Optimization of an Acoustic Cell Filter with a Novel Air-Backflush System

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Increasing worldwide demand for mammalian cell production capacity will likely be partially satisfied by a greater use of higher volumetric productivity perfusion processes. An important additional component of any perfusion system is the cell retention device that can be based on filtration, sedimentation, and/or acoustic technologies. A common concern with these systems is that pumping and transient exposure to suboptimal medium conditions may damage the cells or influence the product quality. A novel air-backflush mode of operating an acoustic cell separator was developed in which an injection of bioreactor air downstream of the separator periodically returned the captured cells to the reactor, allowing separation to resume within 20 s. This mode of operation eliminated the need to pump the cells and allows the selection of a residence time in the separator depending on the sensitivity of the cell line. The air-backflush mode of operating a 10 L acoustic separator was systematically tested at 10⁷ cells/mL to define reliable ranges of operation. Consistent separation performance was obtained for wide ranges of cooling airflow rates from 0 to 15 L/min and for backflush frequencies between 10 and 40 h⁻¹. The separator performance was optimized at a perfusion rate of 10 L/day to obtain a maximum separation efficiency of 92 ± 0.3%. This was achieved by increasing the power setting to 8 W and using duty cycle stop and run times of 4.5 and 45 s, respectively. Acoustic cell separation with air backflush was successfully applied over a 110 day CHO cell perfusion culture at 10⁷ cells/mL and 95% viability.

Introduction

Though not as practical to operate at bench and small scales, semicontinuous or continuous processes are frequently selected for larger-scale clinical production (1). In part this is due to anchorage-dependent cells that are often cultivated in semicontinuous processes where it is relatively simple to exchange the medium while retaining the cells. It is also recognized that perfusion should be selected when products are particularly unstable (2), because in perfusion, after startup, residence times can be reduced to hours from the multiple days typical of fed-batch cultures. It is also simpler, though it can be slower, to optimize the steady-state condition obtained in perfusion bioreactors compared to the continuously changing state of fed-batch cultures. Finally, converting from batch to perfusion provides order of magnitude productivity increases (e.g., ref 3), particularly valuable when an organization is coping with increasing demands for mammalian cell production capacity (4).

Despite considerable use of continuous or semicontinuous processing, more widespread implementation of perfusion cultures has been inhibited by the difficulty of long-term, large-scale bioreactor cell retention. Development and production-scale use of sedimentation and centrifugation, as well as cross-flow and spin filter, technologies illustrate the many efforts that have been pursued to find a simple retention device (5). There is no consensus yet on which cell retention method performs best. Recently, the practical bench-scale acoustic filter technology has been scaled to 200 L/day (6), with some preliminary results also reported at 1000 L/day (7), so it can now be considered a viable alternative for larger-scale production.

Most mammalian cell retention devices used for perfusion processes (5) are based on size (e.g., cross-flow filters (8, 9) and spin filters (10–12)) or a combination of size and density differences between the cells and medium (e.g., inclined settlers (13, 14) and centrifuges (15, 16)). Acoustic separators provide an alternative cell retention system based on forces generated in an ultrasonic standing wave field. Cells exposed to an acoustic field reversibly aggregate at the pressure node planes of the field (17, 18). This separation is based on size, density, and compressibility differences between the cells and smaller particles or medium (19), providing selective retention of viable vs nonviable cells (3, 6, 18, 20). Using an acoustic filter, Chinese hamster ovary (CHO) cells having suspension densities of 2.5 × 10⁷ cells/mL were retained at separation efficiencies of 90% for perfusion rates of 10 L/day (21). With a newly developed scaled-up acoustic separator, perfusion rates of up to 200 L/day were processed at separation efficiencies of 95% for 10⁷ CHO cells/mL (6). A common concern is that increased

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shear stress during pumping might further decrease culture viability, cell-specific productivity, or product quality, especially for shear-sensitive insect cells (22, 23). It would therefore be desirable to develop a cell separation system with minimal shear stresses. An alternative approach suggested by Merten (22) is to backflush cells with the clarified medium, allowing shear-sensitive insect cells to be perfused with the acoustic separator. However, if medium is used for backflush, then it needs to be clarified again, resulting in an unfavorable increase of the harvest pump flow to maintain a constant average perfusion rate.

In this work, a novel air-backflush system for acoustic cell filters was developed, its operating conditions were systematically optimized, and reliable ranges of operations were defined. Most cell retention devices are external (except for spin filters), and the cell suspension is pumped to the separator and back to the bioreactor, exposing cells to uncontrolled, suboptimal medium conditions, for durations of up to hours (e.g., inclined settlers (24) and centrifuges). Air backflush eliminates the need to pump cells and allows the selection of a separator residence time based on the sensitivity of the particular cell line. The new method was successfully applied to a 110 day perfusion culture at 10^7 cells/mL and 95% viability.

**Materials and Methods**

**Cell and Perfusion Culture Maintenance.** CHO cells (CHO 540/24, Cangene, Winnipeg, MB, Canada) expressing human tissue plasminogen activator (t-PA) were thawed and then maintained in T-flasks for 5 days in a humidified incubator at 37 °C and 5% CO₂, before transfer to a 300 mL (working volume) spinner flask. Air backflush eliminates the need to pump cells and allows the selection of a separator residence time based on the sensitivity of the particular cell line. The new method was successfully applied to a 110 day perfusion culture at 10^7 cells/mL and 95% viability.

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**Analytical Methods.** Both cell number and viability were measured by trypsin blue dye exclusion and haemocytometer counting. Culture samples (3–5 mL), trypsin

Figure 1. Schematic of the perfusion system that was 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.

(1)

**Separator Operation.** During separation operation, cells were retained by the acoustic field in the active chamber, while the clarified medium was harvested from the outlet of the separator. The acoustic power and the harvest pump were turned off periodically (duty cycle) to enhance the settling of cells out of the active region of the separator. Two modes of returning the captured cells to the bioreactor were investigated. In the conventional recirculation mode of operation, the contents of the bioreactor were continuously pumped to the base of the acoustic filter via the "recirculation line" (Figure 1). A
portion of this stream was clarified and harvested, while the remainder flowed back to the bioreactor through the "settling tube" (Figure 1) to enhance the return of cells retained in the separator. A novel air-backflush mode of operating the 10L acoustic cell separator eliminated the need to pump cells through the recirculation line and allowed the selection of short cell residence times in the separator. Injection of bioreactor air downstream of the separator periodically returned the captured cells to the reactor by completely emptying the separator while the acoustic power and harvest pump were turned off (Figure 2). The separator was refilled from the bioreactor by reversing the direction of the backflush air pump, followed by turning on the acoustic power and harvest pump. The time from the start of one backflush to the next was called a backflush cycle. Figure 3 shows the status of the backflush pump for a 300 s air-backflush cycle. Also shown is the status of the acoustic power and harvest pump, both of which remain off during backflush as well as during the stop period of the superimposed duty cycle.

The backflush flow rate was set to 160 mL/min so that separation typically resumed within ~20 s. To optimize the separation efficiency, a part of the separator volume (residual backflush volume—typically from the top of the transducer plate up to the backflush air inlet) was not refilled with culture after each backflush. To achieve a defined perfusion rate (net harvest pump flow rate), the harvest pump flow rate was raised to accommodate the down times during the duty and air backflush cycles, as well as the residual backflush volume. The air-backflush mode of operating a 10L acoustic separator was systematically tested at 10^7 cells/mL to define reliable ranges of operation and was applied over a 110 day CHO cell perfusion culture.

Results and Discussion

Perfusion Culture Performance. Starting from a batch culture inoculated at 5 x 10^6 CHO cells/mL, perfusion was initiated on day 8 and adjusted to maintain a glucose concentration of 5.2 ± 1.7 mmol/L for the duration of the culture. Initially, the bioreactor temper-
Both effects caused a rise in temperature of the nearby liquid relative to that of the bulk liquid in the chamber, resulting in a slow but visible free convective flow that increased with increasing power input. This flow apparently disturbed cells from within the acoustic field, decreasing the separation efficiency.

Cooling Air. The transducer is normally cooled by an air flow to remove the heat generated by the partial dissipation of electrical into thermal energy. At the chosen operating conditions, cooling air flows of up to 15 L/min had no significant effect on the separation efficiency (Figure 6). With a further increase in the air flow rate, the separation efficiency dropped due to overcooling of the transducer, which led to a reversal of the free convective flow inside the chamber, once again disturbing the alignment of cells within the acoustic field. (Nonetheless, a cooling air flow should be maintained to reduce heating of the medium and cells.)

Duty Cycle. The duty cycle operation can enhance separation efficiency by periodically stopping the acoustic field and harvest pump, to allow settling of cells from the acoustically active region of the separator. The separation efficiency was investigated for stop time settings from 0 to 18 s for a constant run time of 45 s. Only at stop times of 4.5 and 6 s was a significant increase found in the separation efficiency (from 85.5% to 90.7%) observed (Figure 7). It should be noted that, to maintain a constant perfusion rate of 10 L/day, harvest flow was increased from 12.4 L/day at a stop time of 0 s to 18.4 L/day at a stop time of 18 s to compensate for the harvest pump being off during the air-backflush and duty cycle stop periods. The decreasing separation efficiencies with increasing flow rates above 6 s stop time probably overcome the beneficial effect of increased settling of cells during greater stop periods. Varying the stop time at a constant stop to run time ratio of 10 revealed a slightly beneficial effect of stop times shorter than 4.5 s (data not shown). Similar to observations with the 200L large-scale separator (6), this duty cycle variable had little influence on separator performance, and would appear to be a relatively low priority for optimization.

Backflush Frequency. The air backflush of the separator contents periodically pushed the captured cells back to the bioreactor to minimize cell accumulation in the separator. The separation efficiency increased from 73% at a backflush frequency of 6 h⁻¹ to a maximum of 91% at frequencies between 12 and 20 h⁻¹, where a broad range of robust separator operation can be seen (Figure 8). Interestingly, there was only a drop of SE to 87% with a pump flow rate of 24 L/day at a 40 h⁻¹ backflush frequency (Figure 8). At backflush frequencies higher than 40 h⁻¹, the SE decreases dramatically mainly due to the harvest flow increasing from 24 L/day at a backflush frequency of 40 h⁻¹ to 31 L/day at a backflush frequency of 50 h⁻¹ (to compensate for the harvest operation downtime during the backflush and refill.
Figure 8. Separation efficiency of total cells (●) and harvest pump flow rate (▲) as functions of backflush frequency. The reactor cell concentration was 1.1 × 10^7 cells/mL, perfusion rate 10 L/day, power input 8 W, cycle stop time 4.5 s, cycle run time 45 s, residual backflush volume 3.6 mL, and cooling air flow rate 7 L/min.

Refill Volume. After each backflush the separator was refilled by reversing the air pump (Figure 2). The air-backflush inlet was mounted in the harvest line as close as possible to the separator so that a minimum dead volume of 1.4 mL between the separator outlet and the air inlet was obtained. The residual backflush volume describes the total volume that was not refilled with cell suspension before the harvest pump was restarted (Figure 2). At a residual volume of 1.4 mL, corresponding to a completely filled separator chamber, the SE was suboptimal at 67.6%. This is because, when harvesting recommenced, a portion of the cell suspension was downstream of the active volume of the chamber and hence could not be dislodged by the acoustic field. As the residual backflush volume increased (corresponding to lower refill levels), the SE increased to a maximum of 78.5% at 4 mL and then decreased as the residual backflush volume was increased further (Figure 9). The SE was highest near the point where the transducer was fully covered with cell suspension (3.6 mL) after the refill was complete and before harvesting was resumed. Increasing the residual refill volume at a constant backflush frequency requires a corresponding increase in the harvest pump flow to compensate for the volume of liquid not refilled after each backflush cycle. This increase in harvest pump flow decreased the separation efficiency, as was the case for high duty cycle stop times and backflush cycle frequencies.

Fraction Collection. The time course of SE during one full backflush cycle was investigated to explore the separation dynamics. A net harvest flow rate of 12 L/day and a backflush frequency of 12 h\(^{-1}\) were selected as a suboptimal test case to allow improved modes of operation to be distinguished (as compared to working at closer to 100% separation efficiency where variations in SE would have been more difficult to detect). Every 10–20 s, the separator outflow was pooled for 10 s and the SE was determined. The backflush and refill time prior to the start of each harvest operation was 23 s. At the beginning of the harvest operation, indicated by the dotted line at 0 s, the SE was relatively low at 66.8% but increased within the first 50 s to a maximum of 91% (Figure 10). After 130 s, the SE began to decrease drastically until it plateaued at around 30% after 200 s of operation. The low SE at the beginning of harvest operation might be due to the washout of cells that had not yet been captured in the acoustic field. With increasing sonication time, more cells would have formed loose aggregates that are more effectively retained by the acoustic field (3, 27). The decrease in the SE after 127 s might be caused by the washout of cells that had not yet been captured in the acoustic field. The decrease in the SE after 127 s might be caused by the washout of cells that had not yet been captured in the acoustic field. The decrease in the SE after 127 s might be caused by the washout of cells that had not yet been captured in the acoustic field.

Operation. A comparison of the new air-backflush and conventional recirculation operating modes was performed for perfusion rates from 2 to 25 L/day (Figure 11). The performance of the separator in the backflush mode of operation was improved significantly by applying the optimized settings. Under these conditions, the measured separation efficiencies were somewhat lower (maximum of 6.6%) than those obtained using the recirculation mode of operation for perfusion rates of up to 15 L/day (Figure 11).
Medium backflush (operation provides a higher separation efficiency (Figure 11). At this time the conventional recirculation mode of perfusion (9–11 L/d) is another option but requires limited to as little as 1.5 min. Acoustic cell separation and backflush frequencies from 10 to 40 h⁻¹, wide ranges of cooling air flow rates of up to 15 L/min, increased harvest pump flow rates to compensate for compartmentation with dnase I. The newly developed air-backflush system for acoustic separators eliminated the recirculation pumping of cells while maintaining high separation efficiencies of 90% and above at perfusion rates of 10 L/d and 10⁷ cells/mL. At this time the conventional recirculation mode of operation provides a higher separation efficiency (Figure 11). However, air backflush can be selected when cells are particularly sensitive to pumping or require limited residence times in the separator. Also, it is expected that the air-backflush operation can be further optimized. Medium backflush (22) is another option but requires increased harvest pump flow rates to compensate for medium backflushed. Consistent air-backflush performance was obtained for wide ranges of cooling air flow rates of up to 15 L/min and backflush frequencies from 10 to 40 h⁻¹, demonstrating operational robustness. Residence times of cells in the separator were inversely related to the backflush frequency and within the range of robust operation were limited to as little as 1.5 min. Acoustic cell separation with air backflush was successfully applied to a 110 day, 10⁷ cells/mL perfusion culture while maintaining 95% viability.

Conclusions

Overall, these results demonstrate that the newly developed air-backflush system for acoustic separators increased harvest pump flow rates to compensate for medium backflushed. Consistent air-backflush performance was obtained for wide ranges of cooling air flow rates of up to 15 L/min and backflush frequencies from 10 to 40 h⁻¹, demonstrating operational robustness. Residence times of cells in the separator were inversely related to the backflush frequency and within the range of robust operation were limited to as little as 1.5 min. Acoustic cell separation with air backflush was successfully applied to a 110 day, 10⁷ cells/mL perfusion culture while maintaining 95% viability.

Acknowledgment

A Deutsche Forschungsgemeinschaft Postdoctoral Fellowship supported V.M.G. We express our gratitude to Felix Trampel of SonnSep Technologies (Coquitlam, BC, Canada) for useful discussion contributing to this work. Financial support from Cangene Corp. (Winnipeg, MB, Canada) and the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

References and Notes


Accepted October 21, 2002.

BP025625A