Scale-Up and Optimization of an Acoustic Filter for 200 L/day Perfusion of a CHO Cell Culture

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Abstract: Acoustic cell retention devices have provided a practical alternative for up to 50 L/day perfusion cultures but further scale-up has been limited. A novel temperature-controlled and larger-scale acoustic separator was evaluated at up to 400 L/day for a 10^7 CHO cell/mL perfusion culture using a 100-L bioreactor that produced up to 34 g/day recombinant protein. The increased active volume of this scaled-up separator was divided into four parallel compartments for improved fluid dynamics. Operational settings of the acoustic separator were optimized and the limits of robust operations explored. The performance was not influenced over wide ranges of duty cycle stop and run times. The maximum performance of 96% separation efficiency at 200 L/day was obtained by setting the separator temperature to 35.1°C, the recirculation rate to three times the harvest rate, and the power to 90 W. While there was no detectable effect on culture viability, viable cells were selectively retained, especially at 50 L/day, where there was a 5-fold higher nonviable washout efficiency. Overall, the new temperature-controlled and scaled-up separator design performed reliably in a way similar to smaller-scale acoustic separators. These results provide strong support for the feasibility of much greater scale-up of acoustic separations. © 2002 Wiley Periodicals, Inc.

Keywords: acoustic; separator; scale-up; perfusion; mammalian cell culture; continuous; optimization; Chinese hamster ovary cells (CHO cells); cell retention; separation

INTRODUCTION

Approval of increasing numbers of mammalian cell products is expected to significantly stimulate worldwide demand for production capacity (Garber, 2001). Some limitations will be relieved by converting from fed-batch to perfusion processes to obtain order of magnitude increases in bioreactor volumetric productivity. For efficient perfusion processing, it is necessary to select a reliable and simple to operate cell separation device.

Most mammalian cell retention devices used for perfusion processes (Woodside et al., 1998) are based on size, e.g., cross-flow filters (Van Reis et al., 1991; Mercille et al., 1994) and spin filters (Himmelfarb et al., 1969; Deo et al., 1996; Roth et al., 1997) or combined size and density differences between the cells and the medium, e.g., inclined settlers (Batt et al., 1990; Thompson et al., 1994) and centrifuges (Tokashiki et al., 1990; Johnson et al., 1996). Devices based on filtering have the advantage of clarifying particulates from the permeate. Settlers (Batt et al., 1990) and centrifuges (Tokashiki et al., 1990) are less prone to fouling (Deo et al., 1996) and can provide high separation efficiencies at high flow rates.

Acoustic separators provide an alternative cell retention system based on forces generated in an ultrasonic standing wave field. Cells that are exposed to an acoustic field reversibly aggregate at the pressure node planes of the field (Kilburn et al., 1989; Pui et al., 1995). This separation is based on size, density, and compressibility differences between cells and smaller particles or medium (Coakley et al., 1994), providing selective retention of viable vs. nonviable cells (Doblhoff-Dier et al., 1994; Trampler et al., 1994; Pui et al., 1995). Exposure of mammalian cells to MHz-range acoustic standing wave fields at power inputs of up to 220 W/L had no impact on culture viability, glucose uptake rate, or antibody production (Pui et al., 1995). The first acoustic separator used in a perfusion culture yielded a 70-fold increase in volumetric productivity compared to batch (Trampler et al., 1994). Doblhoff-Dier et al. (1994) reported the highest superficial velocities (720 cm/h) with greater than 90% separation efficiency. CHO cell densities of up to

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25 × 10^6 cells/mL at separation efficiencies of 90% were obtained for perfusion rates of up to 10 L/day (Ryll et al., 2000).

Acoustic separators are relatively simple, reliable, and nearly maintenance-free, but their use has been limited to flow rates up to only ~50 L/day (Woodside et al., 1998). In this work, a new water-cooled and multiple-chamber design for acoustic separations was tested and operations optimized at 250 L/day perfusion. A preliminary 100-L CHO cell perfusion process is presented, followed by the results from investigations to optimize the performance of the new acoustic cell separator. The responses to operational separator settings were explored, including the variation of acoustic power input and its duty cycle and the temperature within the separator. The maintenance of culture viability and selective separation of viable cells were also analyzed. Finally, the performance of 10-L and 200-L acoustic separators were compared and the implications for further modular scale-up discussed.

MATERIALS AND METHODS

Perfusion Culture

A 100-L working-volume stainless-steel bioreactor (Bio Pilot ADI 1075; Applikon, Foster City, CA) equipped with a 150-mm marine impeller was seeded at a volume of 100 L Ex-Cell® 325 PF CHO medium supplemented with 5 g/L glucose and 8 mmol/L glutamine. The CHO cell culture was inoculated at 2 × 10^6 viable cells/mL. Culture temperature, pH, dissolved oxygen (DO), and stirrer speed (100–150 rpm) were automatically maintained using an Applikon ADI 1060 Bio Control controller with an ADI 1040/21 control console interface and data logged by a personal computer. pH (7.0) was adjusted by adding (5 N) NaOH along the side wall of the bioreactor or sparging CO₂. DO was maintained at 50% air saturation by a constant flow of 0.25 L/min air and addition of pure oxygen as needed at up to 5 L/min. Upon inoculation, 50 L/day perfusion was started and increased stepwise when needed to maintain the glutamine concentration above 1.0 mmol/L. Cells were retained in the reactor using a prototype of the newly developed BioSep 200L separator (AppliSens, division of Applikon, Schiedam, Netherlands). A key component of the separator is a four-sectioned, 500 mL, 1.4 MHz BioSep® chamber, powered by an APS 991 controller (AppliSens). The chamber's temperature was controlled by means of a VWR 1162 refrigerated water bath circulator (VWR, West Chester, PA). Perfusion rate was controlled by adjusting the harvest pump rate that was stopped during acoustic filter stop times. To maintain the culture volume the feed pump was triggered by two level sensors. Masterflex L/S peristaltic pumps (digital standard drives models 7523-20 (10–600 rpm) and 7523-30 (1–100 rpm)) equipped with Easy Load II pump heads (model 77201-62) were used for feed, harvest, and recirculation (Cole-Parmer, Vernon Hills, IL).

Analytical Methods

Cell count and particle size analysis were performed using Trypan blue dye exclusion and an automatic cell counter (Cedex AS20; Innovatis, Bielefeld, Germany). Daily bioreactor samples were taken and the glucose, glutamine, lactate, and ammonium concentrations analyzed by a BioProfile 200 Cell Culture analyzer (NOVA Biomedical, Waltham, MA). Recombinant protein concentration was determined by HPLC separation on a polymer-based reversed-phase resin using a Trifluoroacetic acid/Acetonitrile (ACN) gradient at 1% ACN per min. The standard error of the mean (SEM) is provided with data cited in the text and as error bars in the figures.

Separation Experiments

During separation experiments the harvest outlet of the BioSep 200L separator was returned to the bioreactor (Fig. 1) to maintain the cell concentration of the culture while exploring extremes in the ranges of operation. Between 200–800 mL of the separator outlet was sampled for analysis of cell concentration. The reactor cell concentration was determined 3–5 times within one daily set of experiments. In all experiments the separator power input was tuned so that the power limiter was active. The separation efficiency (SE, expressed as a percent) was calculated based on the ratio of the cell concentrations in the outflow and reactor:

\[ SE(\%) = 100 - \left( \frac{C_{\text{Cell, outflow}}}{C_{\text{Cell, reactor}}} \times 100 \right) \]

Figure 1. Schematic of the perfusion system operated in perfusion mode with the harvest line open or, during separation experiments, with the harvest line closed and the separator outflow returned to the bioreactor.

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RESULTS AND DISCUSSION

Perfusion Culture Performance

Perfusion was initiated at 50 L/day from the start of a 100-L culture inoculated at $2 \times 10^6$ CHO cells/mL. After 2 days the viable cell concentration increased exponentially to over $12 \times 10^6$ cells/mL at day 10. The perfusion rate was increased to 200 L/day in order to maintain a glutamine concentration above 1 mmol/L (Fig. 2). The viable cell separation efficiency remained above 95%. Throughout the culture, viability remained between 80–90%. In a manner similar to hybridoma perfusion results (Trampler et al., 1994), the recombinant protein concentration increased over the 16 days of cultivation to a maximum protein concentration of 200 mg/L. The average productivity was maintained at 34 g/day in the last 3 days. During this time period the average volumetric productivity of the perfusion system was 336 mg/L/day, outperforming a 20-L fed-batch cultivation with the same cell-line by a factor of 13 (neglecting greater time needed for turnaround of multiple fed-batches, data not shown). The fed-batch did yield a 27% higher maximum protein concentration at 254 mg/L after 10 days of cultivation.

Acoustic Separator Performance

Within a subsequent 25-day perfusion culture, 8 days of separation efficiency experiments were performed at an average total reactor cell concentration of $\sim 10^7$ cells/mL and a culture viability of 87%. The first sequence of separation efficiency measurements at 139 L/day revealed a pseudo-steady state of performance after 30 min of constant separator operation (data not shown). The separation efficiency was determined for a wide range of perfusion rates from 50–400 L/day based on two samples taken at each flow rate, the first at 40 min after each change and the second after another 20 min to assess the pseudo-steady state reached for each specific condition (Fig. 3).

For the preliminary operations, the average total cell separation efficiency decreased from 96% at a perfusion rate of 50 L/day to 80% at 250 L/day down to 57% at the highest perfusion rate of 400 L/day. The small variation in the separation efficiency at each perfusion rate between 40 and 60 min after a change up to flow rates of 250 L/day demonstrates that clearly distinguished pseudo-steady states were reached within 40 min for each sample point. Higher variability of the separation efficiency at higher perfusion rates was also observed for other conditions and it is not believed that the points at 400 L/day in Figure 3 reflect a reproducible trend in the separation efficiency with time. SEM error bars were derived from the duplicate samples taken at 40 and 60 min for each point in the following investigations of the acoustic separator settings for power input, recirculation flow rate, duty cycle, and temperature. A high constant perfusion rate of 250 L/day starting at $\sim 80\%$ separation efficiency was selected for these studies to allow improved modes of operation to be distinguished (as compared to working at closer to 100% separation efficiency, where improvements would have been difficult to detect). It was also a priority to further optimize the performance at high flow rates to extend the scale-up of the acoustic separator capacity.

Power Input

Operation at three different power settings (60, 75, and 90 W) evaluated the impact of the power input level on the performance of the separator. Changing the power level from 60 to 90 W increased the total separation efficiency from 78 to 82% (Fig. 4). This result is consistent with the need for higher power input at increased harvest flow rates in small-scale acoustic separators (Doblhoff-Dier et al., 1994; Zhang et al., 1998). Nonviable cells were retained with a significantly lower efficiency than viable cells, leading to a selective retention of viable cells, as previously reported for small-scale acoustic separators (Trampler et al., 1994; Pui et al., 1995; Gaida et al., 1996).
Recirculation Flow Rate

Cell culture is continuously pumped out of the reactor and partially recirculated through the lower conical part of the separator to limit the cellular residence times between the bioreactor and separator (Fig. 1). The separation efficiency increased from 81% at 500 L/day to 85% at 1,000 L/day (Fig. 5). Higher separation efficiencies at higher recirculation flow rates could be explained by increased removal of cells and consequently a reduced cell concentration in the lower part of the separator. The decrease in the separation efficiency at a further increased recirculation flow rate of 1,500 L/day might be due to 1) greater turbulence in the lower part of the separator disturbing the cells retained by the acoustic field, or 2) disturbance of separations by a few bubbles observed to be drawn from the bioreactor at this highest recirculation rate. Again, the selective retention of viable cells vs. an enhanced washing out of nonviable cells was observed.

Duty Cycle

The duty cycle operation can enhance the settling of cells out of the acoustically active region of the separator by periodically stopping the acoustic field and harvest pump. The separation efficiency was investigated for stop time settings of 3, 4.5, and 9 sec for a constant run time of 120 sec. No significant effect on the total cell separation efficiency could be seen (Fig. 6). It should be noted that to maintain a constant perfusion rate of 250 L/day, the harvest flow was increased from 262.5 L/day at a stop time of 3 sec to 282.5 L/day at a stop time of 9 sec to compensate for the harvest pump being off during the duty cycle stop periods. The steep descent in separation efficiencies with increasing flow rates above 250 L/day (Fig. 3) probably overcomes the beneficial effect of enhanced settling of cells during greater stop time periods. However, the selective washout of nonviable cells appeared increased at longer stop times. Increasing the run time from 60–120 sec for a stop time of 4.5 sec resulted in only a minor increase in total cell separation efficiency from 78.3 ± 0.1% to 79.2 ± 0.5% (data not shown). Since these duty cycle variables had little if any influence on separator performance, they would appear to be a relatively low priority for optimizations.

Water Bath Temperature

Differences between the separator and water bath temperatures were investigated by adjusting the water bath temperature. During the experiment the water bath temperature was changed from 33.5 to 18.0°C. The highest 90% separation
efficiency was obtained at the lowest temperature difference of 1.6°C and decreased to 72% with increasing temperature difference up to 15.3°C (Fig. 7). This could be explained by increasing free convective flows inside the chamber that would disturb the alignment of cells within the acoustic field and decrease the separation efficiency.

Comparison of Optimized vs. Nonoptimized Operation

The performance of the separator was improved significantly by applying the above optimized settings. For the optimized separation efficiency experiments, the water bath temperature was adjusted so that the separator temperature never exceeded 35.2°C while the water bath temperature was not increased above 35.0°C. The separation efficiency at 250 L/day increased from 80% to a maximum performance of 90% (Fig. 8), which represents a reduction of the amount of washed-out cells by a factor of two. While the separations were not improved at 400 L/day, the optimized separation efficiency was increased at flow rates down to 50 L/day (96–99%). Therefore, the 250 L/day optimized settings could also be used to improve separator performance during earlier stages of the culture, when lower perfusion rates are needed.

Acoustic Separator Effect on Culture Viability

The decreased harvest viability as a consequence of selective retention of viable cells vs. nonviable cells could be suspected due to cell death during separation. This concern was addressed by ensuring that returning the low viability effluent (Fig. 1) to the bioreactor did not decrease culture viability. For instance, in the experiment that yielded the

Figure 6 results the culture viability remained at 90.8 ± 0.3% for 9.1 h despite exposure to the maximum power input (90 W) and a 250 L/day perfusion rate. Over this period of time nearly one culture volume (94.8 L) was processed by the resonator, while 380 L passed through the recirculation pump. Thus, selective separation is responsible for the observed major differences in viability (Figs. 4–7, 9). Previous reports for small-scale acoustic separators also reported no detectable effect on culture viability (Doblhoff-Dier et al., 1994; Trampler et al., 1994; Pui et al., 1995).

Particle Size and Viability

The selective washout of nonviable cells by acoustic separators has the advantage of reducing the need for viable cell bleeding and thereby eases the maintenance of high cell densities. This selective separation was studied in more detail by comparing the particle size distributions in the bioreactor culture to the harvest outflow of the separator. At low perfusion rates of 50 L/day and the highest separation efficiencies the mean particle size of the harvest outflow was 12.4 ± 0.2 μm and significantly lower than the mean particle size of the culture at 13.9 ± 0.04 μm (Fig. 9A). With increasing perfusion rates, the particle size in the harvest outflow increased and approached the particle size of the culture at perfusion rates of 250 L/day and greater. Correspondingly the harvest viability of 56.6 ± 0.9% was lowest at 50 L/day and with increasing perfusion rates approached the culture viability of 87.3 ± 0.2% (Fig. 9B). The separation efficiency as defined above represents a normalized measure of the cell retention efficiency. A washout effi-
Efficiency (WE) can be defined as the percentage of cells washed out (i.e., $\frac{WE}{H_11505} \times 100 - SE$). The ratio of 5.3 between the WE of nonviable and viable cells at a perfusion rate of 50 L/day (Fig. 9B) illustrates the high impact of the selective washout of nonviable cells and hence the selective retention of viable cells. At smaller scales Gaida et al. (1996) reported WE ratios up to 11.0. With increasing perfusion rate the selectivity for the washout of nonviable cells decreased.

CONCLUSIONS

Overall, these results demonstrate the scalability of the acoustic separator principle from 10 L/day up to 200 L/day with $\geq 95\%$ separation efficiencies at $10^7$ cells/mL. The new separator performed simply and reliably, with selective nonviable cell washout and other features of smaller-scale acoustic separators. Part of the 20-fold scale-up from the original 10 L/day perfusion rates was based on successfully using four parallel compartments in one separator with a single controller. A 1,000 L/day system has been developed based on modular scale-up using five 200-L acoustic separator chambers.

Figure 9. A: Separation efficiency of total cells (−○−), viable cells (△), nonviable cells (△), mean particle size harvest (−●−), and bioreactor (−×−) as a function of perfusion rate. B: Separation efficiency of total cells (−○−), viability harvest (−■−), bioreactor (−▲−), and ratio of nonviable to viable washout separation efficiency (−×−). Power input, 90 W; recirculation flow, 4 × perfusion rate; separator-cooling bath temperature difference, $-0.5\text{–}2.2^\circ C$; cell concentration bioreactor, $8.9 \pm 0.9 \times 10^6$ cells/mL.

Figure 10. Comparison of the 10-L and the 200-L performance. Separation efficiency is plotted as a function of the perfusion rate from 0–400 L/day and 0–20 L/day for the 200L (−■−) and 10L (−○−), respectively. Run time 60 sec; stop time: 4.5 sec; 200 L: power input, 90 W; recirculation flow, 4 × perfusion rate; separator-cooling bath temperature difference, $-0.5\text{–}2.2^\circ C$; cell concentration bioreactor, $8.9 \pm 0.9 \times 10^6$ cells/mL; 10 L: power input, 8 W, 80%; recirculation flow, 2.5 × perfusion rate; cell concentration bioreactor, $11.0 \pm 0.8 \times 10^6$ cells/mL.

Comparison of 10L/200L

The optimized performance of the scaled-up BioSep 200L separator was compared to a similarly optimized smaller BioSep 10L separator (Applikon) using a CHO-cell culture, both at approximately $10^7$ cells/mL. The separation efficiencies were plotted as a function of the perfusion rate ranging from 0–400 L/day and 0–20 L/day for the 200-L and 10-L, respectively (Fig. 10). Both separators had similar trends, with greater than 95% separation efficiencies up to perfusion rates of 200 and 10 L/day, respectively. At further increased perfusion rates a sharp decrease in performance was observed.

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