Sunflower

Application Note

Equipment Highlight

Keywords

In-vessel cell retention device (CRD), perfusion fermentation, continuous bioprocess, yeast, fungi

A modular in-vessel cell retention device enables perfusion fermentation across diverse bioprocesses

Kevin Fuentes, Martin Rochefort, Sophie Lee, Laura Crowell Orella

info@sunflowertx.com

Abstract

Sunflower's in-vessel cell retention device (CRD), a key component of the Daisy Petal™ Perfusion Bioreactor System, enables continuous bioprocessing for microbes with higher productivity and enhanced control compared to traditional batch methods. Typical continuous bioprocessing techniques rely on external CRDs which fail to address the respiratory requirements of microbial fermentation, as microbes pose a heightened risk of cell starvation and death when removed from a controlled environment. Sunflower's solution is a novel in-vessel CRD, a membrane-based device that is seamlessly integrated within the Daisy Petal™ Perfusion Bioreactor vessel and control schema. Here, we demonstrate how our in-vessel CRD enables perfusion fermentation of microbes, and highlight the versatility of our CRD, demonstrating compatibility with diverse protein classes and microbial cell types – empowering next generation manufacturing.

Introduction

Continuous bioprocessing for the manufacture of recombinant protein demonstrates various advantages to batch bioprocessing, such as higher volumetric productivity, consistent product quality and better process control.[1,2] Sunflower's Daisy Petal™ Perfusion Bioreactor System is a continuous bioreactor that supports long-duration high-density cell cultures by continuously feeding fresh media while removing spent media and secreted protein products.[2,3] This is an advantageous difference from batch processes, which typically only maintain operation for a short period of time before lack of nutrients and accumulation of waste material deteriorates cell viability.

Following cultivation, secreted recombinant protein products must be separated from the cells to enable purification. In batch, secreted proteins are separated from the cells at the end of a cultivation through additional unit operations employing conventional harvesting techniques such as centrifugation and depth filtration. In a continuous bioreactor, secreted proteins must be separated from the cells throughout the entire duration of the cultivation, making it difficult to adapt and scale separation methods traditionally used for batch.

Cell retention devices (CRDs) can combine cultivation and harvest into a single unit operation by enabling the continuous separation of secreted proteins from cells during a cultivation. CRDs typically fall into two broad categories: 1) membrane-based filters designed to separate cells from smaller components such as secreted proteins and 2) devices which leverage density and/or centrifugal forces to achieve separation. Perfusion is a mode of continuous bioreactor operation that utilizes CRDs to continuously separate secreted proteins from cells. Many perfusion processes employ the use of external CRDs – separation units that are outside of the bioreactor vessel, connected to the rest of the system via a recirculation loop (Figure 1A). In this case, cells and supernatant are constantly removed from

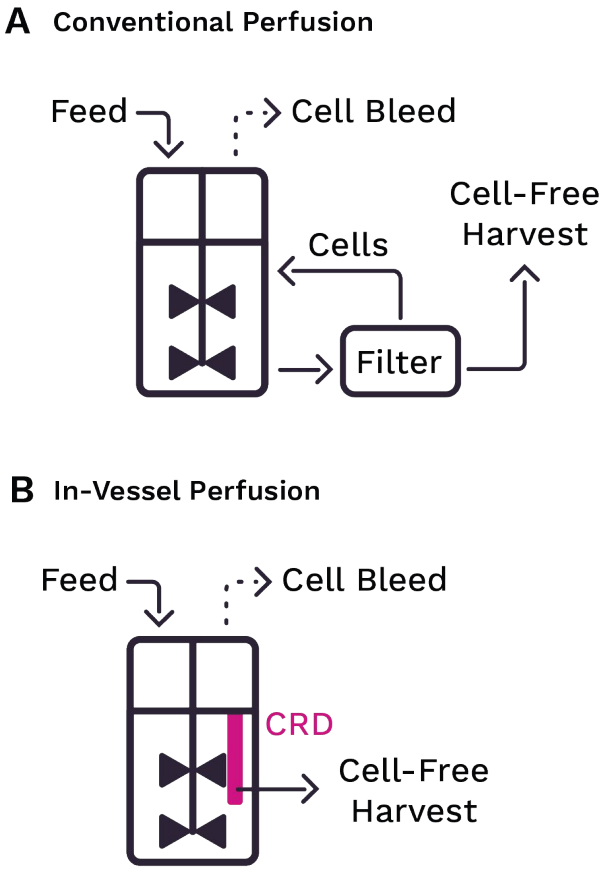

Figure 1. Modes of perfusion using (A) conventional external filtration and (B) Sunflower's in-vessel cell retention device (CRD).

the bioreactor and passed through the CRD to separate the cells from the secreted protein. The cells are then directed back into the bioreactor while the secreted protein is continuously collected or sent directly to the next unit operation.

While external CRDs allow for continuous separation of recombinant protein from cell bulk, they suffer from key design challenges. High shear forces, lack of environmental control within the external loop, and poor gas delivery are among some of the problems that trouble external CRDs. These challenges are exacerbated for cells with high respiratory coefficients, such as microbes, which are particularly susceptible to starvation and death when removed from the bioreactor environment.[2]

To address the challenges experienced when using external CRDs, Sunflower has engineered a proprietary in-vessel cell retention device that keeps biomass within the bioreactor while continuously harvesting secreted protein and removing spent media. Our in-vessel CRD is a membrane based device that sits inside the bioreactor, enabling the separation of secreted proteins and cells to occur without the cells ever leaving the controlled bioreactor environment (Figure 1B). Sunflower's CRD is seamlessly integrated into the control schema of the Daisy Petal™ bioreactor, demonstrating excellent antifouling capabilities, all while maximizing effective membrane area via its geometric configuration. The in-vessel CRD demonstrates an impressive versatility, supporting manufacturing of diverse protein classes of disparate biochemical and biophysical characteristics. Beyond protein diversity, the in-vessel CRD has also demonstrated compatibility with different cell types, enabling the use of several different alternative hosts for recombinant protein production.

Although our standard CRDs demonstrate an impressive range in compatibility with diverse biomolecules, protein-membrane binding interactions are possible and can prevent effective product separation, limiting protein yields. Sunflower therefore has also developed in-vessel CRDs that utilize several membrane types to fit the specific and particular needs of a manufacturing process.

This application note will demonstrate how Sunflower's in-vessel CRD combines fermentation and harvest into a single continuous unit operation, allowing for the expression of a diverse set of recombinant proteins from different cell types, all while sustaining longer, ultra-high cell mass cultivations.

Materials and Methods

Cultivations

Heterologous proteins were expressed in either Sunflower engineered *Pichia pastoris* strains,[4] or the filamentous fungi C1, an engineered strain of *Thermothelomyces heterothallica* (Dyadic Applied Biosolutions). Sunflower's proprietary defined media was used for all *Pichia pastoris* cultivations.[5] Dyadic's custom media formulation was adjusted

for perfusion and used in C1 cultivations. Perfusion fermentations were conducted on a Daisy Petal™ bioreactor as previously described.[2,3] During cultivation, samples of both in-vessel (pre-CRD) and harvested (post-CRD) material were collected. SDS-PAGE was performed on these samples to evaluate recovery of protein products from cell bulk after filtration through the CRD.

A flushing method was developed and used to dislodge protein bound to CRDs if a membrane-protein interaction occurred during cultivation. SDS-PAGE was used to confirm the presence or lack of protein in the flush material.

Membrane compatibility testing

A custom setup was used to evaluate performance of CRDs made with alternative membrane materials. CRDs were manufactured by Sunflower. All supernatant and cell broth material used in CRD membrane material compatibility testing was derived from cultivations which were carried out as previously described.[3] Cell broth or supernatant were passed through CRDs manufactured with different membrane materials. Trans-membrane pressure (TMP) was measured and used as an indicator of fouling. Membrane materials were evaluated on their ability to maximize CRD longevity based on the change in TMP over time.

Results & Discussion

Sunflower's in-vessel cell retention device enables continuous production of high quality protein from microbes

In fed-batch fermentations, nutrients eventually become limiting and toxic byproducts accumulate, which shortens the productive window to just a few days. Perfusion fermentation powered by the Daisy Petal™ unlocks higher achievable biomass by providing a controllable, flexible and continuous supply of fresh nutrients while removing waste. Using our proprietary in-vessel CRD, the Daisy Petal™ bioreactor supports 2-fold higher cell masses than those typically observed in a fed-batch fermentation.[3] Wet cell weights (WCW) of 700 g/L or higher have been demonstrated using the yeast *Pichia pastoris*. In addition to supporting ultra-high biomass, the Daisy Petal™ bioreactor can extend cultivation lengths up to 440% longer than fed-batch processes utilizing the same organism, with Daisy Petal™ cultivations lasting as long as 27 days.

In tandem to supporting cell health and culture longevity, our in-vessel CRD has enabled the successful recovery of proteins from numerous different classes. Comparing supernatant samples before and after filtration through the CRD during the production of a single-domain antibody (sdAb) shows that product is efficiently transferred across the membrane without significant losses (Figure 2A). Similar comparisons across other protein classes at different times

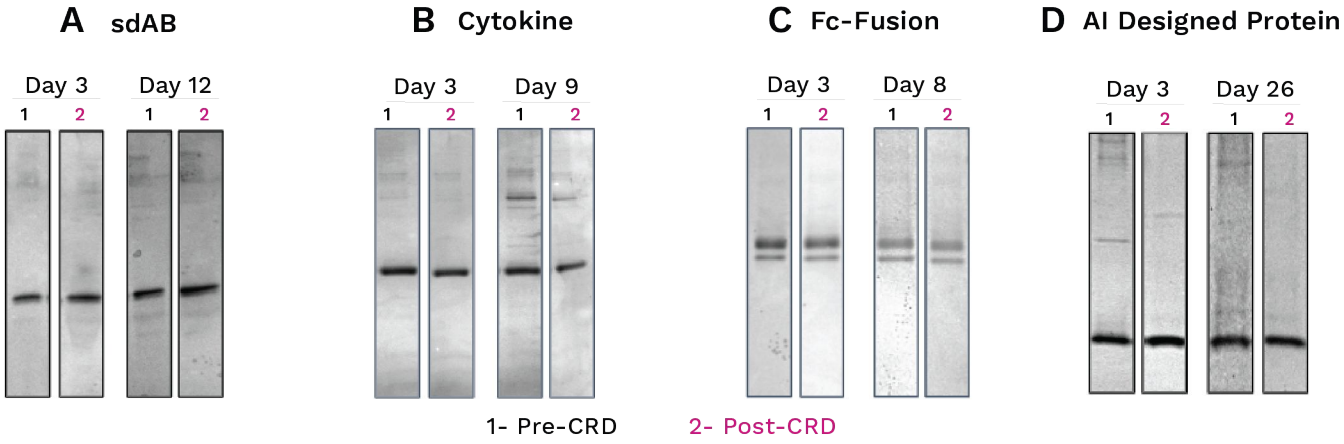

Figure 2. Sunflower's in-vessel CRD enables recovery of (A) a single-domain antibody, (B) a cytokine, (C) a Fc-fusion protein and (D) an AI-designed protein during perfusion fermentation. SDS-PAGE of samples from inside the vessel (pre-CRD - black) and harvest (post-CRD - pink).

during fermentation demonstrate that Sunflower's in-vessel CRD enables diverse biomolecule recovery without sacrificing product yield or quality throughout the entirety of a cultivation (Figure 2 B, C, D). Cytokines, sdAbs, mAbs, Fabs, Fc-fusions, subunit vaccine antigens, and AI-generated molecules; this diverse set of biomolecules are all compatible with Sunflower's in-vessel CRD.[4]

Our modular in-vessel CRD is compatible with a wide range of bioprocesses

Our in-vessel CRD demonstrates inherent flexibility because of its modularity. The CRDs can be made with different membrane materials to meet specific bioprocess needs. Here, we demonstrate the performance of CRDs with different membrane types in the recovery of a protein prone to interaction with Sunflower's standard CRD.

Live pressure data, representing the CRD trans-membrane pressure (TMP), is continuously reported during a perfusion cultivation in the Daisy Petal™. This data serves as a proxy for CRD health. Similar to other membrane-based processes, as a biomass cake layer forms on the CRD membrane, the TMP increases. If cake layer formation prevents free-flow through the membrane, the membrane is considered "fouled," concluding its operational use during cultivation. For this reason, the time it takes for a CRD to foul describes a key CRD performance attribute that determines its compatibility with a given bioprocess.

In a normal perfusion fermentation, the TMP stays constant or increases gradually, as shown for the production of a single-domain antibody (sdAb-1) (Figure 3A). In some cases, however, we have observed fouling of our standard CRD almost immediately upon expression of a biomolecule. This occurred during the fermentation of a similar single-domain antibody, sdAb-2 (Figure 3A). This behavior is atypical and has occurred only once

across the perfusion fermentation of over 45 proteins. Given the immediate increase in CRD TMP as expression of sdAb-2 began, we hypothesized that the protein itself may interact with the membrane, causing premature fouling. We therefore evaluated the potential interaction between the CRD membrane material and sdAb-2. Following the fermentation campaign, the CRD membrane was subjected to a flushing protocol to disrupt potential interactions and release bound protein. SDS-PAGE evaluation of the flushed material revealed that sdAb-2 was released from the membrane during flushing (Figure 3B). As a control, the same flushing protocol was applied to CRDs from the sdAb-1 cultivation (Figure 3B). In this instance, no protein was observed in the flushed material. The flushing protocol's results corroborated the TMP spike observed during the bioreactor cultivation and confirmed a protein interaction between sdAb-2 and Sunflower's standard in-vessel CRD.

To alleviate the process risk associated with protein-membrane interactions as observed with sdAb-2, Sunflower evaluated the use of various membrane material types for the fabrication of the in-vessel CRDs. The selection of membrane candidates was based on their potential to mitigate protein-membrane interactions, while demonstrating compatibility with the perfusion fermentation process. Four membrane candidates were investigated for compatibility with sdAb-2 by continuously perfusing cell culture material containing sdAb-2 through each membrane.

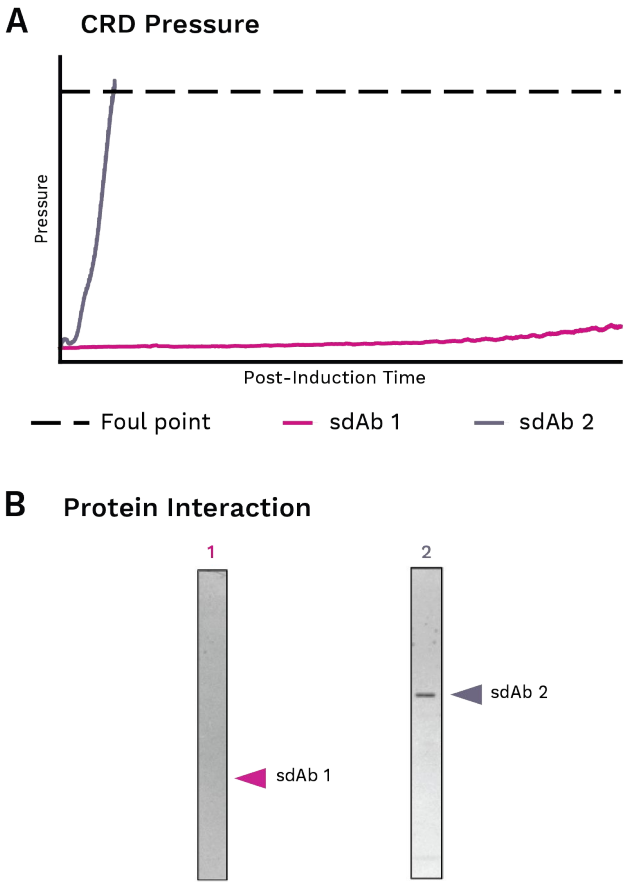

Figure 3. Protein-membrane interaction observed using Sunflower's standard in-vessel CRD. (A) Transmembrane pressure profiles from the fermentation of two single-domain antibodies. (B) SDS-PAGE analysis of protein removed from CRDs during flushing after fermentation with two sdAbs. Arrows indicate the expected molecular weight.

TMP data was used as a quantifiable metric to evaluate fouling of a CRD membrane due to protein interaction. Material A, the material used in Sunflowers' standard CRD, was included as a control and showed a rapid increase in TMP, as expected (Figure 4A). Materials C and D, however, demonstrated a very small change in TMP over time, a 40-fold

smaller change compared to material A. While material B did not exhibit a change in TMP as severe as material A, it still demonstrated a 10-fold greater change in TMP over time relative to materials C and D. Given the results from this testing, materials C and D were selected as promising alternative membrane candidates for use in CRDs due to their low-risk of protein-interaction with sdAb-2.

In-vessel CRD versatility facilitates cultivation of diverse microbes with the Daisy Petal™ bioreactor

The Daisy Petal™ was designed to support perfusion fermentation of diverse microbial organisms. Before cultivating a new organism in the Daisy Petal™, Sunflower can test it's compatibility with multiple CRDs manufactured with different membrane types. We did this testing with the filamentous fungus, C1, an engineered strain generously provided by Dyadic Applied Biosolutions. Similar to the testing previously discussed, cell-containing bioprocess fluid was continuously perfused through several membrane candidates, and TMP data was used to compare performance between each membrane type. This testing aimed to define the interaction between Sunflower's standard in-vessel CRD and the new organism, while identifying alternative material candidates if Sunflower's standard membrane material was deemed incompatible with the new microbe. To this effect, C1 cell broth was evaluated for compatibility with three different membrane types (A, C and E) and compared to a control where *Pichia* cell broth was passed through our standard membrane material, membrane A. The result demonstrated that CRDs made from materials A and C were suitable for use with C1. CRDs made with materials A and C demonstrated a

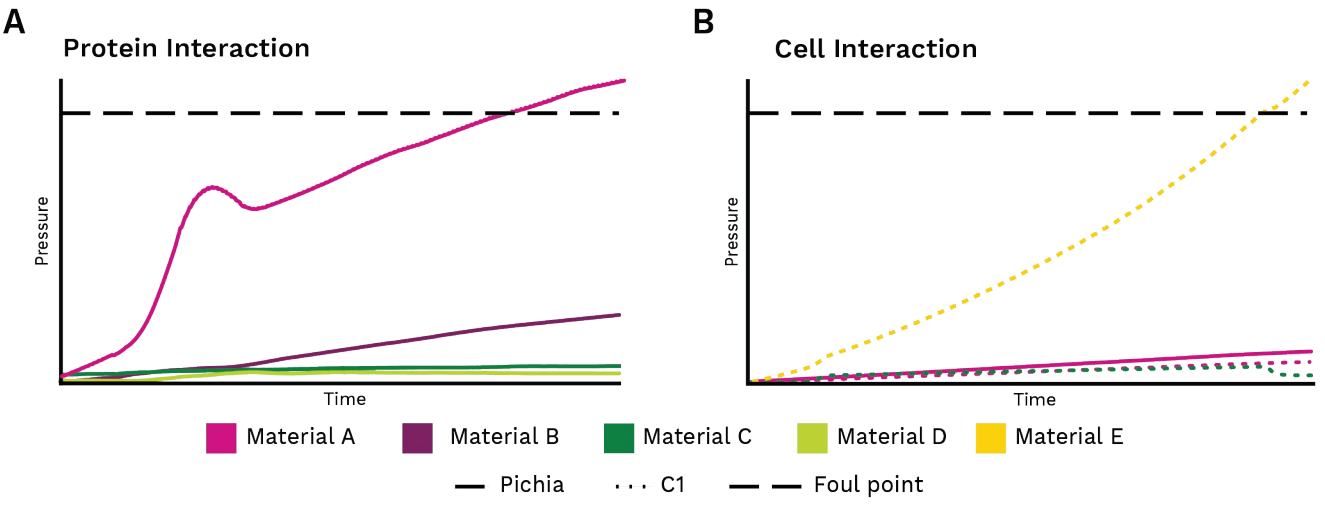

Figure 4. CRD performance using different membrane materials (colors) for perfusion fermentation of (A) an interaction-prone protein sdAb-2 and (B) alternative cell types (Pichia pastoris - solid line; C1 - dotted line). The fouling point is depicted by a black dashed line (--).

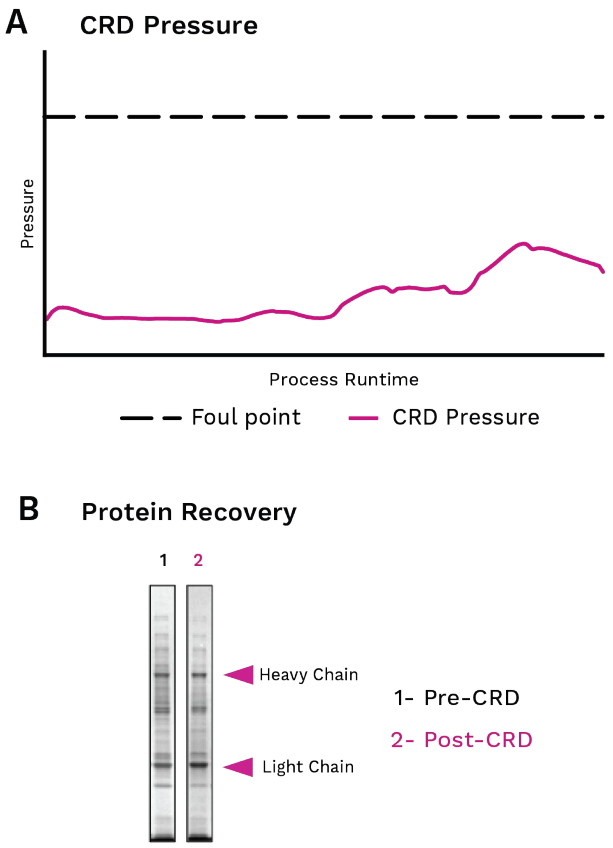

Figure 5. Cell retention device performance with filamentous fungus C1. (A) Transmembrane pressure profile of a perfusion fermentation with C1. (B) SDS-PAGE analysis of samples from inside the vessel (pre-CRD - black) and harvest (post-CRD - pink). Arrows indicate the expected molecular weight of the expressed mAb heavy and light chains.

negligible change in TMP over time, similar to the *Pichia* control (Figure 4B). CRDs made from material E, however, showed 10-fold greater change in TMP over time, indicating incompatibility with C1. This testing led to the use of membrane material A in a 14 day cultivation with C1, in which the in-vessel CRD enabled higher biomass and a longer cultivation compared to fed-batch fermentation, all while successfully harvesting the secreted mAb protein product (Figure 5). The success of membrane material A in a full-length Daisy Petal™ bioreactor cultivation with C1 demonstrates Sunflower's ability to test and adapt its modular in-vessel CRDs to expand its platform compatibility to a diverse array of microbial organisms.

Conclusion

The in-vessel CRD technology developed by Sunflower is the cornerstone of perfusion fermentation using the Daisy Petal™ Perfusion Bioreactor System. Sunflower's in-vessel CRD allows cultivations to achieve remarkably dense cell masses that retain excellent productivity over several weeks to months. As the biotechnology sector continues to evolve and produce new bio-based products with diverse hosts, the adaptability of our in-vessel CRD will be invaluable. Sunflower's CRD design emphasizes modularity, resulting in a separation tool that can be customized to accommodate a diverse array of bioprocesses expressing recombinant proteins with diverse biophysical and biochemical characteristics. Furthermore, the in-vessel CRD enables the use of multiple microbial host organisms within Sunflower's Daisy Petal™ Perfusion Bioreactor System, pushing the horizon for perfusion-compatible bioprocesses and the possibilities for next-generation biomanufacturing.

References

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Related Resources

Application Note: Perfusion fermentation in the Daisy Petal™ bioreactor

Learn more about the Daisy Petal™ Perfusion Bioreactor System