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Preparation, Separation,
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Application Note

Evaluation of Novel Cell Retention Technology and Cell Culture Media for N-1 Perfused Seed Train

Achieve Greater Upstream Productivity with Intensified Processing

Abstract

Perfusion technology can efficiently make use of existing facility assets to increase upstream productivity and reduce costs compared to traditional batch or fed-batch processes. This yields a reduction in process time, or an increase in manufacturing capacity of seed train bioreactors, without increasing volume requirements. Heightened cost pressures are driving the need to increase process efficiency and flexibility, resulting in a renewed interest in process intensification via perfusion. In this study, the Cellicon™ perfusion filter and controller are utilized in an N-1 perfusion process with Cellvento® 4CHO-X Expansion Medium prior to a fed-batch production bioreactor. The results were compared to a control process, with a conventional seed train, and evaluated for cell growth, titer, nutrients and metabolites, and product quality during the production process. Additionally, a high seeding density was evaluated for its effect on these same parameters as well as the possible benefits of implementing process intensification in the upstream process. The cell growth, specific productivity and product quality were very similar for all three processes, showing that the Cellicon™ perfusion filter can increase manufacturing possibilities with process intensification while maintaining product quality.

Introduction

Process intensification is a trend within biopharmaceutical manufacturing that aims to more efficiently bring biologics to market using new technologies and changes in the manufacturing paradigm. Upstream is one area in which manufacturers can accelerate their process and significantly increase volumetric productivity. In order for cells to efficiently produce the target protein in a conventional fed-batch cell culture process, nutrients and/or other feed components must be periodically added to the bioreactor. Alternatively, in perfusion mode, a cell retention device is used to maintain cells inside the bioreactor while continuously removing spent media as it is replaced by fresh media. Perfusion cultures can achieve higher cell density, run for longer duration, reduce turnover time, and improve overall product yield and quality within a facility, which makes perfusion a key enabler of process intensification in upstream.

The cell retention device is a critical technology for optimization of perfusion-based processes. It maintains cells within the bioreactor while removing other components such as spent media, cell debris, and protein products. Many cell retention technologies today utilize membrane filtration to achieve this separation and have demonstrated the success of perfusion in intensifying upstream processes. However, the available cell retention technologies often come with mechanical or performance challenges, e.g. relating to throughput, efficiency, product retention, or process scalability.

Another critical component to a perfusion bioreactor is the media formulation. Many media formulations are optimized for fed-batch processes and may not achieve the best performance when applied to perfusion. It is important to reduce media volume requirements over the course of a run because the logistics of handling large quantities of media for perfusion processes can be a challenge. Media requirements for perfusion are typically described as cell-specific perfusion rate (CSPR), which is the volume of media that each cell requires over time in order to maintain a desired state. As the cells grow, the rate of media turnover is increased to keep a constant amount of media per cell per day. This is achieved by adjusting the flow rate of spent media removed from the cell retention device and continuously replacing what is removed with an equivalent volume of fresh media inside the bioreactor. Perfusion-optimized media allows for operation at lower CSPRs to reduce media volume turnover. Media formulations may provide additional benefits such as improved protein titer in the production process, reduced effects of cell shear, and optimized cell growth rate.

When utilized in a cell expansion in the seed train, perfusion can achieve the targeted cell population while reducing the equipment, time and resources needed as well as mitigating the risk upstream. This study was designed to evaluate new technologies and explore the benefits of process intensification in an N-1 perfusion application. The experimental condition utilized an N-1 perfusion bioreactor to seed a fed-batch production bioreactor, while the control condition utilized shake flasks to achieve the same cell expansion. A high-seed condition in which the production bioreactor was inoculated with ten times greater cell density from the perfused N-1 bioreactor was also utilized in order to evaluate a proposed mode of process intensification on critical process parameters. This study used the Cellicon™ perfusion filter and controller, as well as the Cellvento® 4CHO-X Expansion Medium during cell expansion. The products were evaluated based on the metrics of cell growth, viability, titer, nutrient quantities, and product quality.

Materials and Methods

A CHO cell line was selected for its robust and established fed-batch process performance across scales, high peak viable cell density (PVCD) and titer, and stability over many passages. This cell line was used in the production bioreactor as a baseline for evaluating new experimental conditions. Prior to the experiment, some of the cells were adapted from their original storage medium to the Cellvento® 4CHO-X Expansion Medium and frozen into a working cell bank. The media requirements for the N-1 perfusion condition were then established by finding the lowest CSPR that would support exponential cell growth in perfusion. To evaluate process intensification with a high seed inoculation, a density of 5 E6 vc/mL was selected, ten times higher than the inoculation density of the control at 0.5 E6 vc/mL. Once these steps were complete, the case study plan was executed in order to compare a conventional seed train to a high- and low-seed perfused N-1 seed train.

A. Adaptation of cells to expansion medium and production of working cell bank

CHOZN® GS cells were thawed from a vial that had been frozen in EX-CELL® CD CHO Fusion medium and placed directly into a shake flask with Cellvento® 4CHO-X Expansion Medium. Every three days, the cells were diluted into a new shake flask with fresh media so that the cell density did not exceed 5 E6 vc/mL, keeping the cells in exponential growth phase. The viability and doubling times were monitored over multiple passages until a steady growth rate was reached, at which point the cells were considered as fully adapted to the new medium. A freezing protocol was then followed to make a working cell bank for future use.

B. Determination of CSPR requirements in perfusion

A 3 L Mobius® bioreactor with a 1 L working volume was selected for determining the lowest CSPR able to maintain exponential growth of the selected cell line in perfusion with the Cellvento® 4CHO-X Expansion medium. The 100 cm^2 Cellicon™ filter was used as the cell retention device and attached to the bioreactor by sterile welding. The feed tubing on the filter was attached to the harvest line on the bottom of the bioreactor, while the return line from the filter was attached to a submerged tubing line. Flow through the recirculation loop was maintained at 100 mL/min over the duration of the experiment. The bioreactor was inoculated at 0.71 E6 vc/mL, and cells grew up to about 50 E6 vc/mL before a bleed was initiated to maintain a constant cell density. Bioreactor control was maintained based on the parameters listed in Table 1, and samples were taken daily for analysis of cell growth and nutrient/metabolite levels. From day 3 to 9, an average CSPR of 50 pL/cell/day was maintained, and then from day 9 to 12, the CSPR was reduced to 40 pL/cell/day. Results were analyzed to determine the lowest CSPR at which a consistent cell growth rate was maintained.

Process Variable	Value
Working Volume	1.0 L
Agitation Rate	175 RPM
Temperature	36.5 +/-0.5 °C
pH	7.0 +/-0.05 (CO_2, 1 M sodium carbonate pump)
Dissolved Oxygen (% air saturation)	50% (air, O_2 cascade)

Table 1: Bioreactor control parameters during CSPR determination experiment.

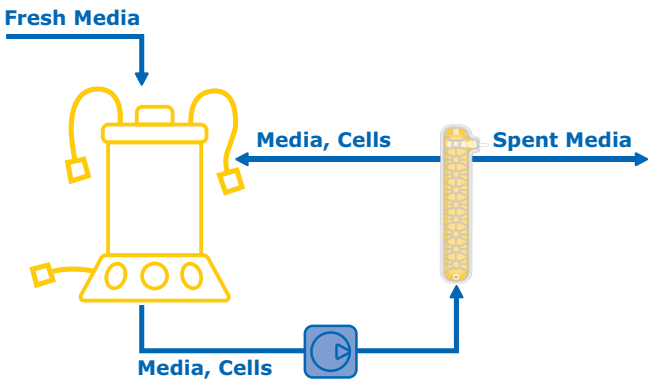


Figure 1: Cellicon™ filter connected to 3 L Mobius® bioreactor.

C. Intensified upstream case study evaluation

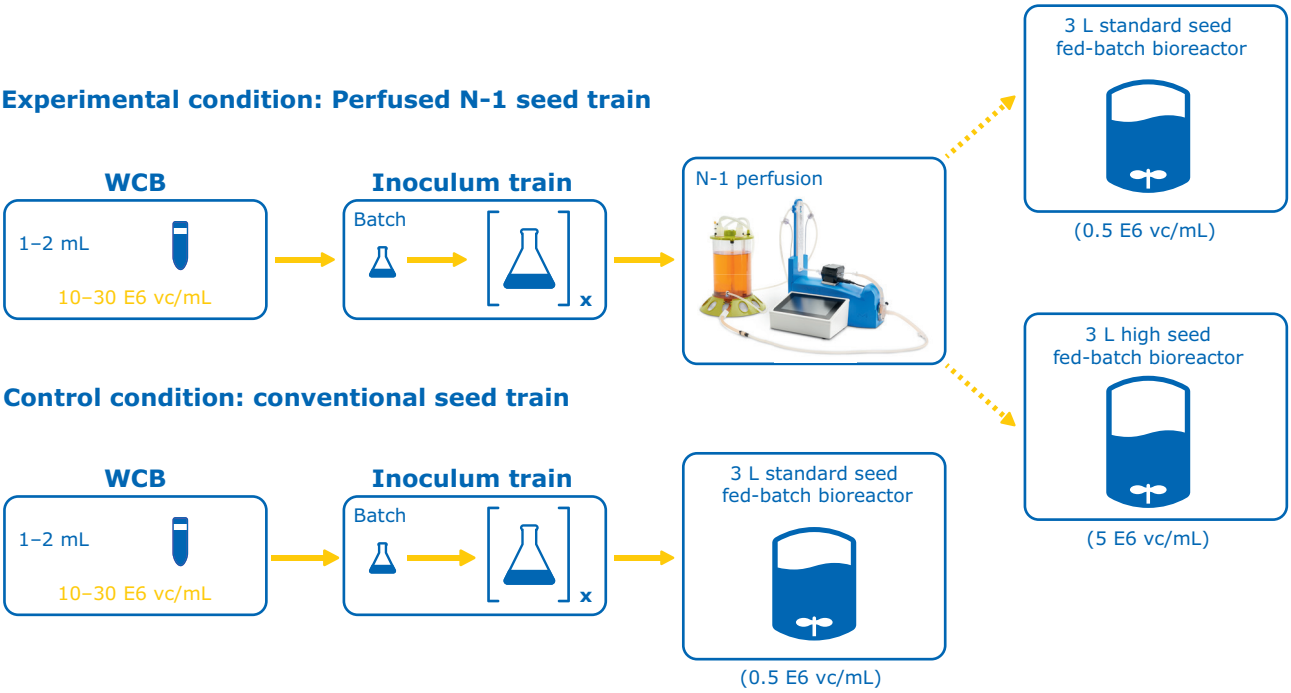


Figure 2: Experimental plan for intensified upstream case study evaluation.

The control condition bioreactor for this study was inoculated with cells frozen in EX-CELL® CD CHO Fusion medium, passaged in flasks inside an incubator at 6% CO_2 and 125 RPM, and then inoculated at 0.5 E6 vc/mL to the production fed-batch experiment. The experimental condition utilized frozen cells from the working cell bank of Cellvento® 4CHO-X Expansion medium, also thawed and expanded in flasks prior to inoculation of an N-1 bioreactor at 0.68 vc/mL. The N-1 step took place in a 3 L Mobius® bioreactor with a working volume of 2.2 L and controlled according to the parameters listed in Table 2. It was connected to a 100 cm² Cellicon™ filter with a flow rate of 100 mL/min, attached in the configuration shown in Figure 1. The CSPR was maintained at 40 pL/cell/day until the cells reached a density of >50 E6 vc/mL, at which point the cells were transferred to inoculate the production bioreactors.

The fed-batch production processes were run in 3 L Mobius® bioreactors with a starting volume of 1.5 L and followed the control parameters outlined in Table 3 and the feed strategy outlined in Table 4. Feed percentages were based on the volume at the time of seeding, with a 50:50 ratio of EX-CELL® Advanced Feed 1 and Cellvento® 4 Feed. The low-seed strategy started at 0.5 E6 vc/mL and followed the historical feed schedule, and the high-seed strategy started at 5.0 E6 vc/mL and followed a shifted feed schedule based on the same strategy as the historical process. Glucose was maintained at 6 g/L with daily additions (increased to 12 g/L on day 5 and 10 g/L on day 12 so that the following day's addition could be skipped).

Each bioreactor run was performed in duplicate with samples taken once a day to evaluate VCD, viability, nutrients, and metabolites. Additionally, HPLC was used to measure titer on days 7 and 9–14, and samples from day 14 were submitted for product quality analysis.

Process Variable	Value
Agitation Rate	200 RPM
Temperature	36.5 +/-0.5 °C
pH	7.0 +/- 0.05 (CO_2, 1 M sodium carbonate pump)
Dissolved Oxygen (% air saturation)	50% (air, O_2 cascade)

Table 2: Bioreactor conditions for the N-1 perfusion process.

Process Variable	Value
Agitation Rate	200 RPM
Temperature	36.8 °C
pH	6.9 +0.1/-0.15 (CO_2, 1 M sodium carbonate pump)
Dissolved Oxygen (% air saturation)	50% (air, O_2 cascade) 50 mL/min
Headspace Air	50 mL/min

Table 3: Bioreactor conditions for fed-batch production bioreactors.

Day	0	3	5	7	10	12	Total
Control	0%	5%	5%	7.5%	7.5%	5%	30%
pN-1 Low	0%	5%	5%	7.5%	7.5%	5%	30%
pN-1 High	5%	5%	7.5%	7.5%	7.5%	5%	37.5%

Table 4: Feed strategy for production bioreactor processes.

Results

A. Adaptation of cells to expansion medium and production of working cell bank

After CHOZN® GS cells were thawed and placed directly into Cellvento® 4CHO-X Expansion medium, their viability and cell density were tracked over time for multiple passages, as shown in Figure 3. The viability remained high, above 98%, for all 5 passages after the thaw. The specific net growth rate was calculated from the cell density readings and reached a plateau at 0.7–0.75 day-1 between passages 3 and 6. After passage 5, cells were frozen in 3 mL vials at a density of 20 E6 cells/vial.

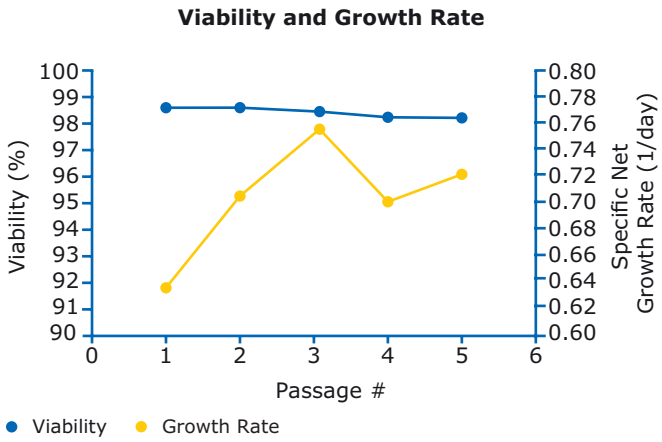


Figure 3: Viability and growth rate of cells after thaw and adaptation in expansion medium.

B. Determination of CSPR requirements in perfusion

A bioreactor was inoculated at 0.71 E6 vc/mL and operated in perfusion mode with a Cellicon™ filter and controller. The cells grew up to 50 E6 vc/mL before a bleed was initiated to maintain a constant cell density based on feedback from a capacitance probe to a bleed pump, which removed cell mass when the density went above the setpoint. At the start of the run, the perfusion rate was chosen based on a target CSPR of 50 pL/cell/day. During this time, as seen in Figure 4, the cells reached steady state with a high viability and constant doubling time. On day 9, after 3 days at steady state, the CSPR was reduced to 40 pL/cell/day. At this point, the concentration of glucose dropped slightly (Figure 5) but remained constant between days 9 and 12. Additionally, the doubling time remained stable between days 9 and 12, indicating that the cells were still growing exponentially. For this process, it was desirable to keep a glucose concentration of above 3 g/L, therefore the CSPR was not further reduced. The target CSPR for the perfused N-1 step was selected as 40 pL/cell/day to maintain exponential cell growth and a high viability during the perfused N-1 step of the intensified process.

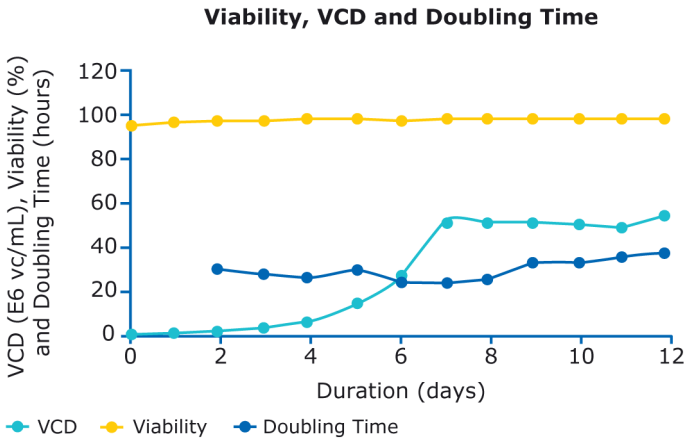


Figure 4: Viable cell density, viability, and doubling time during the perfusion run to determine the target CSPR for the intensified case study.

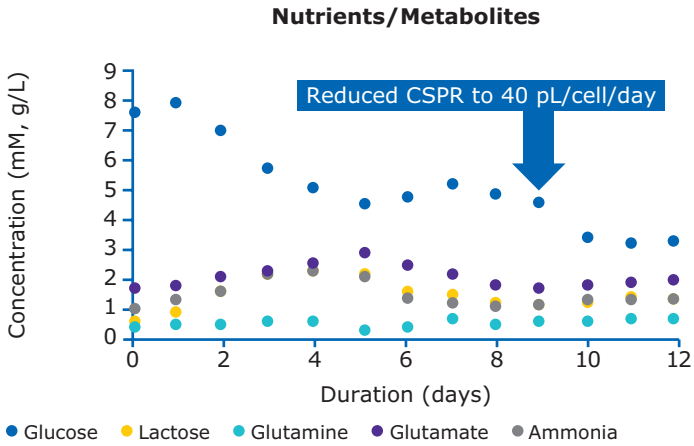


Figure 5: Nutrient and metabolite profiles during the perfusion run to determine target CSPR for the intensified case study.

C. Intensified upstream case study evaluation

For the perfused N-1 step, a bioreactor was inoculated with cells from the prepared cell bank. The cells grew exponentially up to 50 E6 vc/mL and maintained a high viability before being added to the production bioreactors (Figure 6a and 6b) on day 6. Two production bioreactors were inoculated at 0.5 E6 vc/mL from the perfused N-1 bioreactor; two were inoculated at 5 E6 vc/mL from the perfused N-1 bioreactor; and a final two were inoculated at 0.5 E6 vc/mL from a standard seed train process, which served as the control. The feed strategy for each of these conditions is detailed in Table 4.

During the perfusion process, pressure was monitored with the Cellicon™ controller and exported using the data logging feature. Figure 7 shows that there was little change in pressure over the duration of the run. The steady feed pressure of 0.8-0.9 psi indicates there is no accumulation of cells or debris in the feed channel that would result in blockage of flow through the channel and back to the bioreactor. The steady state perfusate pressure indicates that the 100 mL/min crossflow rate is providing adequate sweeping of the membrane surface preventing membrane fouling throughout the 3 days of operation.

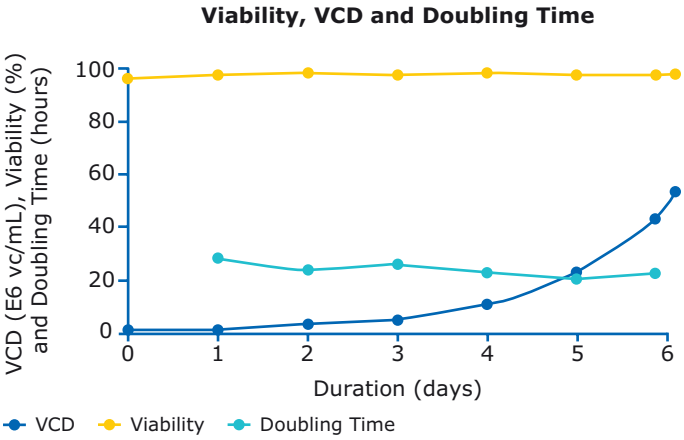


Figure 6a: Viability, viable cell density, and doubling time of cells in N-1 perfusion process.

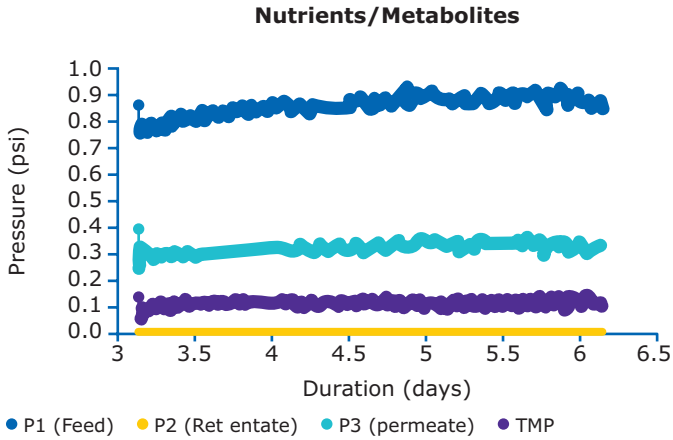


Figure 7: Cellicon™ filter performance during N-1 perfusion process.

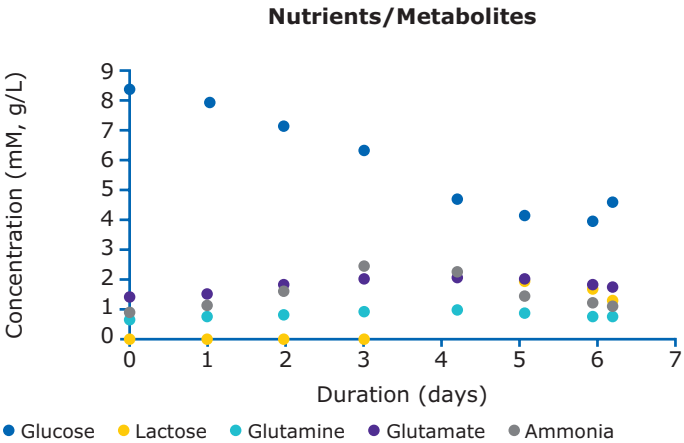
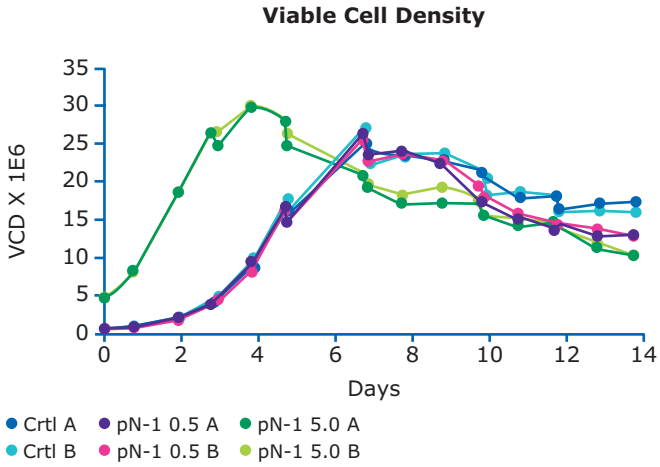


Figure 6b: Nutrients and metabolite concentrations during the N-1 perfusion process.

Figure 8 shows the resulting viable cell densities of each production bioreactor for the 14-day run. The peak VCD was achieved 3 days earlier for the high-seed process, and the peak was slightly higher than for the lower-seeded bioreactors – 30 E6 vc/mL versus 25 E6 vc/mL. Declines in VCD after the peak followed a similar trend under all conditions and were within normal process variability. Looking at viability, the high-seed process began to experience a drop 3 days earlier than the control. This resulted in a final viability of around 50%, lower than the target of 70% ending viability for the high-seed condition. In both cases, the duplicates showed very similar performance.

The titer was measured for each bioreactor on day 7 and days 9–14. Figure 9 shows that the high-seed condition provided an increase in protein production over the run, with a final concentration of just over 5 g/L. There was no difference between the final protein concentrations for the conditions with a standard seeding density, regardless of the inclusion of a perfused N-1 seed train step. The specific productivities were very similar across conditions, at approximately 20 pg/cell/day for each sample. The higher titer in the high-seed condition was a result of the overall increased viable cell count rather than a difference in specific productivity.



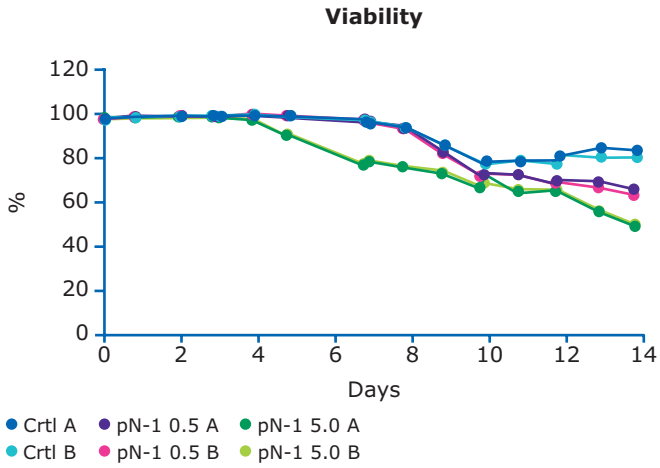
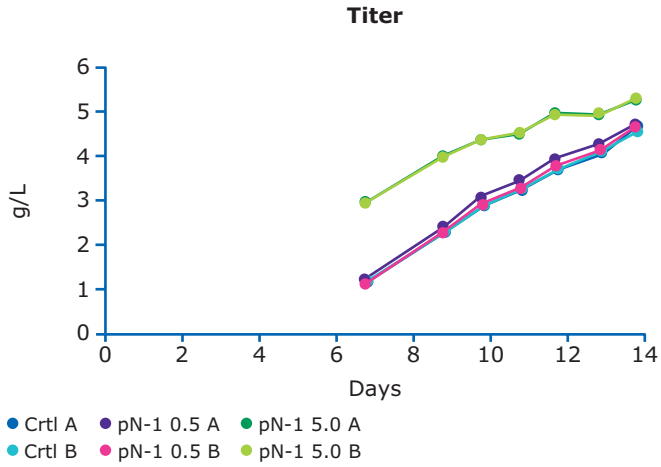


Figure 8: VCD and viability for the fed-batch production bioreactors.



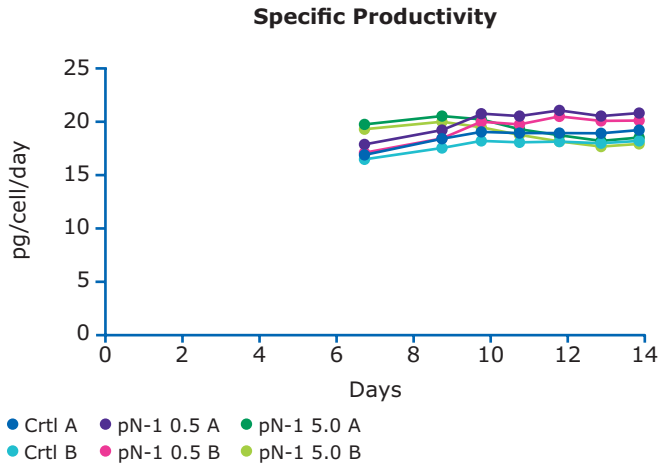
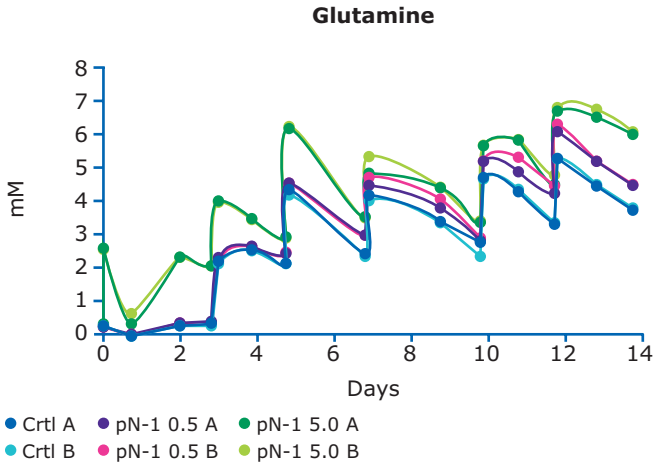


Figure 9: Titer and specific productivity for the fed-batch production bioreactors during the intensified case study.

The glutamine and glutamate concentrations were similar regardless of bioreactor conditions. They were influenced by the time of the feeds, with an increase in both glutamine and glutamate seen just after a feed.

The glucose concentration over the course of the run also followed a similar pattern for each condition. Early in the run, between days 0 and 5, the high-seed condition had a higher rate of glucose consumption due to the higher cell density. However, this consumption rate decreased later in the process as the viability dropped.



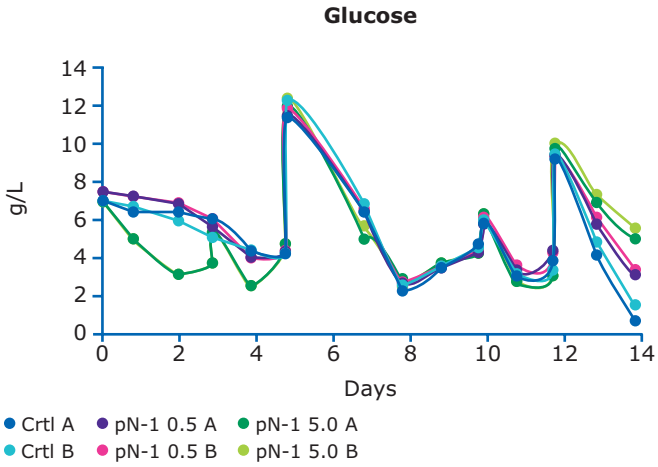


Figure 11: Glucose trend in the fed-batch production bioreactors during the intensified upstream case study.

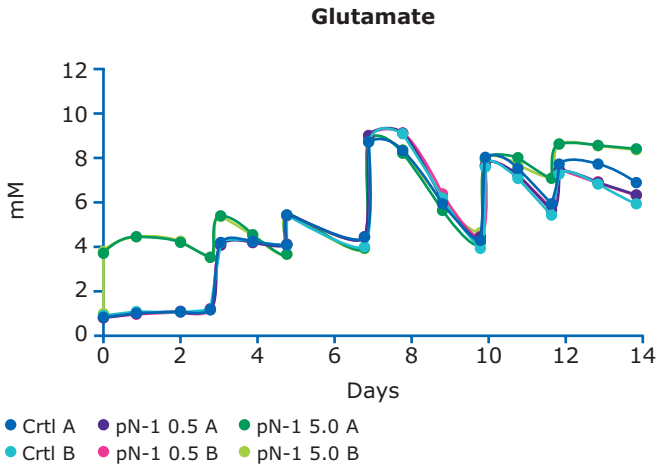


Figure 10: Glutamine and glutamate trends in the fed-batch production bioreactors throughout the duration of the intensified upstream process.

The ammonia trends generally show an increase in ammonia concentration over the course of the run, before a drop on the last few days. The perfused N-1 high-seed condition reached its peak two days earlier than the standard seed conditions. It is unclear why the control saw the highest overall ammonia level, but these results are within a normal process variability for ammonia concentration.

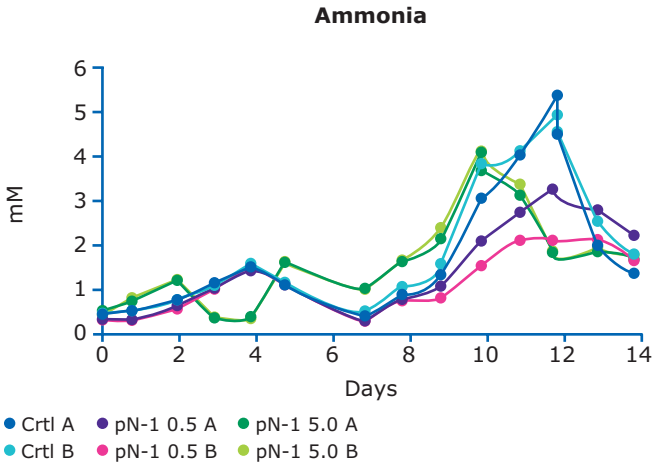


Figure 12: Ammonia concentration in the fed-batch production bioreactors during the intensified upstream case study.

The lactate trends seem to correlate with the VCD trends in that there is a shift from production to consumption when the maximum cell density is reached. At the end of the run, a shift back to production of lactate is observed. Carbon dioxide is used for pH control and thus also correlates with the lactate trend. As the lactate increases, the pH drops, and CO_2 is not needed. Then, as lactate is consumed, pH increases, and CO_2 is required to maintain the pH setpoint. Overall, the trends in lactate and CO_2 correlate to the phase of growth the cells are in and are not significantly affected by the experimental conditions.

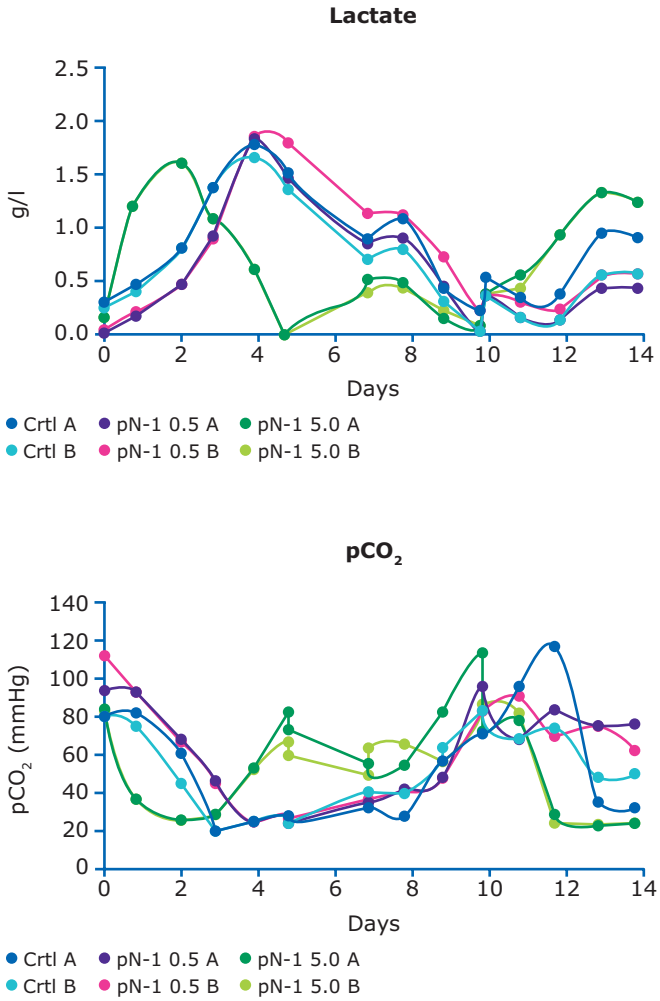
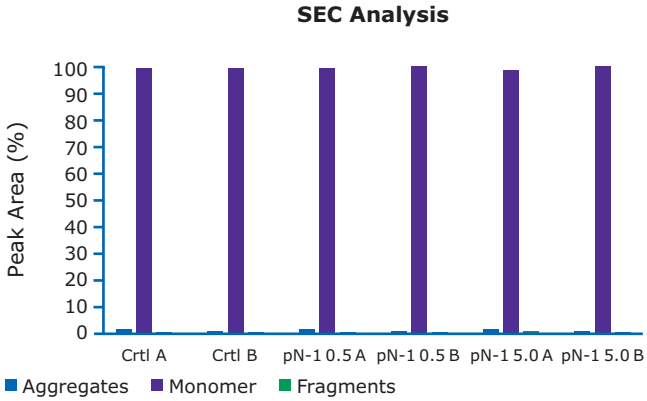


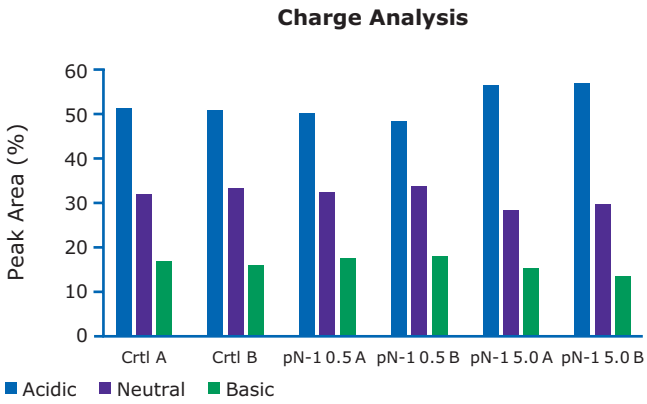
Figure 13: Lactate and carbon dioxide concentration in the fed-batch production bioreactors during the intensified upstream case study.

Samples for product quality were taken on the final day of the production process and tested. The SEC analysis shows consistent product quality across all conditions, detecting almost all of the product as a monomer. A small amount of fragment appears in one of the high-seed perfused N-1 samples, but it was not observed in duplicate. In the charge analysis, relatively consistent charge profiles were observed between conditions. Slightly higher acidic peaks were observed in the high-seed perfused N-1 cultures, and this resulted in slightly lower neutral and basic peaks. This trend was most likely correlated with the lower pH towards the end of the culture, which could be mitigated with process development. A glycan analysis was performed on the samples from each condition to look for any changes to the target molecule. Shown in Figure 14, the glycan profile came back as very consistent between the perfused N-1, high-seed perfused N-1, and control conditions. Overall, the product quality was not affected by adding a perfused N-1 bioreactor in the seed train or using a high seeding density. The use of a Cellicon™ filter and Cellvento® 4CHO-X Expansion medium also did not significantly affect the product quality during the production runs.

Major peaks (only main peaks are considered in calculating % area)

	Area (%)								
Structure	**G0F-N**	**G0**	**G0F**	**Man5**	**G1a**	**G1b**	**G1Fa**	**G1Fb**	**G2F**
Time	**10.9**	**11.5**	**13.2**	**14.0**	**14.6**	**15.1**	**16.3**	**16.7**	**19.7**
Crtl A	1.2	5.2	55.9	2.6	1.5	0.6	21.4	7.9	3.7
Crtl B	1.1	5.3	56.0	2.4	1.4	0.6	21.4	8.1	3.7
pN-1 0.5 A	1.0	5.1	55.5	2.4	1.5	0.6	21.9	8.3	3.8
pN-1 0.5 B	0.9	4.9	54.5	2.2	1.4	0.7	22.8	8.6	4.1
pN-1 5.0 A	1.6	5.2	54.7	3.1	1.7	0.6	21.4	8.0	3.8
pN-1 5.0 A	1.5	5.1	55.4	3.0	1.6	0.6	21.1	8.0	3.7





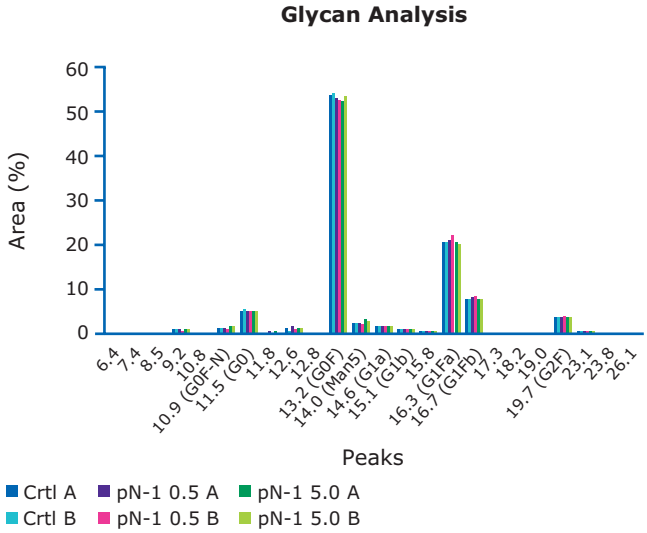


Figure 14: Product quality data from samples on the final day of the fed-batch production bioreactors in the intensified upstream case study.

Discussion

This case study evaluated the implementation of the Cellicon™ perfusion filter and controller and Cellvento® 4CHO-X Expansion medium in a seed train based on the results of a fed-batch production bioreactor. The use of a high inoculation density was determined to be a common method of process intensification, so a high-seed condition was also included in the experiment. The process development steps needed to begin the case study included establishing a working cell bank of the selected cell line in the medium and finding the target CSPR for use during the perfusion step.

The differentiating step between the intensified seed train process and the conventional seed train process was the perfused N-1 bioreactor. Overall, the Cellicon™ perfusion solution enabled a 10x greater cell density in the expansion process that could help to reduce process steps or facility footprint – both key components of process intensification. The Cellvento® 4CHO-X Expansion medium provided an optimized formulation of components for the seed train steps by maintaining an exponential cell growth rate, high viability, and low media turnover requirement during the process. The expansion medium also showed seamless adaptation to the basal media of the fed-batch production bioreactor.

The results of the fed-batch production bioreactor were used to evaluate the effect of the Cellicon™ perfusion filter and controller with Cellvento® 4CHO-X Expansion medium on the overall process. When comparing the production bioreactors seeded at 0.5 E6 vc/mL, one containing the N-1 perfusion step and one using only shake flasks for cell expansion, there were no significant differences. The cell density, viability, titer, specific productivity, nutrient/metabolite concentrations, and product quality were all very similar for the entire process. This demonstrates that the use of the Cellicon™ perfusion filter does not modify process output and drug quality attributes, and is a viable solution for process intensification. When comparing the high-seed bioreactors to both standard seed bioreactors, some process differences were seen. The high-seed bioreactors experienced a greater peak viable cell density and integral viable cell count overall. This led to increased titer since the specific productivity was in the same range as the control conditions. An increased titer was highly beneficial since greater productivity was achieved within the same time period. There were no significant differences in the nutrient/metabolite concentrations or the product quality between the high-seed bioreactors or the standard seed bioreactors. For this specific process, additional development work could be completed, including adjusting the feed schedule or implementing a temperature shift to solve the viability drop mid-run and to further improve the results. This data demonstrates that adding an intensified process in the upstream seed train could provide significant improvements to titer without sacrificing product quality.

Summary

When used at the N-1 stage in a bioreactor seed train, the new Cellicon™ perfusion filter and controller provided high throughput with low shear and zero fouling, and consistent, reliable process control. Cells were inoculated into production bioreactors at normal and high-density conditions, and their production was the same as for those in a conventionally seeded control. The Cellicon™ perfusion filter and controller together with Cellvento® 4CHO-X Expansion medium worked seamlessly for the adoption of a perfused N-1 seed train step into the cell expansion process. This approach could be used to reduce process steps, efficiently utilize volume and facility space, and reduce risk while keeping product quality standards high. When used in combination with a high-seed production process, the intensified expansion steps could also help to achieve greater productivity in the upstream process.

For additional information, please visit MerckMillipore.com/Cellicon.
To place an order or receive technical assistance, please visit www.MerckMillipore.com/contactPS

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