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TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology

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Abstract

Process development with mammalian cells is often limited by the lack of resources and assay capacity to deal with the multiplicity and complexity of interrelated parameters in culture technology. Multi-component analysis, an issue in media development and in screening of newly established production cell lines, is subject to the same limitations. We therefore developed a high throughput system for the culture of mammalian cells in suspension. Our scale-down reactor system called "TubeSpin" allows many experiments to be run in parallel with a minimum of resources and labor. Reactor conditions were readily simulated using 50 ml centrifugation tubes as culture vessels mounted on a rotational shaker installed in a warm room. This system was applied to the development work for a number of CHO based processes for the production of a human recombinant IgG. We show here how such a system can increase process development speed and efficiency. The results presented illustrate the improvement of volumetric productivity using butyrate and temperature shifts.

Keywords: Monoclonal antibody; High throughput; Process development; Optimization; Small-scale bioreactor; Screening; Scale-down; Shaking technology

1. Introduction

Progress in process development in animal cell culture is often limited by the lack of information about the response of cells to different process conditions, the addition of supplements or productivity enhancers. Commonly, to achieve a reliable and robust process, spinner flasks or small bioreactors must be used. These experiments are time consuming and demanding in resources. The limited availability and the high cost for spinner and reactor runs limit the advancement of the process development to a considerable extent. Certainly, for some parameters, such as pH, pO_2, pCO_2, and aeration method there is a need for a controlled environment and hence an obligation to use small-scale bioreactors. However, identification of promising conditions, such as optimal medium composition, productivity enhancers, osmolality and temperature shifts, do not need a set of bioreactors, and so the use of smaller-scale and simpler equipment can be envisaged. The major difficulty for the use of such small- or micro-scale systems lies in their correlation (or lack thereof) to the large-scale industrial process. Ideally, experiments should be executed in conditions resembling as close as possible those found in a bioreactor in order to achieve reasonable correlation. Limitations of the scale-down systems have to be studied carefully, well understood and eventually anticipated in terms of the conclusions drawn. If done properly, a large amount of useful information can be obtained that dramatically reduces the need for work in bioreactors and thus the time spend for the identification of sets of parameters improving productivity and/or cell growth.

Until now shaking technology was used mainly for microbial or yeast cultivation [1]. For the culture of mammalian cells several shaken cultivation systems have been proposed [2,3]. In these systems, shaking the whole culture vessel with an external device provides the power input necessary for agitation. Several systems based on this principle were proposed for different culture scales. Starting with small devices, for volumes for up to 3 ml based on microtiter plates [2], up to larger shaken rigid containers [3] for superior volumes up to 10 l, and finally to culture systems where cells grow in flexible bags [4], many different shaken cultivation systems were developed. In wave bioreactors, the cells are

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grown in sterile plastic bags and the agitation is provided by an up and down movement. The available culture volumes range from 10 ml to 100 l.

We present a high throughput system applicable as a process development strategy based on shaking technology that has met to a very large extent the requirements of parameter studies for bioprocesses with mammalian cells. We show examples such as how to optimize concentration and time of addition of sodium butyrate with a CHO cell line expressing recombinant anti-RhD antibodies as well as the effects of temperature shift on the same cell line in combination with sodium butyrate treatment. In one set of experiments, executed in a "week's" timeframe, important insights on useful ranges of concentration and timing of butyrate addition could be gained, showing potential for 2–5-fold productivity improvements for a cell culture process for a human recombinant antibody expressed in CHO cells.

2. Materials and methods

2.1. Cell culture

CHO cells (AMW1) were stably transfected to express a human antibody as described earlier [5,6,8]. Cells were adapted to grow in suspension in chemically defined, ProCHO5-CDM (BioWhittaker, Walkersville, MD). For routine maintenance, cells were cultivated in 500 ml spinners flasks (Bellco Glass Inc., Vineland, NJ) with filling volumes of 300 ml medium. The cells were split every 3–4 days. Prior to passaging, cells were spun down at 160 g for 5 min and then the cell pellet was resuspended in fresh pre-warmed medium at densities of 2×10^5 cells/ml. The spinner flasks were placed in a warm room (37 °C) with the stirrer speed set to 80 rpm.

2.2. Bench-scale reactor system

The bench-scale glass bioreactors (Fig. 1) were 3 and 7 l Applikon (Schiedam, Holland). The pH was controlled with NaOH 1 M (Fluka Chemie AG, Buchs, Switzerland) and CO_2 (Carbagas, Bern, Switzerland) to pH 7.2. The pO_2 was held constant at 20% (relative air saturation) and controlled by sparging air or pure oxygen into the culture. The stirrer speed was set to 150 rpm. Cell seeding density was of 2×10^5 cells/ml. The final culture volume was 1 and 3 l for bioreactor sizes of 3 and 7 l, respectively.

2.3. Small-scale reactor system (TubeSpin)

Standard 50 ml centrifugation tubes with 28 mm inner diameter (TPP, Trasadingen, Switzerland) were stacked into racks mounted on a rotational shaker (ES-W, Adolf Kühner AG, Birsfelden, Switzerland) (Fig. 1). The set-up was installed in an incubator providing temperature control. For a 25 mm throw, the agitation speed was set to 200 rpm. The culture volume was 5 ml per tube.

2.4. Parameter screening

In order to test multiple parameters in satellite experiments, cells from a bioreactor were transferred into the centrifugation tubes (5 ml cell suspension per tube) at different time points, days 3–5, of cultivation. Centrifugation tubes were either closed or very slightly opened to influence gas mass transfer with the outside environment. The cultures were not maintained longer than 6–8 days to avoid erroneous results due to evaporation losses.

2.4.1. Sodium butyrate induction

A 1 M sodium butyrate (Sigma, St. Louis, MO) stock solution was prepared with UHP water and sterile filtered (0.22 um). Cells from the reactor, were taken out of the bioreactor at days 2–5 of cultivation and exposed to different concentrations of sodium butyrate. The concentrations ranged from 0 to 6 mM. The cells were cultivated for six additional days at 37 °C. The experiments were executed in duplicate.

2.4.2. Temperature shift combined with sodium butyrate induction

Cells were taken out of the bioreactor at days 4–6 and incubated in the presence of different sodium butyrate concentrations ranging from 0 to 10 mM. Cells were then cultivated at 37 or 32 °C. The experiments were executed in triplicate.

2.5. Sampling

Bioreactors were sampled twice a day. The cell count and viability, using heamocytometer and trypan blue, were established. The cell suspension was spun down at 3500 rpm for 10 min and cleared supernatant was either frozen (−20 °C) until analysis, or pre-diluted in ELISA diluent and stored at 4 °C, until antibody titer quantification. For TubeSpin experiments the entire centrifugation tube content was used for sampling. One milliliter was immediately used for metabolite analysis, pH, pO_2 and pCO_2 using the BioProfile 200 Analyzer (Nova Biomedical, Waltham, MA) and also cell count and viability assessment. pH was measured using a hydrogen ion selective glass membrane, the partial pressure of oxygen (pO_2) was measured amperometrically and the partial pressure of carbon dioxide (pCO_2) was measured with a modified pH electrode, where a gas permeable membrane is mounted on a combination measuring/reference electrode. Air and CO_2 saturation (in %) are calculated from the respective measured partial pressure of oxygen and carbon dioxide. The remaining cell culture supernatant, was spun down at 13,000 × g for 5 min and then clarified supernatant was pre-diluted into ELISA diluent and stored at 4 °C until analysis. For

Fig. 1. Classical glass bench-top bioreactor on the left. TubeSpin set-up on the right: 50 ml centrifugation tubes are fixed into standard tube racks mounted on the plate of a rotational shaker to allow mammalian cell cultivation in suspension. An incubator or a warm room provides the temperature control. Cells are transferred under sterile conditions from the bioreactor into the centrifugation tubes.

screening purposes, 0.2 ml samples were taken from each tube and clarified cell culture supernatant were kept at 4 °C pre-diluted in ELISA diluent only for *r* anti-RhD IgG quantification. Quantification of anti-RhD IgG in cell culture supernatants was done by enzyme-linked immunosorbent assay using goat anti-human kappa light chain as capture antibody (Biosource, Luzern, Switzerland) and goat anti-human IgG alkaline phosphatase conjugated as detection antibody (Biosource). Concentration was measured against a 0–40 ng/ml standard of purified human anti-RhD IgG.

3. Results and discussion

3.1. Preliminary characterization of TubeSpin system

In order to partially characterize the TubeSpin system, the two most critical parameters in mammalian cell culture technology, pH and dO_2, were studied. The trends of pH and oxygen levels for 4 days inside the tubes are shown in Table 1.

Open, closed tubes, or tubes equipped with filters showed no oxygen limitation even with cell densities higher than

Table 1
Data collection from TubeSpin cultures

Tube type	Cell density (cells/ml) ($\times 10^6$)	Viability (%)	pH	O_2 saturation (%)	CO_2 saturation (%)
(A)					
"Open"	5.4	95	6.99 ± 0.11	64 ± 3	0.47 ± 0.06
Closed	4.5	98	6.86 ± 0.15	41 ± 7	3.7 ± 0.4
(B)					
"Open"	1.5	23	6.91 ± 0.14	97 ± 0.9	0.27 ± 0.06
Closed	1.5	27	6.85 ± 0.09	93 ± 1.3	0.47 ± 0.06

Tubes were seeded with cell suspension from a bioreactor on day 3 of cultivation at a density of 5×10^6 cells/ml. (A) Data obtained from TubeSpin "reactors" 1 day after cell suspension transfer. (B) Data obtained from TubeSpin "reactors" 4 days after cell suspension transfer.

5×10^6 cells/ml (1 day after transfer from the bioreactor). The ratio of volume of culture to volume of headspace used (1:10) was hence well adapted to the cell culture conditions. Closed caps are preferred in order to avoid water evaporation, up to 10% per day of culture volume lost were measured for open tubes. Concerning pH, no strong acidification was observed as initially expected. The measured CO_2 levels show that the carbon dioxide produced by the cells was efficiently stripped out of the culture (Table 1). CO_2 is in a dynamic equilibrium with the weak acid H_2CO_3 and in a close relationship with the pH. The bicarbonate buffering system was most likely responsible for maintaining a rather neutral pH by CO_2 stripping, using the large surface for transfer into the large headspace available [7]. Another important consideration is evaporation. These losses can be avoided by either closing the tubes entirely or by using partially opened tubes in a humidified atmosphere. For partially opened tubes cultivated under the conditions described earlier evaporation losses corresponded to a volume of 50–70 μl per day, which represented a loss of less than 10% of the initial volume at the end of the culture.

These results show that the major limitations of smaller-scale systems associated with good oxygenation, pH and agitation in mammalian cell culture technology could be overcome also for scaled down system as described. Furthermore the range of changes of oxygen and pH during such TubeSpin cultures are comparable to the conditions observed with more sophisticated and complete bioreactor systems.

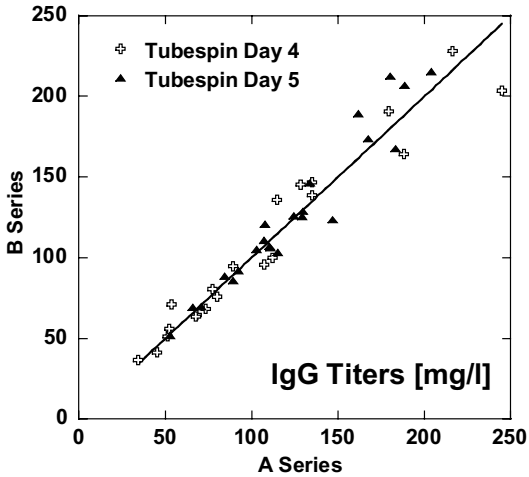

Fig. 2. Data from duplicates of individual experiments were plotted against each other.

3.2. Reproducibility

The reproducibility between experiments with the same conditions in TubeSpin was good with small standard

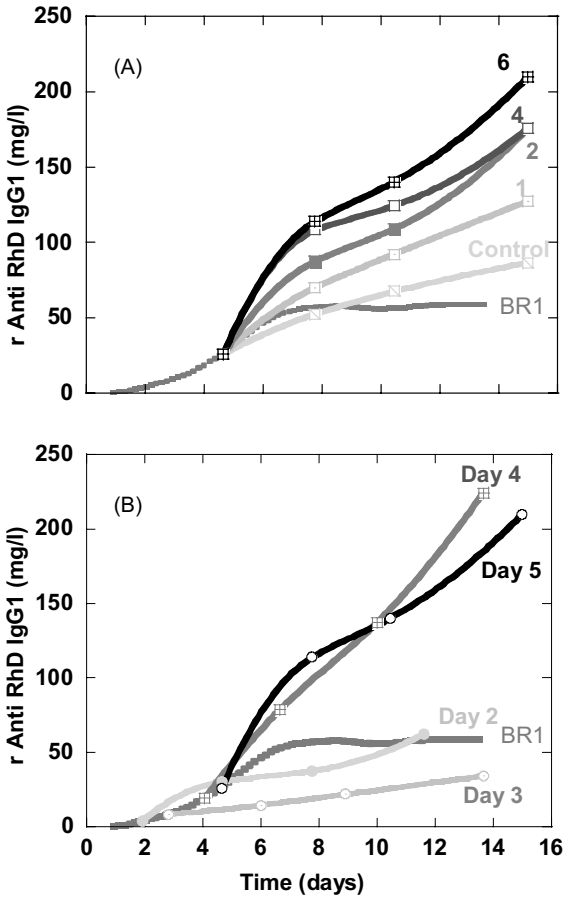

Fig. 3. Effects on IgG titers of sodium butyrate addition to TubeSpin satellite cultures: Sodium butyrate titration (in mM) (A) and timing of addition of 6 mM sodium butyrate (B) on recombinant anti-RhD IgG producing CHO cells coming from a bioreactor (BR1).

deviations (<5%). The overall variation resulting from all operator manipulations, such as sampling and dilution steps for ELISA and the experimental deviations were less than 10%. Fig. 2 shows correlation of titers for duplicates (Series A and B). Sample A *x*-axis was plotted against sample B on the *y*-axis. As the multiplicates behaved identically it was possible to reduce the number of repeats in order to increase the number of conditions to be tested.

3.3. Butyrate experiments

Fig. 3 shows results of a butyrate optimization experiment with respect to timing of addition and concentration applied in cell culture. In Fig. 3A, butyrate additions on day 4 resulted in a clear dose response with the highest productivity enhancement observed with a concentration of 6 mM butyrate, compared with the control (tubespins without butyrate treatment) or productivity observed for the bioreactor (BR1), from where the cells for the experiment were obtained. The same concentration of butyrate (6 mM) was applied for additions to the tubes on different days of the culture (Fig. 3B). The improvement in productivity was only observed on day 4 or 5, whereas days 2 and 3 additions were ineffective and corresponded to productivity observed for the bioreactor (BR1) where no butyrate treatment was applied. The data observed indicates that even higher concentrations of butyrate may be beneficial in overall volumetric productivity.

To verify the TubeSpin results, a set of bioreactor experiments was executed. In this run the culture was exposed on day 5 to 6 mM butyrate. The graphs in Fig. 4 show titers obtained over the run time of 10 days (A) and the viable cell numbers in each of the bioreactors (B). Samples were taken two times per day and continuous lines were fitted through this data points. The two bioreactors control resulted in final titers of about 40 mg/l of recombinant anti-RhD IgG, whereas the butyrate case produced up to 100 mg/l by day 10. The profiles for growth up to day 4 were similar for the two bioreactors with same culture volume (BR2 and BR4). While the chosen butyrate concentration and timing of addition resulted in a 2.5-fold improvement of volumetric productivity in the bioreactor system, it could not exactly reproduce the titers observed in the TubeSpin system (200 mg/l).

In Fig. 5, the specific productivities over time are shown. Fig. 5A shows the observed titers plotted against the accumulated viable cell mass of a non-butyrate treated culture, whereas Fig. 5B shows the same from a butyrate treated culture. The slope of the fitted lines represents the calculated specific productivities. The addition of butyrate resulted in

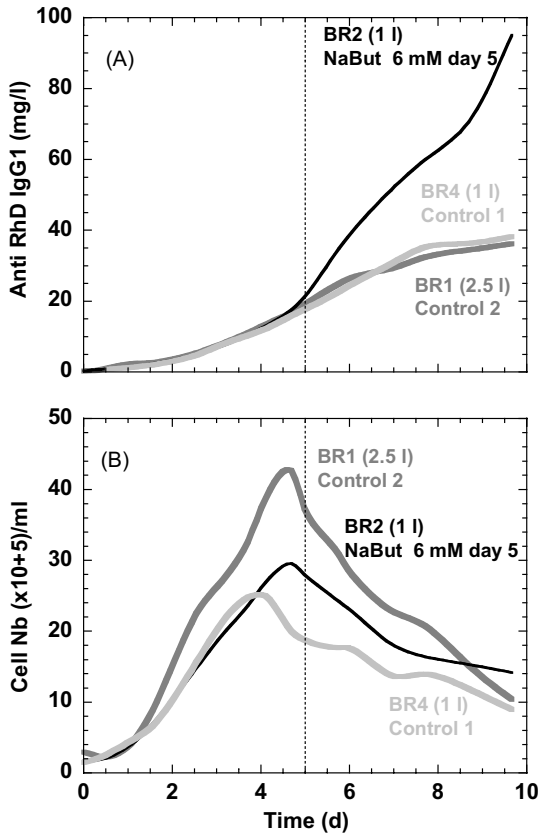

Fig. 4. Productivity (A) and viable cell (B) trends in three bioreactors: treatment with 6 mM sodium butyrate on day 5 in comparison to non-treated controls.

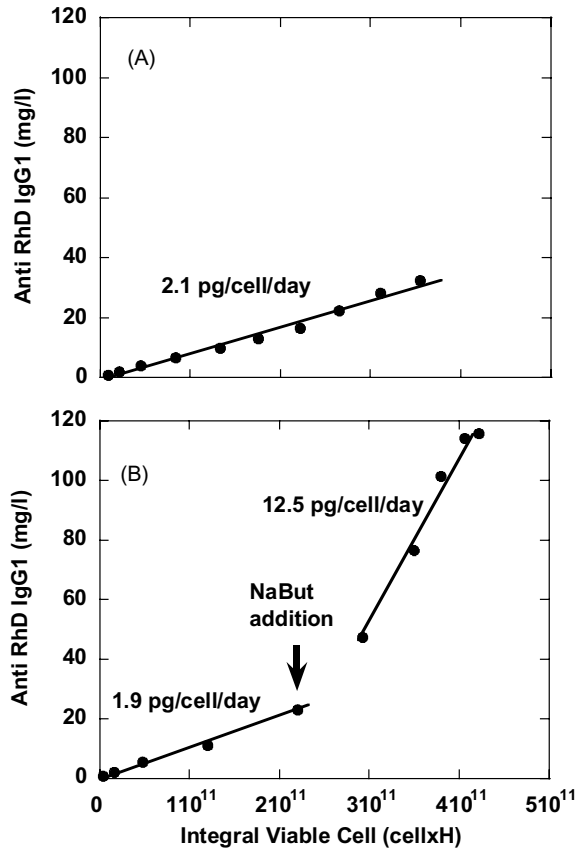

Fig. 5. Specific productivity increase as a consequence of sodium butyrate addition (B) on AMW1 cells compared to cells in a bioreactor without treatment (A).

an increase of the specific productivity from 1.9 to 12.5 pg per cell per day (six-fold increase).

3.4. Temperature shift in combination with sodium butyrate treatment for the improvement of final product titer

Another example of the successful application of the TubeSpin satellite approach is the optimization of a temperature shift combined with a sodium butyrate treatment (Fig. 6). Here, we extended the range of butyrate concentrations under study up to 10 mM. It became evident from the results obtained that shifting the temperature from 37 to 32 °C on day 4 is beneficial for final titer when combined with a butyrate treatment of 7 mM on day 4. Moreover, by applying a stepwise approach in these experiments, we were able to dramatically reduce the number of potential permutations in the optimization program, yet still could afford a rather comprehensive approach for studying these parameters. Thus, we gained considerable confidence in having identified a useful range of conditions for further fine tuning.

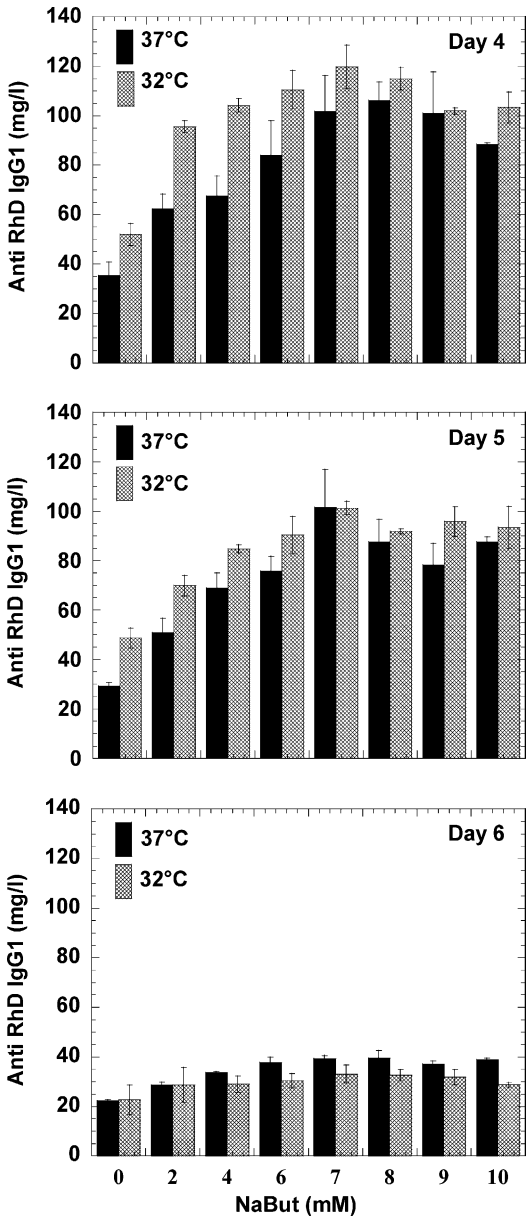

Fig. 6. Satellite experiments executed with cell suspensions from a single bioreactor. A range of butyrate concentration were applied and combined with temperature shift from 37 to 32 °C.

4. Conclusions

Results show a novel approach to cell culture process development, based on satellite cultures derived from standard instrumented bioreactors. These satellite cultures are executed in 50 ml shaken centrifugation tubes. Since each of the cultures in the tubes is initiated with a 5 ml aliquot, a 2 l bioreactor can provide a large number of satellite cultures. These can then be subjected to a wide range of process condition permutations. Due to a very simplified handling and sampling approach, a single operator can execute these experiments, with very little opportunity for errors due to handling. The TubeSpin satellite approach has advantages over other, even smaller volume based systems, for example microtiter based, because it provides larger culture and sample volumes, allowing for more in depth analysis. Applying this approach allows rapid identification of promising process conditions. Final verifications of optimized conditions still have to be done in an instrumented bioreactor, but the data provided here give evidence for the usefulness of this approach.

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References

[1] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory Press, New York, 1989.
[2] P. Girard, M. Jordan, M. Tsao, F.M. Wurm, Small-scale bioreactor system for process development and optimization, Biochem. Eng. J. 7 (2001) 117–119.
[3] C.-M. Liu, L.-N. Hong, Development of a shaking bioreactor system for animal cell cultures, Biochem. Eng. J. 7 (2001) 121–125.
[4] V. Singh, Disposable bioreactor for cell culture using wave-induced agitation, Cytotechnology 30 (1999) 149–158.
[5] S. Miescher, M. Zahn-Zabal, M. De Jesus, R. Moudry, I. Fisch, M. Vogel, M. Kobr, M.A. Imboden, E. Kragten, J. Bichler, N. Mermod, B.M. Stadler, H. Amstutz, F. Wurm, CHO expression of a novel human recombinant IgG1 anti-RhD antibody isolated by phage display, Br. J. Haematol. 111 (2000) 157–166.

[6] H. Amstutz, S. Miescher, S. Moudry, S. Dejardin, M. Imboden, M. Zahn, M. Kobr, I. Fisch, B. Corthésy, N. Mermod, L. Berruex, M. Abdellali, O. Brüggemann, D. Fraboulet, R. Freitag, E. Kragten, S. Nahrgang, P. Ducommun, P. Pugeaud, I. Marisom, U. Von Stockar, M. De Jesus, M. Jordan, M. Bourgeois, L. Hunt, S. Radice, L. Baldi, A. Kulangara, P. Girard, R. Ribiki, F.M. Wurm, The SPP biotech module 1: proteins for medical applications, a multidisciplinary effort for the development of a recombinant anti-RhD antibody for the prevention of hemolytic disease of the newborn, in: A. Bernard, et al. (Eds.), Proceedings of the 16th ESACT Meeting on Animal Cell Technology: Products From Cells, Cells as Products, Lugano, Switzerland, 25–29 April 1999, Kluwer Academic Publishers, Dordrecht, 1999, pp. 455–458.

[7] A. Doyle, J.B. Griffiths, D.G. Newell, Cell & Tissue Culture, Wiley, New York, 1996, pp. 2A:2.1–2.3.

[8] M.J. De Jesus, M. Bourgeois, M. Jordan, M. Zahn, N. Mermod, H. Amstutz, F.M. Wurm, Establishing and developing CHO cell lines for the commercial production of human anti-Rhesus D IgG, in: A. Bernard, et al. (Eds.), Proceedings of the 16th ESACT Meeting on Animal Cell Technology: Products From Cells, Cells as Products, Lugano, Switzerland, 25–29 April 1999, Kluwer Academic Publishers, Dordrecht, 1999, pp. 91–93.