Sunflower

Application Note

Equipment Highlight

Keywords

Perfusion fermentation, cell retention device, single-use bioreactor

Perfusion fermentation in the Daisy Petal™ bioreactor

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Abstract

In-vessel perfusion fermentation is an innovative continuous manufacturing solution that produces high-quality recombinant proteins using smaller volumes than conventional fed-batch fermentors. Perfusion fermentation has not previously been possible due to the lack of stirred tank reactors that can retain microorganisms throughout a fermentation campaign. This application note explores Sunflower Therapeutics' innovative cell retention device currently being leveraged in the Daisy Petal™ Perfusion Bioreactor system. This fully-automated system was designed and built to pioneer in-vessel perfusion fermentation in microbes and accelerate the use of microorganisms for sustainable and accessible heterologous protein production. Here we use two examples, namely the expression of G-CSF and a single-domain antibody, as case studies to demonstrate the versatility and flexibility of the Daisy Petal™ in rapidly generating consistently high-quality, high-yield, low-cost proteins with therapeutic benefits.

Introduction

Continuous manufacturing enables high yields and consistent quality

The benefits of continuous recombinant protein manufacturing have been well demonstrated using mammalian cells. These benefits include lower capital investment and ongoing operational costs, a reduced manufacturing footprint, and increased flexibility, productivity and product quality. With continuous manufacturing, smaller equipment can be used for longer periods of time, increasing the total quantity of protein produced while maximizing facility utilization and lowering HVAC energy consumption. In this operating mode, continuous feeding and waste removal enable a stable and healthy cell environment in the bioreactor, supporting greater viable cell densities and reduced product variability. Together, these benefits ultimately lead to high protein yields and consistent product quality.

Continuous manufacturing historically has been challenging for microbes

The cell mass achievable in legacy continuous manufacturing methods for microbes, such as chemostat (FIG 1A), is limited due to the constant removal of cells. External perfusion (FIG 1B) solves this cell mass limitation for mammalian cells by routing cells and spent media through an external filter, which separates and returns cells to the bioreactor while spent media is harvested. External perfusion is not easily adapted to

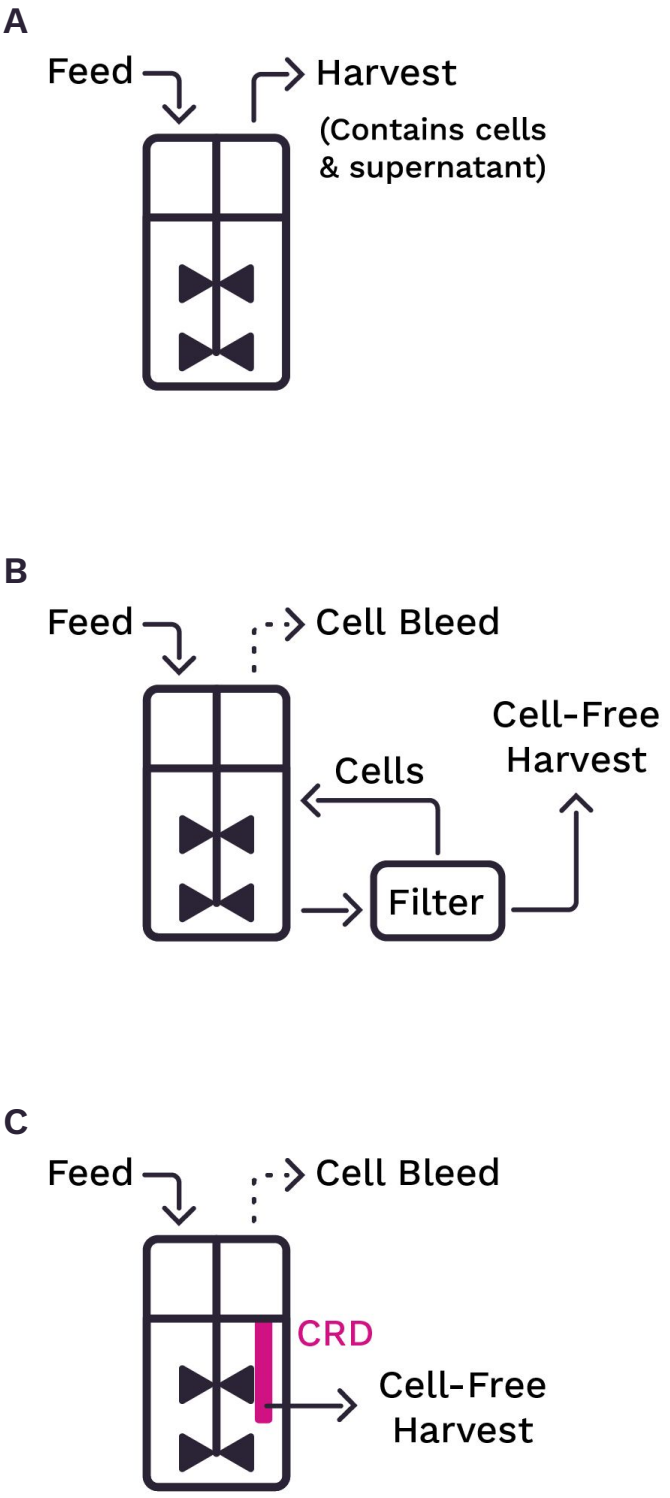

FIGURE 1. Modes of continuous cell cultivation. A. Chemostat. B. Conventional perfusion with an external filtration device. C. In-vessel perfusion with Sunflower's proprietary cell retention device (CRD).

FIGURE 2. Daisy Petal™ Perfusion Bioreactor System.

microbes and cells with high respiratory coefficients, however, since the constant removal of cells from the bioreactor environment can lead to starvation.

Sunflower's pioneering technology enables in-vessel perfusion for microbes

Sunflower has developed an innovative method for in-vessel perfusion to allow for continuous feeding of the biomass in the bioreactor vessel, continuous harvesting of fluids, and continuous retention of cells in the controlled and healthy bioreactor environment (FIG 1C).

Sunflower's proprietary cell retention device keeps the cells inside the bioreactor while continuously harvesting secreted protein and removing spent media. The benefits of this in-vessel perfusion strategy include healthier, consistent cell culture, longer production campaigns, ultra-high cell mass, and high protein yields.

Leveraging our novel cell retention device, Sunflower has developed the Daisy Petal™ perfusion bioreactor system for automated, continuous production of recombinant proteins (FIG 2). The Daisy Petal™ incorporates an exchangeable,

single-use bench-top bioreactor that uses perfusion fermentation to yield grams of protein from a 1 L working volume in 1-2 weeks. The Daisy Petal™ is a bench-top system that performs like a large reactor (FIG 2) to support diverse protein production needs.

Materials & Methods

Heterologous proteins were produced via perfusion fermentation in the Daisy Petal™ bioreactor using a strain of *Pichia pastoris* engineered by Sunflower. The bioreactor was charged with defined growth media containing glycerol to a 1 L working volume. Frozen cell stock was used to inoculate the bioreactor at a starting OD_{600}/mL of 0.15. Defined growth media containing glycerol was continuously fed to the reactor at a perfusion rate of 1.8 vessel volumes per day (vvd) for 32 - 48 hours to build biomass. Cell-free harvest fluid was directed to waste during this time. After accumulating biomass to the desired cell density (typically 400 - 600 g/L), the Daisy Petal™ automatically induced heterologous protein production by switching to a defined production medium containing methanol. The production medium was fed at a perfusion rate 1.8 vessel volumes per day. After an initial media adaptation period (8 - 12 hours), the cell-free harvest fluid was directed to the collection vessel. The HelianthOS™ operating system actively maintained bioreactor level, pH, temperature, and dissolved oxygen at the predefined set points throughout the campaign, and completed a system shut down after the desired fermentation time was reached (200+ hours). Wet cell weight (g/L) was determined using cell-containing samples from inside the bioreactor. Protein yields and quality were determined by SDS-PAGE using samples of the cell-free harvest fluid.

Results

Cytokine Production using the Daisy Petal™

Granulocyte colony stimulating factor (G-CSF), a high value cytokine typically used to stimulate white blood cell production in patients with cancer and neutropenia, was produced from *Pichia pastoris* using perfusion fermentation in the Daisy Petal™. The target wet cell weight range of 300 - 500 g/L was maintained throughout the production phase (FIG 3). The fermentation ran for 9 days until all of the prepared media was

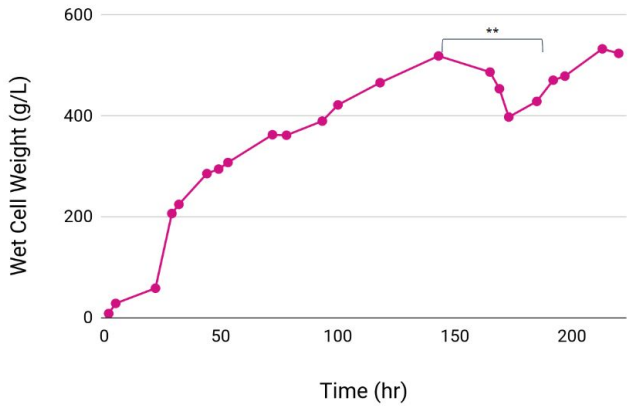

*FIGURE 3. Biomass achieved in the Daisy Petal™ during a 9 day fermentation. **The cell bleed was increased at 150-175h as part of a secondary experiment.*

consumed. Compared to a conventional fed-batch fermentation in *P. pastoris*, the perfusion fermentation in the Daisy Petal™ ran 80% longer due to the in-vessel cell retention device. (NOTE: the HelianthOS™ operating system is customizable and can maintain predefined set points for users interested in longer campaigns.) Over 1.3 grams of G-CSF was collected, resulting in a space-time yield of 145 mg / L / day (FIG 4). Analysis of collection samples by SDS-PAGE showed that the product quality was consistent throughout the cultivation (FIG 5A) and that there was no significant difference in product before and after the cell retention device (FIG 5B).

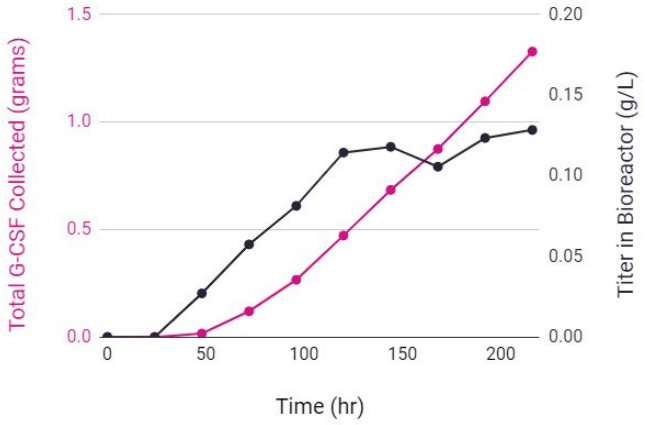

FIGURE 4. Yield of G-CSF over time. Fermenter titer is shown in black; total heterologous protein yield is show in magenta.

Single-domain Antibody Production using the Daisy Petal™

A single-domain antibody specific to a rotavirus antigen was produced from *P. pastoris* using perfusion fermentation in the Daisy Petal™. The target wet cell weight range of 400 - 600 g/L was

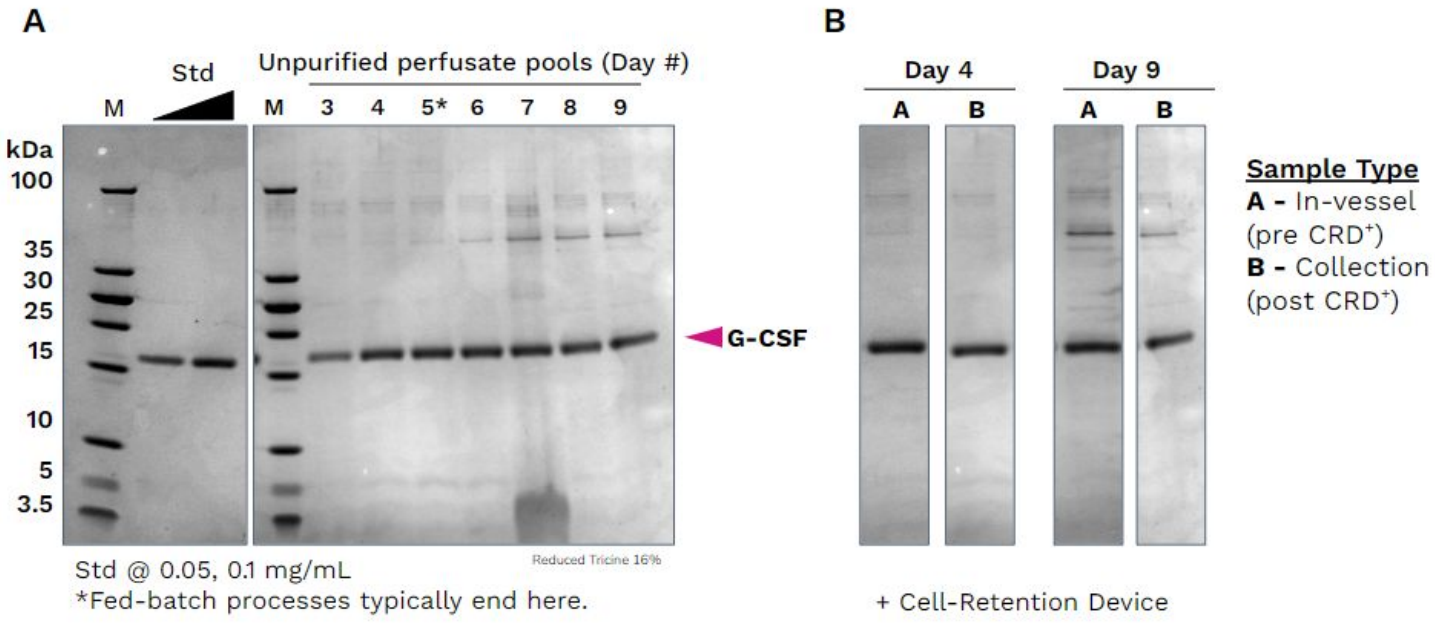

FIGURE 5. Analysis of G-CSF produced by perfusion in the Daisy Petal™. A. SDS-PAGE analysis of perfusate collected during a 9 day perfusion fermentation. B. SDS-PAGE analysis of cultivation fluid samples collected on day 4 and day 9 of the fermentation.

maintained throughout the production phase (FIG 6A). Due to the in-vessel cell retention device, the fermentation ran for 12 days– more than twice as long as conventional fed-batch fermentations producing single-domain antibodies using this host. A total of 3 grams of single-domain antibody was produced, resulting in a space-time yield of 255 mg / L / day (FIG 6B). Analysis of collection samples by SDS-PAGE showed that the product quality was consistent throughout the cultivation (FIG 6C).

Discussion

Efficient production of two different classes of proteins from *P. pastoris* using perfusion fermentation was demonstrated. Both fermentations achieved high cell density (up to 600 g/L WCW) and were maintained for twice as long as standard fed-batch cultivations. A key benefit of these longer fermentations is reduction of downtime and human labor due to equipment turnover. With perfusion fermentation, the bioreactor spends more time running with less setup time, improving equipment utilization and operating costs. The high cell mass and long fermentation time ultimately resulted in high productivity. A space-time yield of 255 mg / L / day was achieved for the single-domain antibody using perfusion fermentation on the Daisy Petal™. For comparison, to achieve the same space-time yield from a 5-day fed-batch fermentation using the same host would require achievement of a protein titer of 1.3 g/L at harvest. Likewise, to achieve the same space-time yield from a 15-day

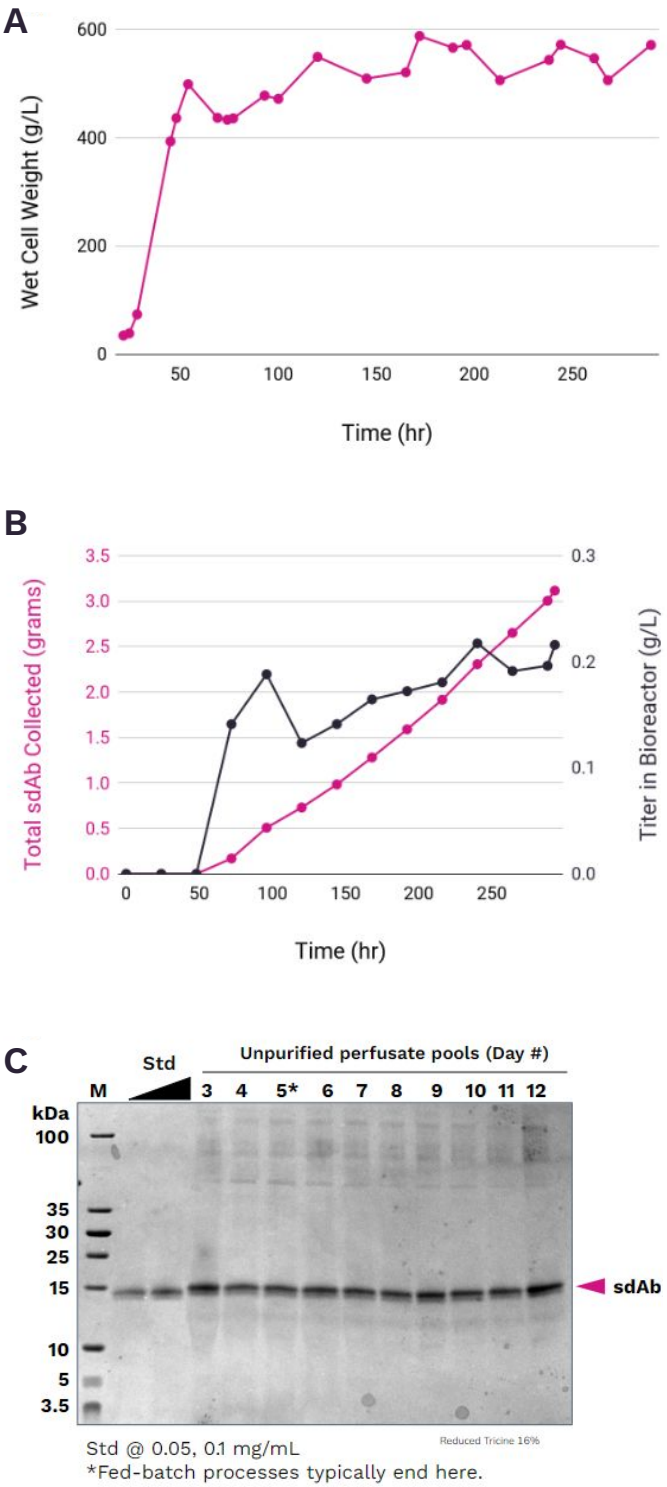

FIGURE 6. Production of a single-domain antibody in the Daisy Petal™. A. Biomass achieved during the perfusion fermentation. B. Space-time yield (magenta) and fermenter titers (black) for heterologous protein expression. C. SDS-PAGE analysis of product quality during cultivation.

fed-batch fermentation using a mammalian host would require a protein titer of 3.8 g/L at harvest.

Beyond the productivity benefits demonstrated here, the Daisy Petal™ enables operational simplicity through intuitive design and automated controls. The unique, single-use, structured consumable used with the Daisy Petal™ is designed for intuitive installation by users of any experience level, which also allows for rapid flexibility and significantly reduced downtime between campaigns. Furthermore, Sunflower's custom control software, HelianthOS™ enabled fully automated operation of the Daisy Petal™ throughout the fermentations, maintaining pH, DO, temperature and bioreactor level to the predefined set points.

In addition to the proteins discussed in this application note, Sunflower has demonstrated proof-of-concept for the production of a wide variety of proteins from *P. pastoris* using perfusion fermentation. These include monoclonal antibodies, single-domain antibodies, Fabs, Fc-fusion proteins, cytokines, hormones, subunit antigens, and virus-like particles. For more information on these or any other protein classes, please contact us.

Conclusion

Sunflower's Daisy Petal™ bioreactor, equipped with our proprietary in-vessel cell retention device, enables the production of high quality protein via perfusion fermentation. The results of two fully automated fermentations of protein-producing yeast strains have been presented here. HelianthOS™, the custom software that controls the Daisy Petal™, demonstrated exceptional process control, leading to high quality, consistent production of protein throughout the fermentations. Using the Daisy Petal™, fermentations can run more than twice as long as in a typical fed-batch mode and grams of protein can be produced from a 1 L working volume in only 1 - 2 weeks.

References

1. Walther, Jason et al. Perfusion Cell Culture Decreases Process and Product Heterogeneity in a Head-to-Head Comparison with Fed-Batch. Biotechnol. J. 14, 1700733 (2018).