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Clarification of animal cell cultures on a large scale by continuous centrifugation

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SUMMARY

Animal cells from 80-L and 2000-L fed batch fermentations were removed by a prototype disc stack centrifuge in order to achieve a fast and reliable separation of solids from large quantities of cell culture fluids. The clarification capacity was excellent for animal cells but particles remained in the liquid phase and affected further downstream processing of the cell-free harvest fluid. No significant loss of product was observed. A number of parameters were monitored to optimize process conditions for use with animal cells.

INTRODUCTION

At the present time the production of biologically active proteins by mammalian cell culture systems is possible up to a working volume of 12 000 L [11]. These cells are able to synthesize even very large and complex molecules [8,10]. Animal cells have been designed to grow in suspension and to secrete their products into the culture medium. Thus, efficient unit operations need to be established for cell separation [4,5] up to the 12 000-L scale to prepare the product-containing fluid for further purification of the products.

Tangential flow microfiltration (TFF) is very efficient for this separation process [6,9]. In contrast to dead-end filtration, high processing capacities with high cell densities can be obtained. TFF systems can be operated as closed systems and achieve a complete clearance of cells and cell debris down to a particle size of the certified pore size. These systems are scalable depending upon available membranes, housings and pumps. They are re-usable and they allow high cell concentration factors without damage to cells and products. Disadvantages of TFF comprise time-dependent pressure and flux profiles during a single and successive operation due to particulate and chemical membrane fouling, the complexity of the system at the large scale and the laborious operation and cleaning procedures.

Hence, an alternative physical principle for the clarification of product-containing cell culture fluid was investigated in this study. Using a disc stack centrifuge it was hoped that the harvest conditions would be less variable and protein adsorption would not be a problem. When compared to crossflow microfiltration [9], the mode of operation as well as integrated cleaning in place and sterilizing in place programs should reduce interference and the necessity of observation by man. A prototype disc stack centrifuge developed for clearance of yeast fermentation fluids was tested. One of our major objectives was to obtain a clarified liquid phase without cells and particles that were generated by cell disruption. The effect of this unit operation on the cells in the sediment and the potential re-use for subsequent fermentation runs or for perfusion procedures was not an objective of this study.

The centrifuge was run at various hardware configurations and process conditions to investigate optima and limits of a dependable operation mode for the cell line used. The scale-up potential of the disc stack centrifuge was evaluated for harvest conditions of 12 000 L cell culture fluid in an appropriate time that can be achieved by conventional microfiltration methods.

MATERIALS AND METHODS

All experimental work reported in this paper was performed with a Westfalia (Oelde, Germany) CSA-1 high speed stainless steel disc stack centrifuge. A sectional drawing of this separator and details of mechanical design are given in various papers [1,7]. The centrifuge was equipped with 43 conical discs that formed a disc gap width of ca 0.5 mm. The clarification surface was 1900 m^2. The cell suspension was fed to the rotating bowl by a central pipe and accelerated to the rotating speed of the bowl (4000–9000 r.p.m.). Separation of solid particles from the liquid phase occurs in the narrow flow channels of the disc stack so that heavy particles only have short distances to travel until they collect on a wall. At the sloping underside of every disc in the stack the cells are deposited in a cohesive layer [7] along the disc surface. At the bottom edge of the

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disc this mass slides further into the sediment holding space [7]. The clarified liquid phase flows to the top of the bowl and is continuously discharged via a nozzle.

The prototype centrifuge used in this study offers two different principles to remove the solid phase from the sediment holding space: discontinuously by activating an intermittent discharge mechanism (standard equipment) [1] or continuously by a special separation disc (modification of this prototype) that allowed the sediment to rise along the inner bowl surface. On the top of the bowl it is then discharged by an additional seizing disc equipped with nozzles for the solid phase in the same way as for the clarified liquid phase. Both types of sediment discharge were explored.

For cell harvesting, the unmodified cell culture fluid was fed to the disc stack centrifuge at a constant pressure of 1.0 bar. The throughput of solid and liquid phases is adjustable by separate pneumatic valves. Hence, the portion of solid phase could be varied in order to minimize product losses via the solid phase share. Throughput, temperature and pressure of the suspension inlet, solid phase outlet and effluent of supernatant phase were monitored periodically using flow meter, temperature and pressure gauges.

Harvest experiments at 80 L and 2000 L batch cultures were performed using one specific hybridoma cell line secreting an IgG 2b monoclonal antibody. Cell concentration and viability were analyzed microscopically by trypan blue exclusion. For the determination of wet biomass ca 30 g of sample were weighted prior to centrifugation at 37 000 × *g* for 20 min. The supernatant fluid was decanted and the pellet was weighted. Wet biomass was calculated as the weight portion of the pellet. Product concentration was measured by an ELISA method.

RESULTS

Impact of centrifugal forces on cell viability

Rotating speeds of 4000 r.p.m. to 9000 r.p.m. were applicable for the CSA-1 centrifuge. Table 1 summarizes results at various rotating speeds and their effect on cell viability during all centrifugation experiments of this study. Viability was not affected at low rotating speeds but clarification was not achieved. With increasing centrifugal forces clarification was better but showed some damaging effect on the cells (see below). For subsequent experiments both extreme parameters of 4000 r.p.m. (lowest shear stress for the cells) and 9000 r.p.m. (highest clarification potential) were chosen for further studies.

Separation at continuous discharge of sediment

In Fig. 1 a typical profile of cell concentrations in the solid phase and clarified liquid phase during a run is shown. The sediment was discharged continuously and rapidly via the separation disc and was not allowed to accumulate in the bowl. Remaining cell concentration in the clarified liquid phase was measured ca $0.03 \cdot 10^6$ cells ml^{-1} or about 1% with respect to the feed concentration. Thus, the continuous discharge via the separation disc was very effective. Within a few minutes after the start the cell density in the solid phase became too high to count (TNTC) and stayed at this level (Fig. 1).

At low cell densities in the feed stock (e.g. $1.47 \cdot 10^6$ cells ml^{-1} at experiment no. 6) the continuous discharge started later (Fig. 2) The separation disc did not remove the sediment quantitatively. Under these conditions, the cells sedimented into the sediment holding space. They accumulated

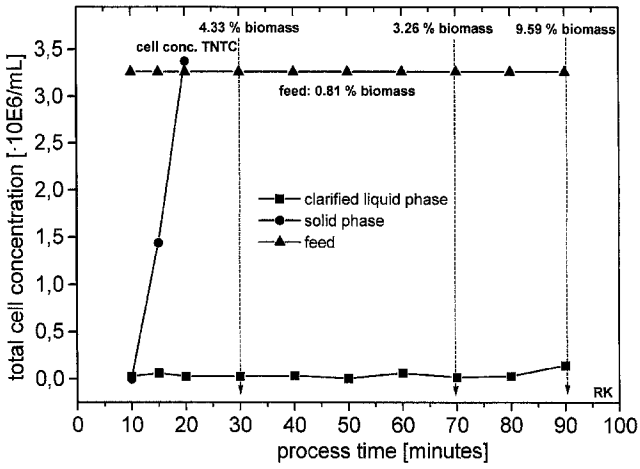

Fig. 1. Mammalian cell separation performance with CSA-1 disc stack centrifuge with continuous solid phase output via separation disc. Parameters: rotating speed 9000 r.p.m., nozzle diameter 1 mm, seizing disc diameter 72 mm, throughput 50 L h^{-1}, sediment discharge 1.5 L h^{-1}. Experiment no. 13.

TABLE 1

Cell viability measured as percent viable cells of total cells relative to that found in the feeding cell suspension at different centrifugal forces. The viability range was measured in specific experimental runs indicated in column 4

Rotating speed (r.p.m.)	Centrifugal force at the outer boundary of the disc stack (*g*)	Cell viability with respect to feed (%)	Experiments no.
4000	983	71.4–100	7, 8, 9, 16
5000	1536	35.5–75.5	4, 7, 9
6000	2211	14.4–43.6	4, 6
8000	3931	0	3, 6, 8
9000	4975	0	5, 10, 11, 12, 13, 14, 17, 18

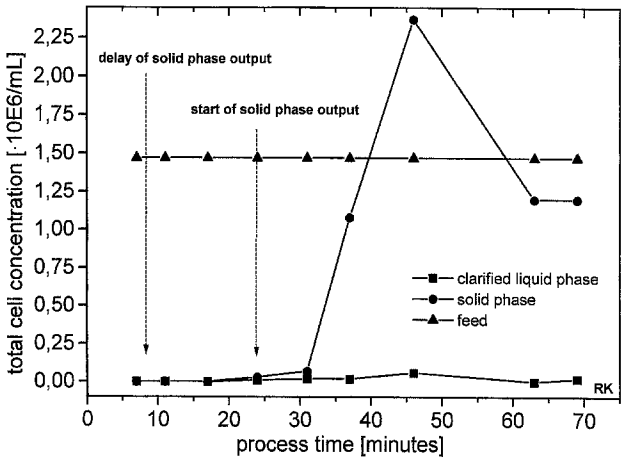

Fig. 2. Mammalian cell separation performance with CSA-1 disc stack centrifuge with continuous solid phase output via separation disc. Parameters: see Fig. 1. Experiment no. 6.

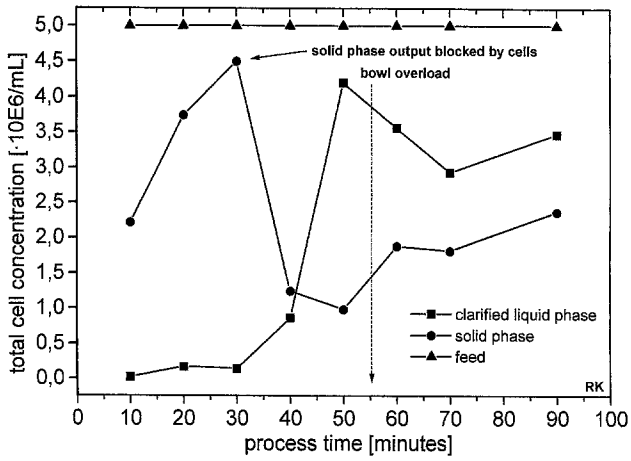

Fig. 4. Mammalian cell separation performance with CSA-1 disc stack centrifuge with continuous solid phase output via separation disc. Parameters: see Fig. 1. Experiment no. 16.

slowly until in a kind of overflow some packed sediment is mobilized and escaped via the separation disc. The time until this first biomass is seen in the efflux is dependent on the cell density of the feed stock and is enhanced when cell density in the feed was decreased (see Figs 2, 3). For example, in experiment no. 6 (Fig. 2) and 14 there was a delay in sediment discharge of ca 20–25 min at $1.5 \cdot 10^6$ cells ml^{-1} in feed and in experiment no. 8 (Fig. 3) a delay of ca 45–50 min at $0.47 \cdot 10^6$ cells ml^{-1} in feed. The clarification capacity however is not impaired and was measured at 98.6% and 99.7% and 97.9% respectively.

At very high cell density (ca $5.0 \cdot 10^6$ cells ml^{-1}) the centrifuge did not cope with the cell separation. Very soon after the start of operation the sediment holding space was filled with cells and clogged the effluent stream retaining all cells, instead cells were discharged in the 'clarified' phase (Fig. 4; experiment no. 16). At 55 min process time the bowl including the disc stack was overloaded completely and most of the cells were ejected via the hydraulic discharge system that opened automatically due to the increasing pressure in the bowl.

Separation at intermittent discharges of sediment

The centrifuge bowl was modified by removal of the separation disc. The discharge nozzles were plugged by stoppers. The hydraulic intermittent discharge mechanism was activated when 30 L clarified liquid phase had been processed. In experiment 17 and 18 cell densities of $1.77 \cdot 10^6$ cells ml^{-1} and $4.38 \cdot 10^6$ cells ml^{-1} respectively were measured in the feed. Cells were not found in the clarified liquid phase, i.e. a clarification capacity of 100% was obtained. In the discharged sediment cells were concentrated 20-fold, because the wet biomass content of the sediment was 10% with regard to 0.51% in the feed stock. A substantial advantage of the discontinuous cell withdrawal mechanism was the insensitivity of the discharge to pressure fluctuations. The sediment was removed reliably at regular intervals and did not accumulate inside the bowl and the separation disc.

Product and cell recovery

Product titers were monitored in feeding cell suspension, the sediment and clarified efflux. As shown in Fig. 5, no sig-

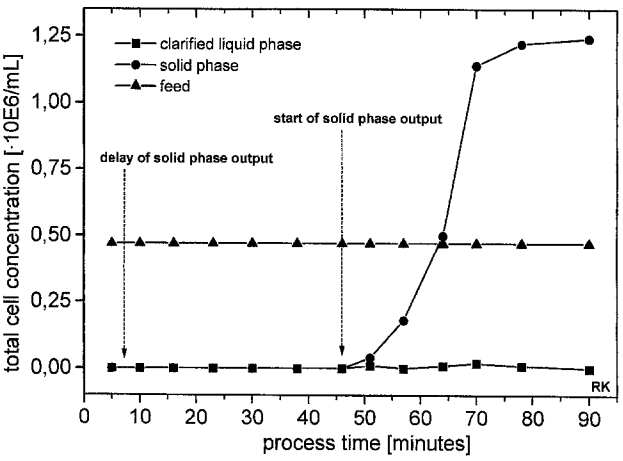

Fig. 3. Mammalian cell separation performance with CSA-1 disc stack centrifuge with continuous solid phase output via separation disc. Parameters: see Fig. 1. Experiment no. 8.

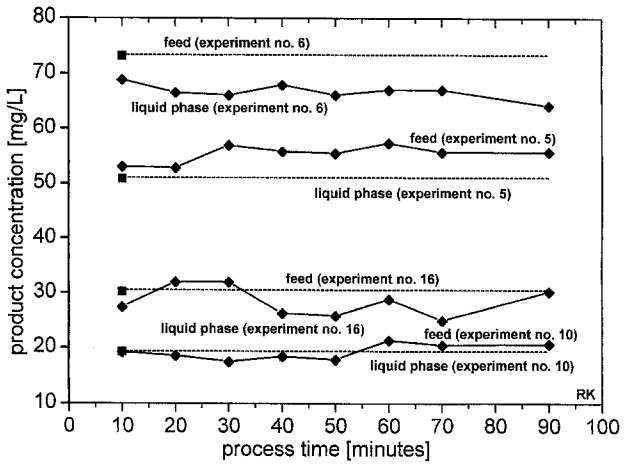

Fig. 5. Course of product concentration in feed and liquid phase during different disc stack centrifugation experiments. Parameters of centrifugation: see Fig. 1.

nificant loss was detected during the entire operation. There was no indication for an accumulation of IgG in any sediment. However, the sediment-containing phase should be a very small part of the throughput (approximately 3% of the throughput in most experiments), because the product there is lost. Thus, the product yield of this type of centrifugal separation process can be very high.

Biomass

The accounting for the cells is complicated by some technical difficulties in obtaining accurate measurement of cell number from sedimented biomass. About 20% of the feed cells were destroyed in case of continuous or discontinuous discharge mode. The debris of these cells increases the particle load in the clarified liquid phase. This is a serious drawback because further downstream processing via ultrafiltration and/or chromatography is impaired by particles. For our specific objective it was not essential that the cells are viable but that they do not burst and release particles and contaminating substances into the product-containing culture fluid and thus into the supernatant. The size distribution was analyzed by Coulter counter and laser light diffraction devices. Presence of particles was demonstrated by dead-end filtration and microscopical observation visible as 'sandy' ground due to light refraction. Detailed results are presented in [3] and reveal that subcellular particles could not be removed efficiently enough by the disc stack centrifuge. The feed stream did not contain particles in this order of magnitude (microscopical examination), did not cause any problems in dead-end filtration experiments and was consistent throughout the experiments. Consequently, at least one filtration step has to be implemented between centrifugation and any further downstream processing such as ultrafiltration or chromatography.

Improvement of process parameters

The rotation speed is the most important feature for separation by sedimentation. As stated above, high centrifugal forces decrease the viability of the applied cell line (Table 1) but improve clarification. Variation of the flow rate led to 1.8–0.72 min residence time of liquid phase in the bowl at a throughput of 20 L h^{-1} to 50 L h^{-1}. The design of separation disc, seizing disc, nozzles and fittings were optimized using various commercially available standard components. In addition to rotating speed other parameters such as throughput, proportion of discharged sediment, pressure and temperature effects have been addressed and optimized somewhat for the continuous operation mode. Table 2 summarizes a set of experiments with continuous solid phase removal but modifications in various operation parameters.

Applying the 72-mm seizing disc within the centripetal pump, constant parameters of 9000 r.p.m. rotating speed, 50 L h^{-1} throughput and 3% sediment discharge portion, more than 95% of the cells were sedimented (Table 2). However, there was a dependency of the clarification capacity on the input of cells (see run 14 and 13 in Table 2). At high cell density there is some spill over into the clarified fraction, while variations in throughputs and proportion of sediment containing discharge did not change the efficient cell removal.

At 4000 r.p.m. the transport of the cell mass via separation disc and seizing disc did not function. Cells were not found in the solid phase output at any feed cell concentration (run 15 and run 16 in Table 2). The discharge of sediment was unreliable and occurred only if fluctuations in the input pressure or the throughput resulted in some dislodgement of sediment (run 8 and 7 in Table 2). The clarification capacity of these runs was slightly lower than the capacity at higher rotating speeds, e.g. at run 5 (Table 2).

In contrast, using a smaller seizing disc for the solid phase discharge cells did not appear in either the solid or liquid phase (run 11, run 12 in Table 2). The same result was obtained even at different experimental process conditions within the experiment (run 10 in Table 2), i.e. slight pressure fluctuations did not expel the cells. At the end of run 10 the cells could be discharged when the liquid phase flow was stemmed at the outlet by a throttling pressure of 2.5 bar, but high pressure cannot be recommended for animal cells. Anyhow a clarification performance of 98.8% was obtained.

In conclusion, an extensive separation of cells was possible in a number of tested conditions. However, the intended regular and continuous discharge of sediment at a very short residence time was rarely realized. Outside a very narrow range of operation conditions the cells were separated from the liquid culture medium but were packed up in the sediment holding space and were not discharged.

Therefore, the applied separation disc, designed and scheduled for use with yeast and bacterial cell suspensions, may have to be modified to permit a satisfactory application to animal cells. Somewhat better results were obtained using the hydraulic intermittent discharge system (see above), which might however further either the disruption of cells when they remain longer in the bowl or the more diluted sediments when the discharge mechanism is activated frequently. Stringent sedimentation at low temperature (e.g. at 4 °C) might improve the separation performance significantly. This process modification was not tested, because aggregation of product and precipitation of substances might occur in the cold and have detrimental effects on the further purification process.

Scale-up potential of the disc stack centrifuge

Generally the scale-up possibility of this technology is promising because large-scale disc stack centrifuges up to 150 m^3 h^{-1} capacity are manufactured in series production [12]. Such clarifiers are designed for use in pharmaceutical industry and have various desirable features for containment such as sealing of feed and discharge pipes, maintenance of vacuum under the bowl, overlay pressure inside the bowl, slide-ring seal insulation of drive and bowl chambers, adequate cleaning-in-place and sterilization-in-place methods, venting by sterile filters, etc. A steam-sterilizable clarifier system for fully automatic operation is described in [2].

Regarding the operation conditions, a number of parameters require close attention. The warm up of material and the pressure profile inside the bowl have to be checked as well as the shear forces at the inlet of the bowl and at the centripetal pump (where liquid phase and possibly sediment containing phase are discharged continuously with help of a seizing disc). It is

TABLE 2

Clarification capacity at different operation modes. All experiments were performed with continuous solid phase discharge via separation disc, seizing disc and centripetal pump. 3% SP indicates that 3% of the throughput was released via solid phase output. The asterisks indicate a variation of solid phase share between 3% and 10% within the experiment

Experiment no.	Rotating speed	Seizing disc diameter	Throughput ($L\ h^{-1}$)	Nozzle size	Cell density in feed ($\cdot 10^6$ cells ml^{-1})	Clarification performance (%)
14	9000 r.p.m.	72 mm	50 (3% SP)	1 mm	1.5	99.7
13	9000 r.p.m.	72 mm	50 (3% SP)	1 mm	3.26	95.4
5	9000 r.p.m.	72 mm	20–50 (*)	1 mm	1.7	99.4
15	4000 r.p.m.	72 mm	50 (3% SP)	1 mm	0.95	no cells discharged
16	4000 r.p.m.	72 mm	50 (3% SP)	1 mm	3.34	no cells discharged
8	4000 r.p.m.	72 mm	20–50 (*)	1 mm	0.47	97.9
7	4000 r.p.m.	72 mm	20–50 (*)	1 mm	1.61	98.8
11	9000 r.p.m.	42 mm	50 (3% SP)	1 mm	0.95	no cells discharged
12	9000 r.p.m.	42 mm	50 (3% SP)	1 mm	1.94	no cells discharged
10	9000 r.p.m.	42 mm	20–50 (*)	1 mm	2.86	no cells discharged

reasonable to assume that the clarification capacity is improved in bigger clarifiers. Larger instruments such as CSA-8, CSA-19 and SB-60 have room for 1.5-, 3.9- and 17-L sediment holding space respectively and provide an equivalent clarification surface of 10 600, 29 000 and 68 400 m^2 respectively [2]. Thereby they permit a scale up of the CSA-1 device (0.25-L sediment holding space, 1900 m^2 equivalent clarification surface). Experiments have not been performed to demonstrate the suitability of such large clarifiers for animal cell cultures.

DISCUSSION

The experiments reported were performed to assess the suitability of an industrial disc stack centrifuge for separation of animal cells from the product-containing cell culture medium. The centrifuge was run both in the standard mode used e.g. for yeast separation and in a modified design with continuous solid phase output. The latter operation mode was chosen to reduce the residence time of sensitive animal cells in the bowl to 1–2 min when compared to the intermittent discharge operation mode (approximately 30 min).

Cell separation and product recovery were very successful both with the standard operation mode and with the special separation disc. 95–100% of the cells were removed from the culture fluid and product yields of ca 90–95% were attained in the clarified liquid phase. However, the prototype device operated well only at a narrow range with continuous discharge of cells. Low cell densities led to a delayed output of the solid phase while very high cell densities caused a bowl overload and spill over of cells into the clarified effluent. In addition the machine was very sensitive to pressure fluctuations. Consequently pressure and throughput had to be adjusted repeatedly by hand during the runs and process automation was impossible.

Thus, centrifugation with intermittent discharge of sedimented cells proved to be the best and most predictable operation procedure in spite of the extended residence time of the cells in the bowl. Furthermore, handling of the machine was more comfortable and less complicated.

A general problem is the impact of centrifugation on cell viability and on particle load in the clarified liquid phase. High rotating speeds affected the viability in this study and up to 20% of the cells were destroyed inside the bowl. Although the hybridoma cell line used is not as robust as some recombinant CHO cell lines and some other hybridoma cell lines in our hands, the maximum tolerated pressure and shear stress has to be examined for every cell line of interest. Possibly some optimization of mechanical engineering could improve this prototype that was initially designed for other applications than for the removal of relatively fragile hybridoma cells. As the purpose of the operation is to generate a suitable feed stock for downstream purification, the high load of subcellular particles has to be reduced significantly. Thus, if the question was to reduce the number of steps in the downstream isolation of a product, the centrifugation does not make a subsequent microfiltration redundant. Rather, the new generated particles add to difficulties for the filtration. Hence, a suitable filtration step may make a preceeding centrifugal clarification redundant. Furthermore the total processing time, during which the product may be at some risk of proteolytic attack by contaminating protease, appears to be shorter for membrane filtration systems [6,9].

The most plausible use of the disc stack centrifugation might be the clarification of very high cell density cultures to relieve the strain on tangential flow microfiltration. Then the clarifier should be operated with intermittent discharges of the solid phase and be followed by a small microfiltration system to remove subcellular particles. However only a comparative experiment can reveal whether the actual separation can be performed as well by single-step microfiltration.

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REFERENCES

1 Hemfort, H. 1983. Separators. Technical Scientific Documentation No. 1 (booklet No. 9997–7133–010/0483) of Westfalia Separator AG, 59302 Oelde, Germany.

2 Hemfort, H. and W. Kohlstette. 1988. Centrifugal Clarifiers and Decanters for Biotechnology. Technical Scientific Documentation No. 5 (booklet No. 9997–8203–000/0688) of Westfalia Separator AG, 59302 Oelde, Germany.

3 Kempken, R., A. Preißmann and W. Berthold. 1994. Assessment of a disc stack centrifuge for use in mammalian cell separation. Accepted for publication in Biotechnol. Bioengin.

4 Lee, S.-M. 1989. The primary stages of protein recovery. J. Biotechnol. 11: 103–118.

5 Mackay, D. and T. Salusbury. 1988. Choosing between centrifugation and cross-flow microfiltration. Chem. Engin. 447: 45–50.

6 Maiorella, B., G. Dorin, A. Carion and D. Harano. 1991. Crossflow microfiltration of animal cells. Biotechnol. Bioengin. 37: 121–126.

7 Mannweiler, K. and M. Hoare. 1992. The scale-down of an industrial disc stack centrifuge. Bioproc. Engin. 8: 19–25.

8 Spier, R.E. 1988. Animal cells in culture: moving into the exponential phase. Trends Biotechnol. 6: 2–6.

9 van Reis, R., L.C. Leonard, C.C. Hsu and S.E. Builder. 1991. Industrial scale harvest of proteins from mammalian cell culture by tangential flow filtration. Biotechnol. Bioengin. 38: 413–422.

10 Werner, R.G. and C.-H. Pommer. 1990. Successful development of recombinant DNA-derived pharmaceuticals. Arzneimittelforschung/Drug Research 40: 1274–1283.

11 Werner, R.G., F. Walz, W. Noé and A. Konrad. 1992. Safety and economic aspects of continuous mammalian cell culture. J. Biotechnol. 22: 51–68.

12 Westfalia Separator Report. Introduction to Westfalia Separator AG and Some of the Fields of Application of the Machines, Installations and Processes. Booklet no. 9997–4776–010/0189 (engl.) of Westfalia Separator AG, 59302 Oelde, Germany.