Sunflower

**Application Note**

*Equipment Highlight*

**Keywords**

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# Daisy Petal™ simplifies fermentation optimization

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## Abstract

Optimizing a biomanufacturing process for high product yield typically requires extensive exploration of process conditions. This optimization is inefficient in legacy batch or fed-batch bioreactor systems, requiring high upfront costs for multiple bioreactors or a long series of experiments. Through continuous perfusion fermentation, the Daisy Petal™ simplifies upstream optimization by enabling evaluation of multiple conditions in a single fermentation. By programming a series of individual steps to test different parameters, the bioreactor will automatically adjust to different steady states. Protein expression and quality can be evaluated under each set of conditions and compared to determine the optimal conditions for high product yield. Here, we demonstrate the evaluation of multiple process conditions in a single experiment using the Daisy Petal™ Perfusion Bioreactor System through two case studies, including a seven-condition DOE to identify influential process parameters, and another fermentation evaluating the impact of four nutrient additives on productivity. These case studies show that cells can quickly respond to a new bioreactor steady state and even recover productivity after non-optimal states. Through the advantages of perfusion fermentation, the Daisy Petal™ system enables accelerated, cost-effective process optimization for biomanufacturing.

## Introduction

The development of biomanufacturing processes that meet cost targets typically requires significant optimization of conditions to achieve the highest product yields. The optimization of cultivation parameters for expression of high quality heterologous proteins can be very costly and time consuming due to its large design space. There are many important process levers to explore to improve protein expression and/or quality, and most of these experiments must be performed in a bioreactor to enable sufficient process control for collection of representative data. Several impactful methods of experimental design, such as Design of Experiments (DOE) or Bayesian optimization, are utilized to maximize the data output while minimizing the number of experiments to be performed. Still, tens to hundreds of experiments may be required to explore the wide biological design space and identify the critical cultivation process parameters such as nutrient feed rates, pH, dissolved oxygen, temperature, etc., that lead to the best yield of high quality protein.

Consider, for example, a 3-level, 2-factor DOE that allows for a deep evaluation of the design space of two distinct cultivation conditions, such as pH and temperature. This DOE requires testing nine sets of process conditions. For batch or fed-batch cultivation processes, this would require nine independent small-scale bioreactor experiments. For a fast-growing organism, such as the yeast *Pichia pastoris*, each of these experiments would be 4 to 5 days long. Using a conventional single benchtop bioreactor, it would take at least 45 days to complete this DOE. Higher throughput systems are available that enable several bioreactors to be run in parallel, but the capital and operating costs of such systems are high. Using a parallel bioreactor approach with nine bioreactors, the timeline for this DOE can be reduced to 1 week, but it would still require the materials and operational resources for nine separate bioreactor runs. This approach using a single condition per bioreactor quickly becomes infeasible when testing larger design spaces.

Sunflower's Daisy Petal™ Perfusion Bioreactor System operates in a continuous mode[1] and has two key properties that enable many cultivation conditions to be evaluated in a single bioreactor campaign. First, in perfusion operations the fluid inside the bioreactor is completely replaced at a known rate. Fresh nutrients are continuously supplied to the biomass in the bioreactor, while secreted proteins and waste products are continuously removed through the cell-free harvest. Over time, this fluid replacement effectively "washes out" all components associated with the previous condition, including secreted protein, unused metabolites, and waste products, providing a clean environment for the cells in the next condition. Second, the Daisy Petal™ system is built for use with eukaryotic microbes, such as *Pichia pastoris*, that rapidly adapt to changing environments. When process parameters such as pH or temperature are adjusted during a fermentation, these cells can

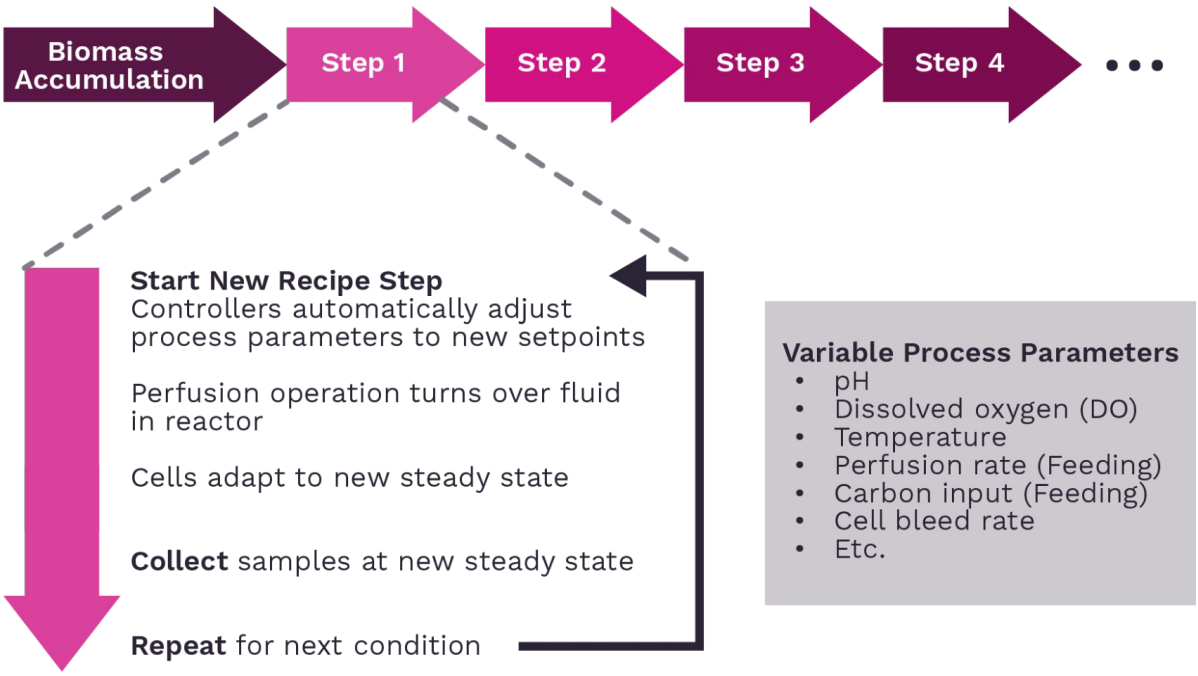


*Figure 1. Automated evaluation of multiple process parameters in a single fermentation using the Daisy Petal™ Perfusion Bioreactor System.*

quickly adapt their metabolism to the new conditions. By applying new process parameters and allowing the replacement of the cell culture fluid, the bioreactor achieves a new steady state operating environment. Samples of the perfusate and biomass collected during this steady state are reflective of the active process conditions, and can be analyzed to understand which conditions are correlative with desired fermentation performance metrics. Connecting several of these process "shifts" during a fermentation can enable broad design space exploration during a single experiment. Using the Daisy Petal™, the nine conditional DOE described above can be completed in less than 12 days using the materials and operational resources for only one bioreactor run.

In this application note, we describe how to utilize the Daisy Petal™ to evaluate multiple sets of conditions during a single automated bioreactor experiment and demonstrate this capability through several case studies.

## Approach

Using HelianthOS™, the software that automates Daisy Petal™ system operations, a user can design a multistep recipe to test a variety of process conditions in a single experiment executed without any manual intervention (Figure 1). A separate recipe 'step' is created for each new set of process conditions or media inputs to be deployed by HelianthOS™. Control ranges for process parameters, like pH, dissolved oxygen, temperature, perfusion rate, nutrient feeding rate (e.g. carbon feeding

rate), cell bleed rate, and others, are selected for each step during recipe development ahead of process deployment on the system. During recipe deployment, HelianthOS™ instructs the controllers in the Daisy Petal™ to automatically adjust the set points or feed rates to the pre-programmed conditions while the perfusion operation continuously turns over the fluid in the bioreactor. Depending on the selected rate of fluid delivery and removal, or the "perfusion rate", the fluid in the bioreactor can be completely replaced up to 2.5 times per day. Once the bioreactor fluid has been replaced and the cells have adapted to the updated process conditions, the cells' biological performance is a reflection of the new steady state achieved in the bioreactor.

Sampling the culture after the new steady state is reached can enable a correlation between a particular set of process parameters applied to the cells and the resulting biological behavior, including heterologous protein expression, protein post-translational modifications or secreted wastes and metabolites. Samples can be taken by setting specific time ranges for automated perfusate collection, or by manually sampling inside the bioreactor vessel through the sample port as desired. After the length of time preset by the user in the recipe, HelianthOS™ will automatically move on to the next recipe step to enable evaluation of the next set of conditions. In this way, the Daisy Petal™ and its software streamlines evaluation of many process conditions in a single experiment.

## Materials and Methods

Perfusion fermentations were executed on Sunflower's Daisy Petal™ bioreactor[1] using a Sunflower-engineered *Pichia pastoris* strain producing a recombinant Fc-fusion protein.[2] Process recipes were created using Nursery™, Sunflower's process planning and visualization software tool, and deployed by HelianthOS™, the central operating system for the Daisy Petal™. Sunflower's proprietary defined media formulations were used to support biomass growth and protein production.[3]

### *Case Study 1: Identifying Influential Process Parameters Using Design of Experiments (DOE)*

JMP® 18.1.0 was used to design a seven condition design of experiments (DOE) investigating the influence of dissolved oxygen (DO), temperature, and an additive on protein production (Table 1). 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/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 48 hours to build biomass. Cell-free harvest fluid was directed to waste during this time. After 48 hours, the Daisy Petal™ automatically induced production of the recombinant Fc-fusion protein by switching to a defined production medium containing methanol, fed at 1.8 VVD. The production phase consisted of seven 24 hour steps, each

carrying out one set of conditions from the DOE. DO and temperature setpoints were automatically adjusted by the Daisy Petal™ software. Additive addition was facilitated by automatically switching the input media between a production media containing the additive and a production media without the additive. For the first 14 hours of each step, the cell-free harvest was directed to waste. For the last 10 hours of each step, the cell-free harvest was directed to a collection vessel. Wet cell weight (g/L) was determined using cell-containing samples from inside the bioreactor. Protein yields were determined from samples of the cell-free harvest fluid collected at the end of each step using a quantitative immunoturbidimetric assay specific to human IgG. Average volumetric productivity for each collection was calculated according to equation 1 and represents the average productivity over the 10 hour collection period. Cell-specific productivity was calculated according to equation 2 from samples taken in the last hour of each step and represents the instantaneous rate of protein production per gram of cells under each condition. Following the campaign, JMP® 18.1.0 was used to fit a model to the data. Cell-specific productivity and volumetric productivity were fit simultaneously using standard least squares methodology. No interaction or second-order model terms were found to be significant.

*TABLE 1: Conditions tested in Case Study 1 DOE.*

| Step | % DO | Temperature | Additive |
|---|---|---|---|
| 1 | Center | High | No |
| 2 | Low | High | Yes |
| 3 | High | Center | Yes |
| 4 | High | High | No |
| 5 | Low | Center | No |
| 6 | Center | Center | Yes |
| 7 | Center | Low | Yes |

## *Case Study 2: Evaluating Nutrient Additives*

A Daisy Petal™ fermentation was performed as described in Case Study 1, except for the following adjustments. After the initial 48 hours of biomass accumulation, a 22 hour transition period was utilized to allow the cells to fully adapt to the methanol containing media. Following adaptation, the first 24 hour production step served as a control where no additional nutrients were added. Next, key nutrients were delivered to the bioreactor every 24 hours in bolus via injection through a port in the bioreactor headplate. Process samples, including samples of biomass from inside the vessel and samples of cell-free harvest fluid from the collection vessel, were collected during the last hour of each step. Growth rate was calculated based on the change in wet cell weight (WCW) over time according to equation 3. Instantaneous volumetric productivity was calculated according to equation 4 from

$$\text{Average Volumetric Productivity (mg/L/day)} = \frac{\text{Total Protein of Interest Collected (mg)}}{\text{Bioreactor Volume (L)} * \text{Collection Length (day)}} \quad (1)$$

$$\text{Cell-Specific Productivity } (\mu g/g_{cells}/h) = \frac{\text{Protein Titer } (\mu g/L) * \text{Perfusion Rate } (h^{-1})}{\text{Wet Cell Weight } (g_{cells}/L)} \quad (2)$$

$$\text{Growth Rate } (h^{-1}) = \frac{\ln(WCW_{t1} \div WCW_{t0})}{t_1 - t_0} + \text{Cell Bleed Rate } (h^{-1}) \quad (3)$$

$$\text{Instantaneous Volumetric Productivity (mg/L/day)} = \text{Protein Titer (mg/L)} * \text{Harvest Rate } (day^{-1}) \quad (4)$$

samples taken in the last hour of each step and represents the instantaneous rate of protein collection from the bioreactor under each condition. Cell-specific productivity was calculated as described above.

## Results

### *Case Study 1: Identifying Influential Process Parameters Using Design of Experiments (DOE)*

The Daisy Petal™ was used to identify cultivation process parameters that impact productivity of a secreted protein by conducting a simple DOE within one fermentation. The protein was an Fc-fusion, an engineered protein containing the Fc (fragment crystallizable) region of an antibody fused to another protein of interest. A seven condition DOE was designed to evaluate the significance of dissolved oxygen (DO), temperature, and a media additive on the productivity of the recombinantly expressed Fc-fusion (Table 1). A recipe was created to automate the change in process setpoints and media inputs every 24 hours, after an initial 48 hour phase of biomass accumulation (Figure 2A). The resulting fermentation campaign progressed for 216 hours sequentially testing 7 different process conditions. The bioreactor was operated at a perfusion rate of 1.8 vessel volumes per day (VVD). At this perfusion rate, it takes 13.3 hours to add one vessel volume of new fluid to the bioreactor. The perfusate was directed to waste for the first 14 hours of each step, comprising the biological transition phase for the cells while they adjusted to the conditions of a given recipe step. For the final 10 hours of each step, the perfusate was automatically directed to a collection vessel to enable straightforward sampling of the steady state achieved in each step. Collected samples were evaluated for protein expression and used to determine volumetric and cell-specific productivity during each condition (Figure 2B,C).

Both cell-specific and average volumetric productivity were used as outputs in the DOE evaluation. DOE models showed that all parameters tested were influential on productivity, with the top productivity attributed to increased levels of dissolved oxygen (DO), higher temperatures, and the addition of the additive, though DO was the most influential parameter overall (Figure 2D).

## *Case Study 2: Evaluating Nutrient Additives*

Next, the Daisy Petal™ was used to evaluate the impact of several key nutrients on cell growth and productivity for the Fc-fusion protein. Four distinct nutrients were evaluated in a single experiment. After the initial biomass accumulation and transition phases of the fermentation recipe, five 24 hour steps were used to test different conditions during heterologous protein expression (Figure 3A). The first 24 hour step served as a control condition where no additional nutrients were added. After that, a new nutrient was added in bolus to the bioreactor once every 24 hours. In the perfusion system, the new nutrient can be consumed or "washed out' due to the continuous harvest of spent cell culture fluid. In the unlikely case that the nutrient is not consumed at all by the cells, 83.5% of the bolus nutrient will still be washed out after 24 hours at the selected perfusion rate of 1.8 VVD.

Samples of both cells and cell-free harvest were collected at hour 23 of each step, after a new steady state had been reached (Figure 3A). These samples were used to calculate the cell growth rate, instantaneous volumetric productivity, and cell-specific productivity following each nutrient addition (Figure 3B,C,D). Nutrients 1, 2 and 3 led to reduced growth rate and productivity compared to the control. Nutrient 4 increased growth rate and substantially increased productivity. The volumetric productivity achieved with Nutrient 4 was over 3-fold higher than the control condition established in step 1.

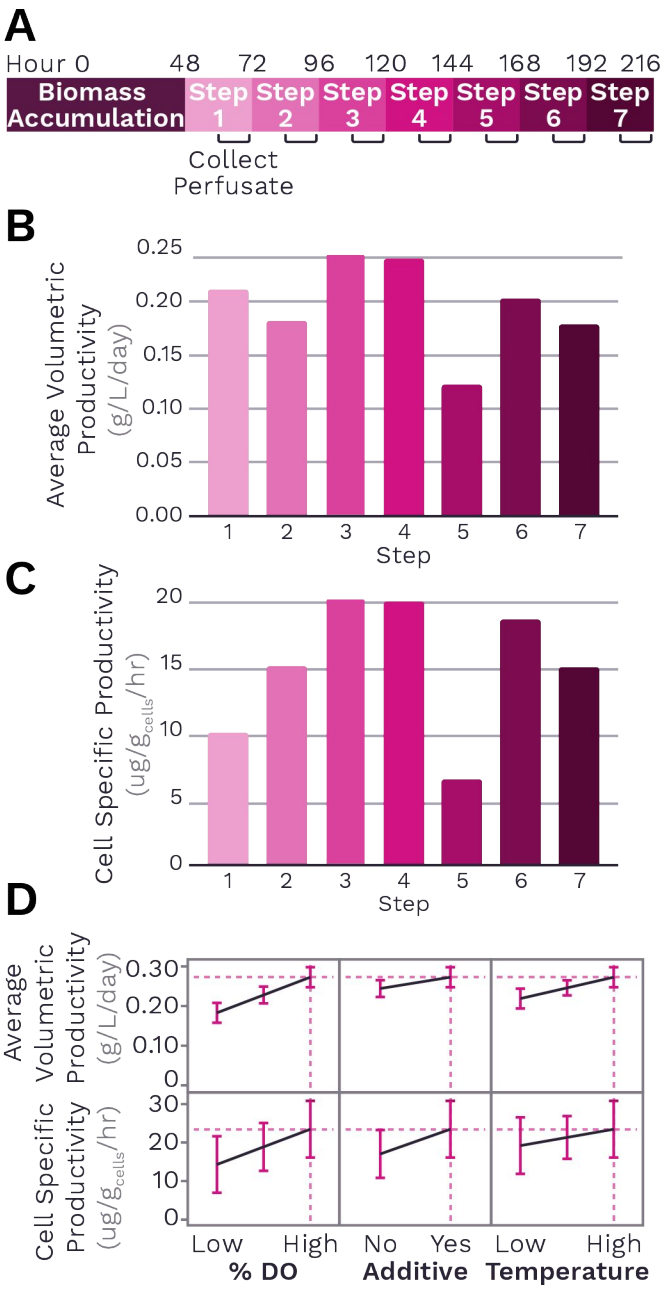


*Figure 2. Identifying influential process parameters in a single fermentation using DOE. (A) Process timeline. (B) Average volumetric productivity achieved at each set of conditions. (C) Cell specific productivity achieved at each set of conditions. (D) Changes in productivity with changing process parameters. Dotted lines indicate the conditions at which the outputs are maximized.*

## Discussion

Here, we showcase how the Daisy Petal™ Perfusion Bioreactor System can be used to evaluate multiple process conditions in a single experiment through two case studies. In one experiment, a 7 condition DOE was conducted in a single fermentation to identify the process parameters most important for the secreted expression of an Fc-fusion protein. DO was found to be the most important parameter. In a separate experiment, the impact of adding four different nutrients was evaluated (in addition to a control condition with no additional nutrients). Addition of Nutrient 4 led to a three-fold improvement in instantaneous volumetric productivity compared to the control condition demonstrated in the same experiment.

Recipes for both campaigns were designed in Nursery™, Sunflower's process planning and visualization software tool, and deployed by HelianthOS™, the central operating system for the Daisy Petal™ Perfusion Bioreactor System. Process parameters were automatically adjusted based on the predefined recipe, effectively carrying out multiple experiments during a single fermentation with minimal to no manual intervention. Process samples were collected after a new steady state had been reached following changes to process parameters. Sample timing was based on the rate of fluid turnover in the bioreactor and the expected time for yeast cells to transition to a new metabolic state and express and secrete heterologous proteins accordingly.[4]

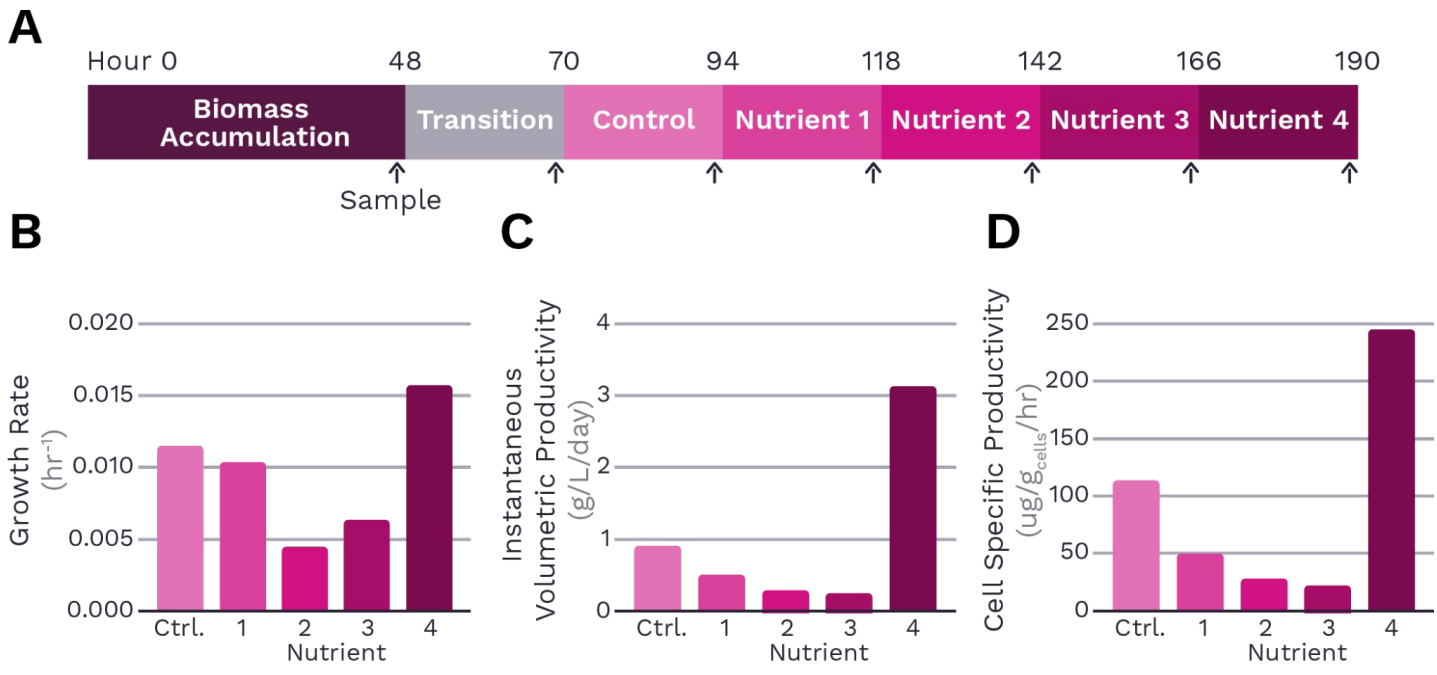


*Figure 3. Evaluating multiple nutrients additives in a single experiment on the Daisy Petal™. Process timeline (A) and impact of nutrient additions on growth rate (B), instantaneous volumetric productivity (C), and cell-specific productivity (D).*

## Conclusion

Sunflower's Daisy Petal™ bioreactor simplifies fermentation process optimization by enabling users to test multiple process conditions in a single experiment. Using the system software tools Nursery™ and HelianthOS™, automated recipes can be designed and deployed to move through several conditions during a single experiment without the user ever making a manual in-process change. This method of evaluating multiple conditions within a single fermentation cannot be performed in batch or fed-batch reactors. The continuous removal of bioreactor fluid enabled by perfusion is critical to enable the removal of secreted proteins, unused metabolites, and waste products, and provide a clean environment to measure key outputs correlative with the next condition. Evaluating a wide range of the design space within a single, automated experiment can significantly reduce the capital and operating costs associated with upstream process optimization.

## References


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## Related Resources

Application Note: Perfusion fermentation in the Daisy Petal™ bioreactor

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