_{max} , was calculated by plotting natural logarithm of viable cell concentration versus time during exponential phase (Augusto et al. 2010).

Proportional samples at different times during kinetics studies in static and agitated systems were taken. They were centrifuged at 750g for 4 min (Sorvall Biofuge Primo R Centrifuge, Thermo

Electron Corp., Germany). Subsequently, supernatants were filtered through 0.22 μm filter (Millex-GV filter unit, Sao Paulo, Brazil) and frozen at $-20\text{ }^{\circ}\text{C}$ until analysis of nutrients and metabolites.

Glutamine and glucose concentrations from supernatant samples were measured using enzyme-coupled reaction and electrochemical detection, in a YSI 2700 Select Bioanalyzer (YSI Life Sciences, Yellow Springs, OH, USA).

Statistical data analysis

Multilevel factorial design analysis and sample average comparisons (analysis of variance (ANOVA), Tukey's multiple range test and Student t test) were performed in Statgraphics Plus 5.0 (Statistical Graphics, Fairfax, VA, USA). The statistical significance level (α) for decisions was 0.05. The other graphic data treatments were carried out in Microsoft Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA).

Results

Static culture in T-flasks

The cell growth experiments performed in 25 cm^2 T-flask allowed for determining the influence of culture medium height on μ_{max} and X_{max} . The parameters: μ_{max} ($p = 0.00004 < 0.05$, ANOVA) and X_{max} ($p = 0.00004 < 0.05$, ANOVA) were significantly influenced by the liquid column height in static culture (Fig. 1a). The relationship between μ_{max} and liquid column height was inverse (as expected). An increase of 69 and 17 % for μ_{max} was observed from 15 to 10 mm and from 10 to 5 mm liquid column height, respectively. The growth phase for static culture performed at 5 mm liquid column was characterized by two intervals; this finding was confirmed by other experiments in our lab (data not shown). Then, the exponential phase was defined as the interval with highest value of maximum specific growth rate (Fig. 1a). The X_{max} for 10 and 15 mm liquid column height were similar, whereas this cell growth parameter was 52.7 % higher for that confirmed in 5 mm liquid column height (Tukey's test) (Fig. 1a). On the other hand, cell viabilities for the three liquid column heights were kept at values higher

Fig. 1 Influence of liquid column height at static culture on BHK-21 cells growth kinetic parameters. **a** Cell growth curve. **b** Viability profile. Experiments were performed in 25 cm^2 T-flask (vertical position). Number of repetitions: 3. Error bars represent standard deviation

Table 1 Influence of liquid column height in static culture on cell concentration ($C_{x\text{ mm}}$, cell mL⁻¹) at 72 h post-inoculation

Repetition	Liquid column height	
	5 mm	10 mm
1	1.83×10^6	1.16×10^6
2	2.25×10^6	1.30×10^6
3	2.00×10^6	1.31×10^6
Average	2.03×10^6	1.26×10^6
Standard deviation	2.14×10^5	8.32×10^4

Experiments were performed in 75 cm^2 T-flask (vertical position). Number of repetitions: 3. Comparison of sample averages (Student t test, $C_{5\text{ mm}} > C_{10\text{ mm}}$ $p = 0.002$)

than 97 % over the course of the 120 h cell culture time (Fig. 1b).

The cell concentration at the end of the exponential phase in static cultures performed in 25 cm^2 T-flask (72 h post-inoculation) for 10 mm liquid column height (Fig. 1a) was confirmed in a 75 cm^2 T-flask,

Table 2 Summary of multilevel factorial design for identifying significant effects of working volume and inoculum volume percentage on some kinetic parameters in cell cultures performed in spinner flask (100 mL, 25 rpm)

Working volume (mL) (A)	Inoculum (%) (B)	$X_{\max}$ (10^6 cell mL^{-1})	$\mu_{\max}$ (day^{-1})	$V > 90$ % (day)	Final pH
35	10	3.39	0.792	4	5.99
35	30	3.27	0.897	4	6.08
50	10	4.10	0.852	4	6.47
50	10	4.10	0.934	4	5.90
50	30	4.16	0.879	4	6.15
65	10	3.49	0.865	5	6.28
65	30	3.66	0.851	3	6.40

Fig. 2 Statistical analysis for maximum viable cell concentration response (spinner flask culture system). **a** Standardized Pareto chart for maximum viable cell concentration. **b** Main effects plot for maximum viable cell concentration. **c** Cell growth kinetics corresponding to a working volume of 50 mL and an inoculum volume corresponding to 30 % of the working volume. Experiments were performed in 100 mL spinner flask at 25 rpm. AA and AB represent the quadratic term of parameter A, and interaction of parameters A and B, respectively

with scale increase factor of three (Table 1) ($p = 0.7021$, Student t test). Nevertheless, a significant increase of 27 % was detected for this parameter ($p = 0.0278$, Student t test) when the same scale increase factor for a 5 mm liquid column height was used (Fig. 1a; Table 1). As a consequence, the relative decrease of cell concentration with liquid column height increase was also confirmed (Table 1).

Agitated culture in spinner-flasks

The working volume (A) and inoculum volume percentage with respect to working volume (B) were not significant in statistical modeling for $\mu_{\max}$ and bioconversion time with higher than 90 % cell viability ($V > 90$ %). The $\mu_{\max}$ values for the experiment performed in spinner flasks were in the 0.792–0.934 day^{-1} range (Table 2); they were higher than those for static culture (Fig. 1a). Post-inoculation time with cell viability higher than 90 % oscillated between 3 and 5 days for all evaluated combinations between A and B under study (Table 2). However, the $X_{\max}$ was statistically influenced by A (directly), its quadratic interaction (inversely) and its interaction with B (directly) (Fig. 2a). Among assessed combinations of A and B at 25 rpm agitation, A = 50 mL and B = 10 or 30 % ensured the highest $X_{\max}$ (4.10 – 4.16×10^6 cell mL^{-1}) (Fig. 2b; Table 2). This cell growth parameter was two-fold higher than that observed for 5 mm liquid column height in experiments performed using static culture (Fig. 1a; Table 2).

The exponential growth phase was defined between the first and the third day, during this period, the cell viability was higher than 98 %. For A = 50 mL and

Fig. 3 Cell growth kinetics in a spinner flask. They were operated at 60 rpm, working volume 50 mL and inoculum volume percentage 30 %. Three repetitions were performed in 100 mL spinner flask. **a** Cell growth and viability curves. **b** Glutamine and glucose consumption. Error bars represent standard deviation

B = 30 %, one of the best combinations in order to obtain high $X_{\max}$ values, the cell viability decreased extensively until 37 % from day four of cell culture to day six (Fig. 2c). At the end of the cell culture, the pH was always lower than 6.5 for this block of experiments.

The experiments performed in spinner flask (A = 50 mL and B = 30 %) at 60 rpm showed analogous $\mu_{\max}$ values analogous with those observed for 25 rpm (Table 2; Fig. 3a). Cell growth and viability profiles at 25 and 60 rpm were almost similar. The main difference was the absence of deceleration phase in the cell growth curve for the run carried out at 60 rpm (Table 2; Fig. 3a). The end of exponential growth phase was 2.9 days after inoculation and $X_{\max}$ was reached at the same moment ($3.26\text{--}4.16 \times 10^6 \text{ cell mL}^{-1}$) (Fig. 3a). The total glutamine consumption was associated with the end of the

exponential growth phase. Seventy % of glucose was metabolized by the cells at the end of the cell culture (Fig. 3b).

Agitated culture in benchtop stirred tank bioreactor

Ten days from cryopreservation tube thawing were necessary to the prepare inoculum for this benchtop bioreactor (Table 3). The $\mu_{\max}$ in bioreactor experiments was at least 86.2 % higher than the values observed in the spinner flasks (Table 2; Figs. 3a, 4a). On the other hand, the end of exponential phase was earlier in the bioreactor (2 days) with respect to the spinner flask runs (3 days) (Figs. 2c, 3a, 4a). The $X_{\max}$ was $4.39 \pm 0.21 \times 10^6 \text{ cell mL}^{-1}$ at 3 days after inoculation. The glutamine and glucose in the culture broth were exhausted. The end of exponential phase was associated with the moment of the cell culture when glutamine concentration was reduced to 0 mg L⁻¹. The beginning of death phase was related to the total glucose consumption (Fig. 4a, b).

Discussion

No matter if biotechnology professionals are facing lab or industrial challenges with mammalian cells; the shortest time from cell thaw to proper cell population is desirable. In the present work an inoculum preparation strategy was developed to reach a benchtop bioreactor considering these factors.

At large scale, there are two main methods for cell expansion, the conventional seed train expansion which uses a seed train bioreactor operating in continuous mode over the course of manufacturing campaigning and the other one is the frozen accelerated seed train for execution of a campaign (FASTEC). FASTEC is based on freezing large quantities of mammalian cells taken from perfusion bioreactors in cryobag (50–100 mL) and thawing those directly into an inoculation bioreactor (Seth 2012). However, a cell bank ampule will be the true beginning for both methods.

In our study case, no additional heat inactivated fetal bovine serum in the cell preservation medium was used, its original proportion in the cell culture medium (5.0 %) was maintained. Historically, fetal bovine serum concentrations for cryopreservation mixtures are usually defined in a 10–95 % (v/v) range (Kloth et al. 2010; Kleman et al. 2008). This change

Table 3 Schedule for 1 L bioreactor inoculation from a cryovial

Operation	Culture system (unit number)	Addition of fresh culture medium (mL)	Inoculum dilution in final volume of the culture system	Time (day)
Thaw	–	–	–	Not considered
IE-1 ^a (Static culture)	75 cm ² T-flask (1)	30.0	16.0	4
IE-2 (Static culture)	75 cm ² T-flask (2)	20.6	3.2	3
IE-3 (agitated culture)	Spinner flask (2)	70.0	3.3	3
IE-4 (agitated culture)	Bioreactor (1)	930.6	14.4	3
Total		1,051.2		13

^a IE means inoculum expansion **Fig. 4** Cell growth kinetics in a benchtop stirred tank bioreactor. It was operated at 80 rpm, 37 °C and 1 L working volume. Two repetitions were performed. **a** Cell growth and viability curves. **b** Glutamine and glucose consumption. Error bars represent standard deviation

has a positive economic and regulatory impact for the cell banking procedure. Besides, 55 % of the conditioned culture medium (medium in which the cells have been culture for a period of time) was also used in freezing mixture. Conditioned culture medium contains metabolites, growth factors, platelet-derived

growth factors and extracellular proteins that were secreted by the cells (Freshney 2005). These substances facilitate cryoconservation and have a helpful effect on the cell viability post thaw (Muller et al. 2004, GIBCO Cell Culture protocols, SAFC Biosciences). It is noteworthy to note that in order to ensure reproducibility for this cryopreservation step it is necessary to keep constant the moment of growth kinetic in which cells are withdrawn for freezing. This guarantees similarity in the chemical composition of cryoconservation culture medium.

After cell thawing, DMSO concentration was 0.63 % (v/v). This parameter is important in order not to reach cell inhibitory concentrations. The limit of tolerated DMSO concentration after thawing depends on the individual cell line. For example for different CHO cell lines 0.1–0.4 % DMSO concentration have been reported (Kloth et al. 2010). Cell centrifugation was not performed after thawing to avoid stress-related loss of cells and cell viability. This was possible because freezing and inoculum expansion medium are identical (without considering DMSO) (Kloth et al. 2008). The initial BHK-21 cell concentration after thawing was 0.13×10^6 cell mL⁻¹. The cell population increased in 6.15-fold after 96 h culture in static culture.

In static cultures systems, oxygen transfer is limited (Bambrick et al. 2011). Thus, the definition of optimal liquid column height in inoculum preparation is focused on finding a relationship of compromise between maximum viable cell concentrations and reducing culture medium exchange and its associated contamination risks. The recommended liquid column height is 2–5 mm range. For cell lines with high oxygen requirements, 2 mm is suggested, meanwhile 5 mm is proposed for cells with low oxygen

necessities (Freshney 2010). In our study, it was demonstrated that BHK-21 cell line can grow satisfactorily in static cultures at 5 mm medium height, the maximum viable cell concentration were comparable with others previously reported using agitated systems (Handa-Corrigan et al. 1989; Cruz et al. 2002; Moreira et al. 1995; Ishaque et al. 2007). Increase of liquid column height is not recommend, this is based on unnecessary culture medium consumption (around 50 % for 10 mm liquid column height) and increasing cost of inoculation procedure.

When the scale factor in static culture was augmented from 25 to 75 cm² by keeping a 5 mm liquid column height, an increase of cell concentration was observed after 72 h cell culture. This result could be explained by the inoculum size and DMSO concentration. For 25 cm² T-flask, the inoculum size was twofold higher with respect to that used for the 75 cm² T-flask; as a consequence, nutrient concentration and toxic metabolites at inoculation were lower and higher, respectively. The DMSO concentration was reduced by 84 % by passing from 25 (0.20 % v/v) to 75 cm² (0.03 % v/v) T-flask cultures. The cell concentrations after 72 h cell culture at 10 mm liquid column height were not dependent of the scale, suggesting that growth is limited by oxygen. In all static culture experiments, no cell loss caused by cell attachment to plastic surface was observed. Nor, cell aggregation was confirmed.

The maximum viable cell concentration in spinner flasks at low agitation speed (25 rpm) was found at 50 mL working volume. For this BHK-21 cell line, the surface aeration area/broth volume ratio in a 100 mL spinner flask corresponding to 50 mL working volume could be optimal for suitable gas transfer. Oxygen transfer through the headspace is the most common and easy method of gas exchange for small vessels like spinner flasks. The maximum volume for a defined surface area in spinner flasks depends mainly on cell concentration and whether or not the headspace gas is enriched with oxygen (Godoy-Silva et al. 2010). Probably, the dissolved oxygen concentration at 35 mL working volume was higher than the optimal value for favoring BHK-21 cell growth and metabolism. Instead, no significant difference in cell concentration at the end of the exponential phase was detected when agitation increased (60 rpm), keeping constant working volume. Thus, inoculum for the bioreactor was prepared from a culture performed at

25 rpm in order to protect cells from unnecessary higher shear forces. The non-influence of inoculum volume–working volume percentage (10–30 %) on maximum viable cell concentration was useful for reducing handling, cell loss and contaminations risks associated to centrifugation operation in lab scale. Maximum viable cell concentration ($>4 \times 10^6$ cell mL⁻¹) in optimal conditions was higher than usual values of this kinetic parameter for BHK-21 cell lines cultured in batch mode ($1\text{--}3.2 \times 10^6$ cell mL⁻¹) (Handa-Corrigan et al. 1989; Cruz et al. 2002; Moreira et al. 1995; Ishaque et al. 2007). Glutamine was exhausted at the end of the exponential growth phase (72 h post inoculation). This confirms that this substrate limits BHK-21 cell growth (Radlett et al. 1971). Therefore, this moment of the growth curve was selected to transfer the cell suspension from the spinner to bioreactor.

The scheduling to inoculate the bioreactor (1 L working volume) was defined using previous experiments. The moment to pass to the subsequent step was the end of the exponential phase of the previous step, in order to ensure high viable cell concentration and viability near 100 %. In general, this suggested transferring cells for the subsequent inoculation step in the middle of the exponential phase, but this could lead to increase the number of steps, consuming time and resources.

The total time and culture medium required to obtain 1 L with a cell concentration of $4.39 \pm 0.21 \times 10^6$ cell mL⁻¹ were 13 days and 1,051.2 mL, respectively. The time required for this purpose was similar to others for mammalian cell cultures (15 days). However, the culture medium consumption was low. The use of cell culture to medium volume ratios higher than 1:10 is not frequent (Kloth et al. 2010). In our study case, this parameter for bioreactor inoculation was 14.4 in our study case (Table 3). The exponential growth phase was reached 12 h post-inoculation and the culture medium consumption decreased at least by 30 % compared with the 1:10 ratio of cell culture to medium volumes. Improvement in kinetic parameters observed for the experiments in bioreactor with respect to those from spinner are justified by the control of important variables for cell growth and metabolism, such as: pH, and dissolved oxygen concentration. Specifically, the maximum specific growth rate in bioreactor was two-fold higher than the values obtained for this parameter in spinner flasks

(present work) and others, commonly reported for mammalian cell culture in bioreactors using serum free medium (Platas-Barradas et al. 2012). Probably, fetal bovine serum addition to the culture medium justified this finding (Sidoli et al. 2004). In that sense, the culture medium composition must be considered in order to adapt the present strategy for other cell lines and industrial applications.

Conclusion

The critical parameters in static (liquid column height) and agitated cultures in spinner flasks (working volume, inoculum volume and agitation) as well as cell transference schedule across the inoculation stages were optimized in order to decrease procedure time, culture medium consumption and contamination risks. These results allowed multiplying by thousand the original cell amount in 13 day with 1,051 mL culture medium.

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