Influence of alterations in culture condition and changes in perfusion parameters on the retention performance of a 20 μm spinfilter during a perfusion cultivation of a recombinant CHO cell line in pilot scale

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Received 6 December 1999; accepted 6 June 2000

Key words: CHO cells, culture condition, model particles, operation cut off, perfusion cultivation, retention, spinfilter, tip speed

Abstract

Since 1969 much attention has been devoted to the use of spinfilter systems for retention of mammalian cells in continuous perfusion cultivations. Previous investigations dealt with hydrodynamic conditions, fouling processes and upscaling. But hydrodynamic conditions and fouling processes seem to have a secondary importance in spinfilter performance during authentic perfusion cultivations. Obviously, alterations in culture condition are more relevant especially during long-term processes. Therefore, our practical approach focussed on the performance quality of a commercially available 20 μm spinfilter during a perfusion cultivation of a recombinant CHO cell line in pilot scale regarding the following issues: 1) retention of viable cells in the bioreactor; 2) removal of dead cells and cell debris from the bioreactor; 3) alterations in culture condition; and 4) changes in perfusion mode. Furthermore, we tested the performance of 20 μm spinfilters in 2 and 100 l pilot scale using solid model particles instead of cells. Our investigations showed that retention of viable cells in pilot scale was independent of spinfilter rotation velocity and perfusion rate; the retention increased from 75 to 95% corresponding to operation time, enlarging cell diameter and enhanced formation of aggregates in the culture during the perfusion cultivation. By means of the Cell Counter and Analyzer System (CASY) an *operation cut off* of 13 μm was determined for this spinfilter. Using solid model particles in 2 l scale, optimal retention was achieved at a tip speed of 0.43 m s^{-1} (141 rpm) – further enhancement of spinfilter rotation velocity up to 0.56 m s^{-1} (185 rpm) decreased the retention rapidly. In pilot scale best retention performance was obtained with tip speeds of 0.37 m s^{-1} (35 rpm) and 1.26 m s^{-1} (120 rpm). Hence, significant retention in pilot scale could already be achieved with low agitation. Therefore, the addition of shear force protectives could be avoided so that the purification of the target protein from the supernatant would be facilitated.

Abbreviations: ATTC, American Type Culture Collection; CHO, chinese hamster ovary; D, dilution; R, retention; rpm, revolutions per minute; SRV, spinfilter rotation velocity

Introduction

For the pharmaceutical industry there is a high value market for the cultivation of mammalian cells for production of native or recombinant, diagnostic or therapeutic proteins, e.g. monoclonal antibodies, erythropoietin, etc. Generally, the highest product output

can be achieved in continuous stirred tank bioreactors performed in perfusion mode (Esclade et al., 1988). Therefore several devices for cell retention were developed accordingly. The use of external systems including filtration (Rebsamen et al., 1987) or centrifugation (Takamatsu et al., 1996; Johnson et al., 1996; Tokashiki et al., 1990) can cause contamination prob-

Figure 1. Spinfilter retention performance. Particles are retained by acceleration effect (case A and C) and by sieve effect (case D). Particles with smaller diameter than pore size pass the filter mesh if perfusion force is higher than centrifugal force (case B).

Figure 2. The setup of the 100-l bioreactor performed in perfusion mode. Basically the setup of the 2-l system was the same.

lems and enhances mechanical stress towards the cells. Therefore, internal cell retention systems like hollow fibers (Büntemeyer et al., 1987), ceramic matrices (Lydersen, 1987), microspheres (Vournakis and Runstadler, 1991), polymer fleeces (Sittinger et al., 1997) and spinfilters (Deo et al., 1996; Favre and Thaler, 1992; Avgerinos et al., 1990) are more favourable.

Since the pioneering work of Himmelfarb (Himmelfarb et al., 1969) spinfilter systems are preferred as they are simple and convenient for continuous perfu-

sion cultivation in stirred bioreactors. Fouling of the sieve mesh is partly prevented by rotation of the filter attached to the stirrer shaft and using a mesh with a pore size slightly larger than cell diameter. Thus, two different ways of particle retention during perfusion can be distinguished: acceleration effect and sieve effect. Each particle is accelerated at the mesh surface by spinfilter rotation. This acceleration removes the particle from the surface as far as centrifugal force (induced by spinfilter rotation) is higher than perfu-

Figure 3. Perfusion cultivation of recombinant CHO cells in 100-l pilot scale. Actual and theoretical cell growth according to maximum specific growth rate ($\mu_{\max}$ -cell growth). The setting of the perfusion parameters (dilution rate [D] and spinfilter rotation velocity [SRV]) are added.

Figure 4. Performance of the 20- μm spinfilter in pilot scale due to retention of CHO cells determined with CASY data (10–50 μm) and with results of the Neubauer chamber (by the means of crystal violet staining and trypan blue staining = viable cells), respectively.

sion force (induced by perfusion flux). In this case the particle is retained in the reactor, regardless of particle diameter and pore size. This acceleration effect corresponds to the radius of the spinfilter and its rotation velocity and can be enhanced by a conical design of the spinfilter (Fenge et al., 1993) and the addition of a draft tube (Esclade et al., 1988). In contrast, if the per-

fusion force is higher than the centrifugal force, only particles with a diameter larger than the pore size are retained (sieve effect). Otherwise they pass the filter mesh and are removed from the reactor (see Figure 1).

Hence, retention performance can be influenced by perfusion flux (perfusion rate, D) and spinfilter rotation velocity (SRV). But it should be considered

Figure 5. Formation of CHO cell aggregates during cultivation in pilot scale. Comparison of cell growth determined with CASY (10–50 μm) and Neubauer chamber (viable cells), respectively.

Figure 6. Removal of dead cells and cell debris in pilot scale. Development of retention performance due to operation time and particle size.

that, in case of CHO cells, culture conditions (cell diameter, formation of aggregates, amount of dead cells and debris) vary during cultivation (Seewöster and Lehmann, 1997). In our investigation we focused on the quality of spinfilter performance due to SRV, perfusion rate and especially referring to removal of dead cells and cell debris during a perfusion cultiv-

ation of a recombinant CHO cell line in 100-l pilot scale.

To circumvent the effects of alterations in culture condition we tested the performance of the spinfilter in 100-l pilot scale and in 2-l bench scale using solid bead particles as a model.

Figure 7. Development of particle size distribution of the CHO culture in pilot scale due to cultivation time in relation to the operation cut off of the 20 μm -spinfiler. The diameter of single living cells is increasing from 13 μm to 16 μm . Furthermore, the number of aggregates ($>15 \mu\text{m}$) is enlarging. The average single cell diameter of the hybridoma cell line HB-58 (ATTC 187.1) is at 13 μm – the operation cut off of the spinfiler.

Material and methods

Cell line, medium

Our investigations were performed with a recombinant CHO cell line producing prothrombin (Lütkemeyer et al., 1999). The cells were cultivated in a serum free medium based on a 1:1-mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (both Life Technologies, Paisley, U.K.). The basal medium was supplemented with 200 mg l^{-1} human serum albumin (Bayer, Elberfeld, Germany), 10 mg l^{-1} bovine insulin and 10 mg l^{-1} human transferrin (both Sigma, Deisenhofen, Germany). Due to the special nutrient requirement of this particular cell line the medium was optimised with 200 μM ethanolamine (Merck-Schuchardt, Hohenbrunn, Germany), 29 nM sodium selenite (Sigma), 20 mg l^{-1} sodium pyruvate (Serva, Heidelberg, Germany), 3 g l^{-1} glucose (Merck, Darmstadt, Germany) and 2 mg l^{-1} vitamin K3 (Sigma). Furthermore, the concentration of glutamine (500 mg l^{-1} ; Merck), proline (20 mg l^{-1} ; Merck), methionine and tryptophan (each 10 mg l^{-1} ; Merck) was enhanced. To prevent shear stress towards the cells 0,1% w/w Pluronic® F-68 (Sigma) was added.

Spinfilters

Spinfilters with an identical mesh structure (20 μm pore size, 5-layered stainless steel; Porostar Standard by Haver and Boecker, Oelde, Germany) provided by B. Braun Biotech International were used in both scales. The filtration surface of the spinfiler in 100-l scale was 2670.35 cm^2 with an outer diameter of 20 cm. The dimension of the spinfiler in 2-l scale was filtration surface = 209.54 cm^2 and outer diameter = 5.8 cm. Thus, according to filter surface, the up-scaling of spinfiler from 2-l scale to 100-l scale was approximately 13-fold.

Bioreactor Setup

The setup of the 100 l system is shown in Figure 2. It consisted of a 100 l bioreactor (UD 100; B. Braun Biotech International, Melsungen, Germany) connected with silicone tubes to a 5-l inoculation bottle, a 200-l medium storage tank and finally a 50-l harvest tank. A membrane tube basket was inserted into the 100-l vessel for bubble free aeration. This basket also acted like a draft tube. The culture was stirred by a 3-blade segment impeller (Fenge et al., 1993). The 20- μm spinfiler was attached to the stirrer shaft. Therefore, stirrer speed and spinfiler rotation velocity

Figure 8. Retention performance profile of the 20- μm spinfilter in 2-l scale using solid bead particles as a model (Superose 6). Best retention (88%) was achieved with a tip speed of 0.43 m s^{-1} (141 rpm).

(SRV) were coupled. Perfusion was performed with two peristaltic pumps installed between the 200-l tank and the reactor and between the 50-l harvest tank and the reactor, respectively.

Basically, the setup of the 2-l bioreactor system due to its volume-reduced containments (inoculation bottle, harvest bottle, pumps, etc) was the same.

Cell counting

CHO cells show a tendency to form aggregates. Therefore, only single dead cells could be determined in a Neubauer chamber using the trypan blue exclusion method (Tennant, 1964) (0.4% trypan blue solution; Sigma). To calculate the viable cell concentration all cells of a sample were osmotically shocked with 0.1 M citric acid (Sigma) containing 0.08% crystal violet (Merck). By this procedure cell membranes were totally disintegrated so that all nuclei could be stained (Sanford et al., 1950). The viable cell concentration was calculated by means of the total and the dead cell concentration.

Determination of particle size distribution

Particle size distribution was performed with the Cell Counter and Analyser System (CASY TTC, Schärfe, Reulingen, Germany). This device measures particle sizes according to the resistance principle combined

with an additional pulse area analysis and a signal scanning frequency of 1 MHz. Prior to measurement an aliquot of a sample was diluted 1:100, respectively 1:400 in a phosphate-buffered saline (CASYton buffer, Schärfe). Then an exactly adjusted aliquot of the diluted sample was automatically measured by the system. Each measurement was repeated threefold. In the case of the CHO culture the obtained distribution can be subdivided into four areas: cell debris ($<5 \mu\text{m}$), single dead cells ($5\text{--}10 \mu\text{m}$), single viable cells ($10\text{--}15 \mu\text{m}$) and aggregates ($>15 \mu\text{m}$).

Analysis of glucose, lactate, ammonium and amino acids

The concentrations of glucose and lactate were measured with the automatic analyser YSI 2700 S (Yellow Springs Instruments, Yellow Springs, U.S.A.). Ammonium was determined by o-phthaldialdehyde (OPA)/thioglycolate derivatisation (Büntemeyer, 1998) and fluorescence detection (spectrofluorimeter RF-551, Shimadzu, Kyoto, Japan). The determination of extracellular amino acid concentrations was carried out using an automated reversed phase high-performance liquid chromatography (HPLC) system (D450/KromaSystem2000, Kontron Instruments, Neufahrn, Germany) equipped with an ODS C_{18} column (Ultrasphere, $5 \mu\text{m}$, Beckman Coulter, München, Germany) after OPA precolumn derivatiza-

Figure 9. Retention performance profile of the 20- μm spinfilter in pilot scale using solid bead particles as a model (Superose 6). Best retention was achieved with a tip speed of 0.37 m s^{-1} (35 rpm) and 1.26 m s^{-1} (120 rpm).

tion, essentially described by Büntemeyer et al. (1991) and Seewöster and Lehmann (1995).

Calculation of retention

During the perfusion cultivation samples were routinely taken twice each day in the same way from the vessel and from the perfusate. The retention was calculated by following equation:

$$R_c(\%) = 100 - 100 \cdot \frac{X'}{X}$$

Retention was 0% if the cell/particle concentration in the reactor and perfusate were the same ($X' = X$). In this case all particles could pass the spinfilter mesh without resistance. If there were no cells/particles in the perfusate ($X' = 0$) retention was complete ($R_c = 100\%$).

Test with model particles

To circumvent effects of alterations in culture condition on the spinfilter performance the retention of solid particles was investigated. A gelfiltration matrix (Superose 6, kindly provided by Amersham Pharmacia, Uppsala, Schweden) was used. These highly cross-linked porous agarose beads were very suitable because they correspond to the average diameter of

single living CHO cells ($13 \pm 2 \mu\text{m}$). Experiments were performed both in 2-l and in 100-l scale.

Each experiment was started with a distinct initial particle concentration in the bioreactor determined with CASY. In 2-l scale we used 7×10^6 particles ml^{-1} . In 100-l scale the initial concentration was set to 2.0×10^6 particles ml^{-1} . Perfusion was performed with a perfusion rate of 1.0 reactor volumes per day ($D = 1.0$) in 2 l scale and with 0.5 reactor volumes per day ($D = 0.5$) in 100-l scale.

After 24 h the perfusion was stopped and the particle concentration in the bioreactor was determined with the CASY-system. The retention was calculated by following formula:

$$R_P(\%) = 100 \cdot \frac{X_e}{X_s}$$

with:

- X_s = Initial particle concentration.
- X_e = Particle concentration after 24 h of perfusion.

In this case, retention was 100% if X_s and X_e were the same. In contrast, retention was 0% if all particles were removed from the reactor ($X_e = 0$).

Results

Actual and theoretical possible cell growth

In Figure 3 the perfusion cultivation in 100-l scale is illustrated. The bioreactor was inoculated with 5 l of a seed culture in a volume of 40 l and a cell concentration of approximately 2.5×10^5 cells ml^{-1} with 90% viability. The culture was expanded to a concentration of approximately 1.0×10^6 cells ml^{-1} for 2.9 days (first expansion phase). Afterwards the reactor was filled up to 100 l. The perfusion started with $D = 0.3$ (30 l per day) and SRV set to 100 rpm at the end of the second expansion phase at day 5.2. During 14 days of cultivation the perfusion rate and SRV were changed manifold. The combination of perfusion rate and SRV resulted in at least seven perfusion modes. $D = 0.5$ (50 l per day) in combination with SRV = 100 rpm was set for two times. The cells reached a maximum concentration of 6.0×10^6 cells ml^{-1} after 11.9 days. Maximum specific growth rate (μ_{max}) was obtained during both expansion phases prior to perfusion cultivation ($\mu_{\text{max}} = 0.65 \text{ days}^{-1}$) although aggregate formation could be observed during the second expansion phase between day 2.9 and day 5.2 as described below. Viability kept stable at more than 90% during the complete cultivation.

Starting the perfusion the cell concentration was not increasing with μ_{max} but only with a decreased growth rate ($\mu = 0.27 \text{ days}^{-1}$). This effect could be observed immediately after starting the perfusion and continued until the end of the cultivation process. As far as could be detected both aggregate formation, limitation of an essential substrate (glucose, amino acids) and accumulation of an inhibitory component (lactate and ammonia) were not causal. As mentioned before, aggregate formation already occurred during the second expansion phase where the culture grew with μ_{max} . Lactate concentration increased to approximately 20 mmol l^{-1} during both expansion phases. Starting the perfusion, this concentration kept stable for another three days of cultivation. Subsequently, the lactate concentration increased slightly to a maximum of 30 mmol l^{-1} after 11.9 days. Ammonia concentration increased during the expansion phases to 3.5 mmol l^{-1} and kept stable during the perfusion cultivation (both data not shown). Hence, a putative rapid toxic effect of lactate and ammonia concentrations on the cells at the beginning of the perfusion can be excluded. During several previous batch cultivations with this CHO cell line in bench and pilot scale

lactate and ammonia concentrations of 25 and 4 mmol l^{-1} , respectively, were achieved without any harmful effect on cell growth (data not shown). In the case of hybridoma cells toxic effects were observed above 30 mmol l^{-1} lactate (Chen et al., 1992) and also above 4 mmol l^{-1} ammonia (Glacken et al., 1988; Ozturk et al., 1992; Chen et al., 1992). Thus, an insufficient retention performance of the spinfilter due to removal of cells from the reactor (cell bleed) was the obvious cause for the instantly reduced growth rate. Nevertheless, a significant cell accumulation in the reactor was achieved.

Development of retention and alterations in culture condition

Figure 4 shows the retention of single living cells and aggregates determined by CASY and with the Neubauer chamber (crystal violet and trypan blue staining). The retention increased independent from perfusion parameters in a linear way from 75 to 95% (Neubauer chamber). CASY determines an aggregate only as one particle. Due to aggregate formation of CHO cells, cell concentrations determined by CASY were lower than determined with the Neubauer chamber. A comparison in cell concentration development of both methods indicates that aggregate formation started at day 3.7 (during the second expansion phase) and increased rapidly until the end of the cultivation process (see Figure 5). Lower retention calculated with CASY data documents that the concentration of aggregates in the bioreactor was much higher than in the perfusate. Thus, spinfilter performance increased merely corresponding to aggregate formation and operation time. Also reduction of mesh pore size of the spinfilter due to fouling could be causal even if significant fouling or cell settlement onto the spinfilter mesh could not be observed as the spinfilter surface was examined at the end of cultivation. However, cells and aggregates were obviously retained by sieve effect more than by acceleration effect.

Removal of dead cells and cell debris

Spinfilter performance is optimal if debris and dead cells are removed from the bioreactor and if living cells and aggregates of living cells are retained. With the help of CASY data we examined retention of particles due to particle diameter and operating time of the spinfilter. In Figure 6 retention of culture samples from the beginning (sample No. 8, day 5.9) the middle (sample No. 15, day 10.2) and the end of the perfusion

cultivation (sample No. 21, day 14) is documented (refer to the sample Nos. in Figure 5). Particles were summarized in classes of each $2\ \mu\text{m}$. Retention of particles with sizes larger than $13\ \mu\text{m}$ increased corresponding to enlarging particle size and to longer spinfilter operating time. In contrast, particles smaller than $13\ \mu\text{m}$ were increasingly removed corresponding to these parameters. $13\ \mu\text{m}$ seems to be the *operation cut off* of this particular spinfilter even if the pore size is slightly larger ($20\ \mu\text{m}$). Thus, debris and dead cells are removed while living cells and aggregates are retained. But even particles with a size larger than pore diameter (aggregates $>20\ \mu\text{m}$) were not retained completely. Flexibility of aggregates could be causal so that they are squeezed through the mesh pores by perfusion flux.

Referring to Figure 7 the used spinfilter is more suitable for perfusion cultivation of CHO cells than of hybridoma cells (HB-58, ATCC 187.1). The maximum peak of HB-58 (single living cells) is at $13\ \mu\text{m}$ – the *operation cut off* of the spinfilter. Thus, most of these cells would be washed out during perfusion cultivation. Hence, a spinfilter with a pore size smaller than $13\ \mu\text{m}$ is required for perfusion cultivations of hybridoma cell lines. This is confirmed by results from perfusion cultivations of hybridoma cell line HB 58 using the same set up and spinfilter system. In this case only a maximum retention of 60% was achieved at a SRV of 100 revolutions per minute (data not shown). In the case of CHO the diameter of single living cells was increasing from $13\ \mu\text{m}$ (sample No. 8) to $16\ \mu\text{m}$ (sample No. 21) corresponding to cultivation time which led to a growing retention of the cells in the bioreactor during perfusion cultivation.

Retention of model particles

The present work has shown that spinfilter performance corresponded to aggregate formation and not to perfusion parameters (perfusion flux, SRV). To avoid such effects of alterations in culture condition we examined retention of solid model particles (Superose 6). Investigations in 2-l scale illustrated that retention could be increased corresponding to enhanced SRV's in a range of 51–141 rpm (tip speed = $0.15\text{--}0.43\ \text{m s}^{-1}$) from 64% to 88% but decreased rapidly between 141–185 rpm (tip speed = $0.43\text{--}0.56\ \text{m s}^{-1}$) to 74% (see Figure 8). Comparable results were received in previous investigations (Fenge et al., 1993). Interference of SRV and acceleration of particles towards the spinfilter surface could be causal, so that the time window for

the particles to pass the spinfilter mesh is smaller with 141 rpm than with other SRVs. In 100-l pilot scale retention decreased from 87 to 80% between 35 and 70 rpm (tip speed = $0.37\text{--}0.73\ \text{m s}^{-1}$). With further enhancement of SRV up to 120 rpm (tip speed = $1.26\ \text{m s}^{-1}$) the retention increased from 80 to 92% (see Figure 9). Finally, the optimal achieved retention with a tip speed of approximately $0.4\ \text{m s}^{-1}$ in 2-l scale was nearly the same in 100-l scale (retention = 87–88%).

Summary

Investigations in perfusion cultivations of a recombinant CHO cell line using a $20\ \mu\text{m}$ spinfilter in 100-l scale were performed. Spinfilter performance (retention) did not correspond to changes in perfusion parameters (SRV, perfusion flux). Formation of aggregates during cultivation, typical for CHO cells in suspension cultures, was the main reason for the increasing retention. $13\ \mu\text{m}$ was obviously the *operation cut off* for this spinfilter even if the pore size was slightly larger ($20\ \mu\text{m}$). Particles smaller than $13\ \mu\text{m}$ (especially debris and dead cells) were removed from the bioreactor as desired. An accumulation of living cells in the bioreactor was obtained although a cell bleed occurred. The presented method for determination of the *operation cut off* should facilitate the decision for this spinfilter system according to the average cell diameter of each cell line without any further investigations. Additionally, this procedure might be applicable to determine the *operation cut off* of other spinfilter systems as well. Hence, perfusion cultivation of CHO cells with this particular spinfilter would be more suitable than perfusion cultivation of hybridoma cells with a smaller average cell diameter. Regarding the *operation cut off* of the spinfilter those hybridoma cells would be washed out more rapidly as far as they do not show aggregate formation. Therefore, spinfilters with pore sizes smaller than $20\ \mu\text{m}$ are necessary in the case of hybridoma cell perfusion cultivation to avoid significant cell bleed. Retention performance of a $20\ \mu\text{m}$ spinfilter in 2-l scale using solid model particles (Superose 6) instead of cells resulted in an optimum curve with best retention at 141 rpm SRV (tip speed = $0.43\ \text{m s}^{-1}$). A high retention of these particles in pilot scale could already be achieved with a low SRV of 35 rpm (tip speed = $0.37\ \text{m s}^{-1}$) but a distinct optimum curve could not be achieved. Thus, $20\ \mu\text{m}$ spinfilter perfusion cultivation in 100-l pilot scale with low agitation would be

possible. Hence, the addition of shear force protectives (e.g. Pluronic® F-68) could be avoided so that the purification of the target protein from the supernatant would be facilitated.

Acknowledgement

We kindly acknowledge the free provision of Superose 6 by Paul Engberg from Amersham Pharmacia Biotech.

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