Sunflower

Application Note

Equipment Highlight

Keywords

Bioreactor, high cell density cultivation, perfusion fermentation, yeast, fungi

Perfusion fermentation in the Daisy Petal™ bioreactor sustains ultra-high biomass

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Abstract

High cell density fermentation processes are advantageous in biomanufacturing due to the increased volumetric productivities achievable with more cells. To reap the benefits of high biomass production, significant cell mass must be adequately supported in a bioreactor. Legacy batch and fed-batch bioreactor systems are capable of reaching high biomass, but struggle to support the high nutrient and oxygen demands brought on by high cell densities. Sunflower's Daisy Petal™ perfusion bioreactor overcomes limitations of legacy systems via advanced programmable feeding strategies with continuous product harvest and waste removal while preserving biomass. Here, we demonstrate the advantages of using the Daisy Petal™ perfusion bioreactor to reach and extend ultra-high cell density fermentations involving multiple microbes producing recombinant proteins.

Introduction

In precision fermentation, the main goal is to produce large amounts of high-quality heterologous proteins. High cell density fermentation is commonly used to achieve this goal because increasing the number of cells in a bioreactor typically results in higher amounts of secreted protein, and therefore higher volumetric productivity.[1] In typical batch and fed-batch fermentations, microbial cultures will usually reach biomass concentrations of ~300 g/L wet cell weight (WCW) (~100 g/L dry cell weight (DCW)). Cell densities upwards of 600 g/L WCW (~200 g/L DCW) are achievable in some cases,[2-4] but limitations on nutrient availability and accumulation of metabolic byproducts do not generally enable maintenance of such high biomass concentrations for more than a few days (Figure 1A).

Continuous fermentation processes overcome the hurdles of fed-batch processes by enabling constant feeding of fresh medium and removal of waste products. Several different strategies exist for continuous fermentation. In chemostat cultures, cell bulk (containing both cells and supernatant) is removed at the same rate as media is supplied into the

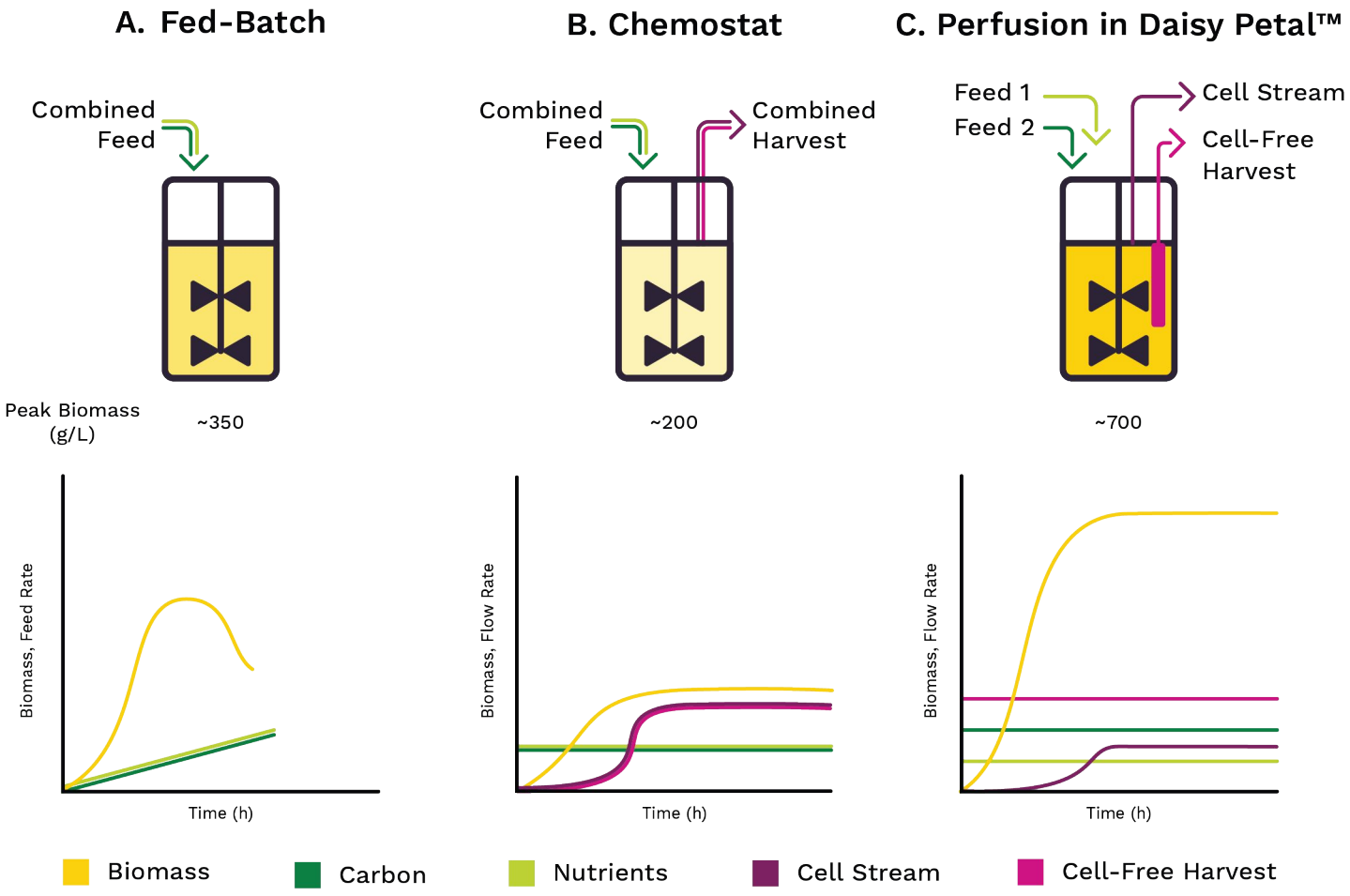

Figure 1. Comparison of biomass, feeding, and harvesting in (A) fed-batch, (B) chemostat, and (C) in-vessel perfusion fermentation modes.

bioreactor, which reduces biomass concentrations within the bioreactor and generally prevents cultures from reaching very high cell density (Figure 1B).

Alternatively, perfusion systems keep cells within the bioreactor environment via cell separation devices while waste products and secreted proteins are continuously removed and fresh medium is continuously fed. In mammalian and microbial systems, perfusion has been achieved by circulating cell bulk to an external device (e.g., tangential or alternating flow filters) where cells are separated from supernatant and recirculated back to the bioreactor in a concentrated cell stream. However, this removal from the controlled bioreactor environment puts stress on cells, limiting cell viability and protein production. For microbes and cell types with high respiratory coefficients, removing the cells from the bioreactor, even for a short time, can induce starvation and is not a feasible strategy for achieving and maintaining high biomass.

Sunflower's Daisy Petal™ Perfusion Bioreactor System uses an in-vessel cell retention device (CRD) that enables continuous removal of waste products and secreted proteins without the cells ever leaving the bioreactor (Figure 1C). Using this in-vessel perfusion strategy, cells grow to higher cell densities and the culture is maintained in a healthy state for extended periods while sustaining productivity. Additionally, the Daisy Petal™ utilizes a multi-pump setup which enables the use of various feeding modes – linear gradient, constant ratio, and exponential – that can be used strategically to build and sustain biomass. Feeding strategies can be tailored to support an organism's growth kinetics, enabling better control over growth rate. Ultimately, strategies can be developed to obtain ultra high biomass and deliver sustained productivities and high protein yields.

In this application note, we demonstrate the advantages of continuous fermentation with Sunflower's Daisy Petal™ perfusion bioreactor to sustain high biomass cultures expressing recombinant proteins through multiple case studies. We analyze the performance of the methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) and the filamentous fungi C1, an engineered strain of *Thermothelomyces heterothallica* (Dyadic International) in perfusion fermentation, and demonstrate sustained productivity of a single-domain antibody in a high-density perfusion fermentation of engineered *P. pastoris*. We also illustrate how the programmable feeding strategies in the Daisy Petal™ can be used to achieve and maintain high cell density cultures. Extended perfusion campaigns spanning 200–300 hours were performed in Sunflower's Daisy Petal™ bioreactors, enabling the stable maintenance of high density cultures with biomass concentrations exceeding 700 g/L WCW throughout the production phase.

Materials and Methods

High biomass fermentation with *P. pastoris*

A *P. pastoris* strain engineered to express a single-domain antibody was cultivated via perfusion fermentation in the Daisy Petal™ bioreactor. The bioreactor was charged with chemically defined growth media containing glycerol to a 1 L working volume. A frozen cell stock was used to inoculate the bioreactor starting at an OD/mL of 0.15. Defined growth media containing glycerol was continuously fed to the reactor at a fixed concentration or exponentially with a perfusion rate of 1.8 vessel volumes per day (VVD) for 32 - 48 hours. After this period, 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 of 1.8 VVD. 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 cultivation, and completed a system shut down after the desired fermentation time was reached (200-300 hours). WCW was determined using cell-containing samples from inside the bioreactor. Briefly, 1 mL samples were transferred to pre-weighed 1.5 mL tubes and centrifuged to obtain cell pellets. The supernatant was carefully discarded and the cell pellet weighed. The empty tube weight was subtracted from the tube-plus-pellet mass to obtain the net weight of the cells. This cell weight was then used to determine the WCW in g/L. Averages were calculated from triplicates. Protein yield and quality were determined by SDS-PAGE using samples of the cell-free harvest fluid.

High biomass fermentation with C1

A C1 strain (Dyadic International) was cultivated via perfusion fermentation in the Daisy Petal™ bioreactor. The bioreactor was charged with a custom growth media. Following a 2-day seed train, the bioreactor was inoculated to a 1 L working volume. Custom growth media was continuously fed to the bioreactor for 12 hours. After 12 hours, the Daisy Petal™ automatically induced heterologous protein production by switching to a custom production medium. A separate pump was used to control carbon feeding independently from the production medium and keep carbon concentrations within a defined range. The production medium was fed at a perfusion rate of 1.8 VVD, and 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 cultivation, and completed system shut down after the desired fermentation time was reached (300+ hours). Dry cell weight (DCW) was determined as described above for WCW, except cell pellets were dried in an incubator prior to measuring weight.

Results

Daisy Petal™ bioreactor supports sustained fermentation of high density biomass cultures

Perfusion fermentation enables the support of highly productive ultra-high biomass cultures through consistent feeding and waste removal without the need to remove biomass from the bioreactor. Here, we show the advantages of in-vessel perfusion for recombinant protein-producing strains of *P. pastoris* and C1, cultivated in the Daisy Petal™ for over 250 hours (10+ days), approximately twice as long as typical fed-batch cultivations for these organisms.

In a perfusion system, *P. pastoris* can quickly gain high biomass due to tight control of feeding and waste removal. Here, a *P. pastoris* cultivation in the Daisy Petal™ achieved a biomass concentration of 436 g/L WCW at the start of induction (48 hours) without the use of a seed train, and was kept between 500-600 g/L WCW using a cell bleed strategy for the remainder of the campaign (Figure 2A). The culture reached a peak of 587 g/L WCW at 172 hours during the induction phase, and ended the cultivation at 571 g/L WCW (Figure 2A). In contrast, fed-batch bioreactors are limited both in biomass levels and duration. Typical fed-batch cultivations of *P. pastoris* reach ~300 g/L WCW and can not be maintained beyond 5 - 6 days due to physical and biological limitations including nutrient limitation and build-up of waste products (Figure 2A).[4-6]

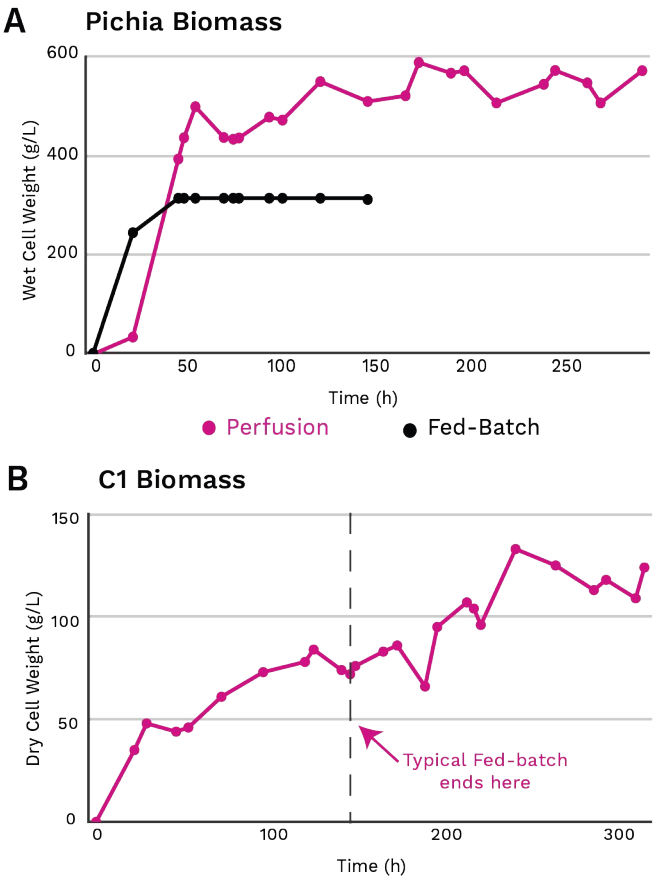

Figure 2. Biomass achievable with various microbes via fed-batch or perfusion fermentation. (A) Wet cell weight of P. pastoris achieved in fed-batch (black) and perfusion (pink) fermentation. (B). Dry cell weight of C1 achieved in perfusion fermentation.

C1 also showed advantages when cultivated in perfusion fermentation. In the Daisy Petal™, the C1 cultivation was maintained at high biomass for over 300 hours (12+ days) (Figure 2B). DCW was maintained well over 75 g/L for the majority of the campaign, with the highest concentration reaching 133 g/L at 241 hours. This is about 2-fold higher biomass sustained for twice as long compared to typical fed-batch performance with this organism.

Perfusion processes in the Daisy Petal™ bioreactor with both *P. pastoris* and C1 outperformed their fed-batch counterparts, reaching 1.5 - 2-fold higher biomass concentrations which were sustained for at least twice as long.

High density *P. pastoris* fermentation yields sustained production of a single-domain antibody

While the Daisy Petal™ perfusion bioreactor supports high biomass for extended fermentation lengths, its core strength is enabling high space-time yield of recombinant proteins via sustained productivity during long-term cultivations. Building on the demonstration above focused on sustaining high biomass for over 10 days, we investigated the productivity of the culture during this extended high biomass cultivation.

After a 48 hour biomass accumulation phase, cells were fed methanol-containing media to enable recombinant protein production for the remainder of the campaign, lasting over 245 hours. For the duration of the induction phase, the instantaneous titer of single-domain antibody was maintained between 0.08 - 0.13 g/L, which resulted in a total yield of 3.1 g and a space-time yield of 257 mg/L/day (Figure 3A). Visual analysis via SDS-PAGE showed consistent production throughout the run (Figure 3B).

Although achieving and maintaining high biomass is key to increasing protein yield, it is insufficient without sustained productivity. This demonstration further

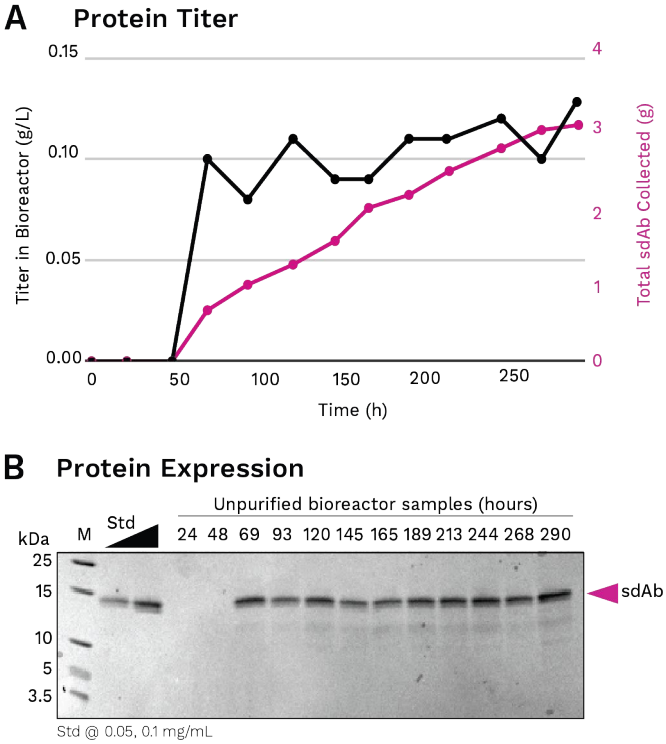

Figure 3. Sustained production of a high-quality single-domain antibody (sdAb) through extended high biomass cultivation. (A) Instantaneous protein titer (black) and cumulative protein (pink) collected as measured by densitometry on SDS-PAGE. (B) SDS-PAGE of unpurified bioreactor cultivation samples.

confirms that, even in an unoptimized process, the Daisy Petal™ is capable of sustaining high density cultures to achieve high protein productivity.

Daisy Petal™ enables fermentation with ultra-high biomass

In addition to supporting biomass for prolonged cultivations, perfusion fermentation is a powerful technique that allows for ultra-high cell densities, exceeding 600 g/L WCW. Here, an engineered *P. pastoris* strain reached

nearly 750 g/L WCW in the Daisy Petal™ perfusion bioreactor, continuously producing a single-domain antibody in a 150 hour campaign.

After a 36 hour biomass accumulation phase, cells were fed concentrated methanol-containing media to encourage continued growth and enable recombinant protein production for the remainder of the campaign, lasting over 150 hours. The biomass concentration was roughly 200 g/L WCW at the start of the induction phase, and reached a peak of 744 g/L WCW at the end of the campaign (Figure 4A). To our knowledge this biomass of nearly 750 g/L WCW is one of the highest ever reported for *P. pastoris*. Notably, the campaign ended as scheduled without any failures of the hardware or in-vessel cell retention device due to the ultra-high biomass. Production of the single-domain antibody started at 48 hours, and remained consistent throughout the campaign, demonstrating that sustained productivity can be obtained from an ultra-high density *P. pastoris* fermentation (Figure 4B). These results are key to taking high cell density fermentation to the next level, enabling the potential to reach previously unattainable cell densities and volumetric productivities.

Typically, higher biomass leads to increased volumetric productivity. In this case, however, although the total cell volume expanded, the total collected sdAb was similar to the previous experiment at this fermentation length, likely due to a non-optimized feed strategy that caused nutrient limitation.

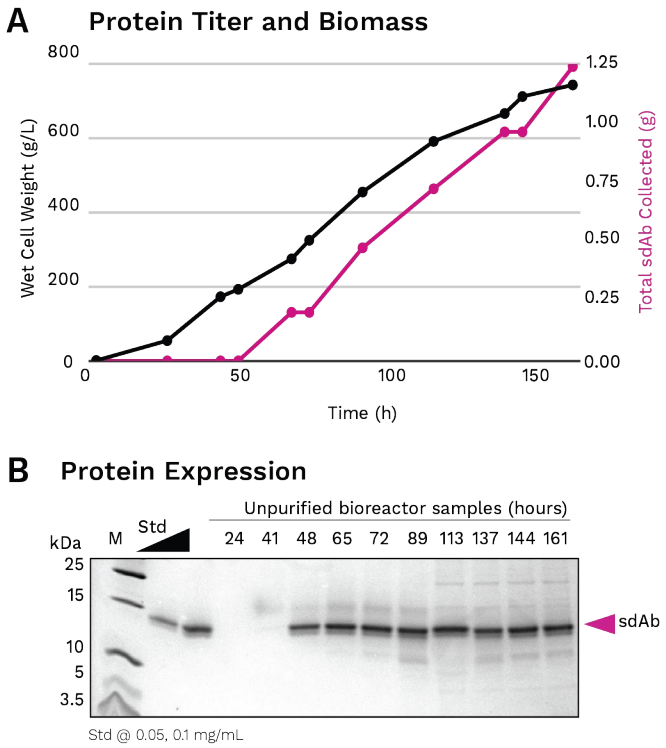

Figure 4. Ultra-high biomass culture yields high-quality single-domain antibody. (A) Wet cell weight (black) and cumulative protein (pink) collected as measured by densitometry on SDS-PAGE. (B) SDS-PAGE of unpurified bioreactor cultivation samples.

Strategic carbon feeding enables higher biomass and increased growth rates

Another defining feature of the Daisy Petal™ perfusion bioreactor system is its multi-pump setup which enables the use of various feeding modes, including linear gradient, constant ratio, and exponential feeding. Cultivations were carried out on the Daisy Petal™ using a *P. pastoris* strain to compare outgrowth feeding schemes, including a constant carbon feed and an exponential carbon feed. A traditional perfusion process was carried out first, where feeding was controlled by a single

feed pump. Standard glycerol-containing media was fed for 24 hours, followed by an automatic switch to feed high concentration glycerol-containing media for another 24 hours (Figure 5A).

A second perfusion process utilized an exponential feed controlled by two pumps. One pump (pump A) was connected to highly concentrated glycerol-containing media, and the second (pump B) was connected to standard glycerol-free media. To ensure a consistent perfusion rate, the total feed rate from pumps A and B was maintained at 1.8 vessel volumes per day. The ratio of feed from pumps A and B was automatically varied to achieve the desired glycerol feeding profile (Figure 5A, B).

Feeding was initiated with a low glycerol feed (low% Pump A : high% Pump B) and the ratio increased exponentially for a duration of 48 hours, resulting in a final ratio with high glycerol (high% Pump A : low% Pump B) (Figure 5A, B). Feeding the

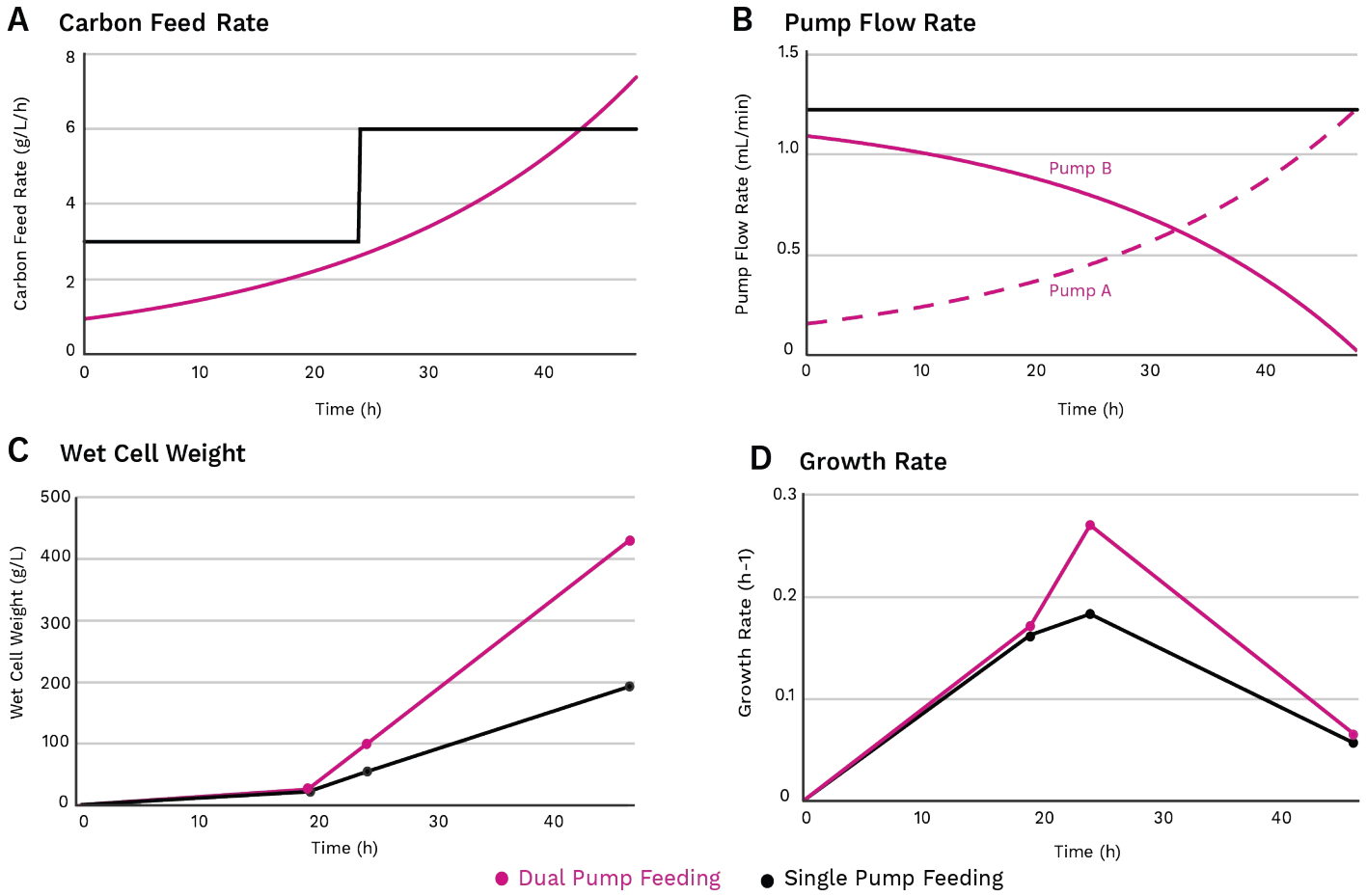

Figure 5. Comparison of standard single pump (black) and exponential dual pump (pink) feeding strategies on Daisy Petal™ and impact on biomass growth. (A) Carbon feed rates (g/L/h). (B) Flow rates (mL/min) of feed pump(s). (C) Wet cell weight (g/L) and (D) growth rates (h-1) of P. pastoris cultures with each feeding scheme.

right concentration of glycerol is important to avoid glycerol depletion or buildup, both of which can lead to metabolic stress and affect subsequent protein expression during the methanol induction phase.

After 24 hours of growth, both cultivations yielded contrasting growth rates and biomass concentrations. The single-pump feeding strategy yielded a WCW and growth rate of 55 g/L and 0.13 h^{-1} respectively, whereas the exponential feed yielded a WCW and growth rate of 100 g/L and 0.19 h^{-1} (Figure 5C, D). After 48 hours, biomass in the cultivation with the single-pump feed reached only 193 g/L WCW. The exponential feed showed improved growth with the biomass concentration increasing by more than 4-fold from 100 to 430 g/L WCW (Figure 5C). Notably, the exponential feed resulted in a maximum growth rate of 0.27 h^{-1} (Figure 5D) following the lag phase, which helped build significantly more biomass compared to the single-pump strategy which resulted in a maximum growth rate of 0.18 h^{-1} (Figure 5D). The exponential feeding strategy resulted in a 2.2-fold increase in WCW and a 1.5-fold increase in maximum growth rate compared to the standard feed. Building biomass early on is important for transitioning into the induction phase, where growth slows down and cell metabolism switches to a focus on heterologous protein expression.

The exponential feed is particularly advantageous because it enables the capacity to properly match nutrient supply to the organism's metabolic demand. This allows for more constant growth and biomass accumulation, which subsequently yields healthier and highly productive cultures.

Conclusion

High biomass continuous fermentation processes provide a cost-effective approach to achieve high protein yields through extended cultivations with increased volumetric productivities. Sunflower's Daisy Petal™ perfusion bioreactor was demonstrated to support up to two-fold higher biomass for at least twice as long as conventional fed-batch fermentations with sustained protein productivity. This exceptional performance is enabled by our in-vessel CRDs that continuously harvest protein and remove waste products while retaining cells inside the bioreactor. Additionally, the dual pump feature in Daisy Petal™ allows users to utilize various feeding modes that can be tailored to an organism's metabolic demand, which can enable precise feeding control for optimization of fermentation processes. Through its unique in-vessel cell retention device and programmable feeding strategies, the Daisy Petal™ perfusion bioreactor enables sustained high cell density fermentation for diverse microbes.

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Related Resources

Application Note: Perfusion fermentation in the Daisy Petal™ bioreactor

Learn more about the Daisy Petal™ Perfusion Bioreactor System