BIOREACTOR PROCESS IMPROVEMENTS IN A PERFUSION-BASED PROCESS

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Abstract
Use of a perfusion bioreactor is one of the dominant operation modes in animal cell culture at commercial scales. This article provides an overview of cell retention technologies, and assesses the advantages and disadvantages of the perfusion process as compared to the fed-batch process. Strategies for bioreactor perfusion process are discussed to support continued process improvement after commercial launch. A case study is presented demonstrating the impact of a minor process change which had a beneficial impact on the perfusion operation and achieved a desired output improvement.

Introduction
The biotechnology industry has grown significantly in the past three decades and continues to grow at a rapid rate. Recombinant therapeutic proteins have yielded major advances in the pharmaceutical field. Their impact may even rival antibiotics, whose discovery and clinical applications transformed modern medicine.
Proteins that require post-translational modifications such as glycosylation and phosphorylation demand the use of mammalian hosts. The primary mammalian hosts for commercial production are Chinese hamster ovary (CHO) cells, and Murine myeloma lines SP2/0 and NS0. These cell lines can be cultured to either adhere to microcarriers or adapted to grow in suspension. The bioreactor type can be stainless steel or single used unit, ranging from 20 Liters to 10000 Liters (Schenerman, Hope et al. 1999). The common commercial culture mode can be batch, fed-batch, or perfusion. The choice of the reactor type and mode of operation depends on the protein titer, protein stability, waste product concentration, timeline for product development, projected market size, and business decision. This article will focus on the perfusion-based mammalian cell culture.

What is Perfusion Cell Culture Process?
Perfusion process is defined as continuously feeding the cells with fresh media and removing spent media while maintaining cells in culture. This provides a steady source of fresh nutrients and constant removal of cell waste products. A typical perfusion bioreactor system consists of a cell separation device, a bioreactor unit, a fresh medium tank, and a spent medium tank.
The most common use for the perfusion process is to manufacture the protein to be further purified. If the process is a serum containing process, the cell culture procedure will involve a growth phase in batch mode to attain certain cell densities and inhibitor levels before starting perfusion to save on the use of medium. This is followed by a transition stage using a chemical defined medium to wash out the serum prior to starting the collection of the

harvest material. If the process is a serum free process, the process will only involve the growth phase and harvest phase with no transition phase. The transition phase in a serum containing process can be used to wash out the serum prior to the collection of material to be further processed. The perfusion bioreactor has the ability to last a long time, even months (Gorenflo, Angepat et al. 2003).
The other application reported for perfusion is a high cell density banking process. This provides a way to reduce the time required for culture seed train expansion and scale up by eliminating the need for multiple small to intermediate scale steps. Figure 1 shows one application case reported by Biogen Idec (Tao, Shih et al. 2011). This perfusion process lasts for 5 to 7 days with the primary goal to reach high cell density between 18 E6 and 27E6 cells/ml. The cells were centrifuged down to prepare the large quantity high cell density inoculum to inoculate directly to 20L wave bag process. This new process has been implemented in a GMP cell banking facility in support of several clinical manufacturing campaigns. This application also allows the reduction of at least 9 days turnaround time at manufacturing scales.

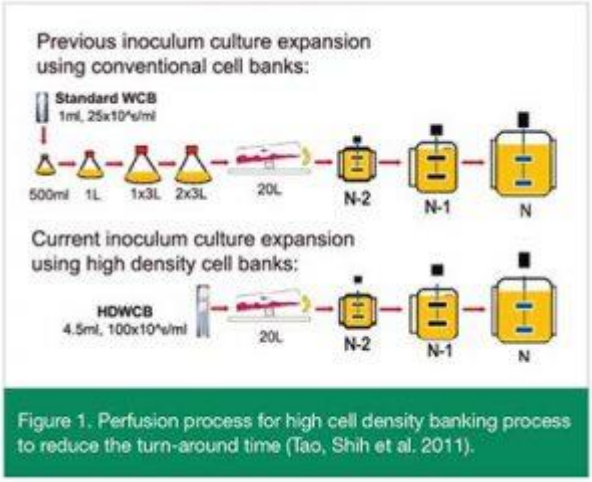


Figure 1. Perfusion process for high cell density banking process to reduce the turn-around time (Tao, Shih et al. 2011).

Cell Retention Devices
Many different techniques are employed to keep the cells in culture while removing spent media. The common devices used are settling cone, membrane filtration, centrifuge, and acoustic settler.

Settling Cone
Settling cone cell separation devices are used in Genzyme's commercial perfusion production. This design was described in US patent 5,733,776 (Genzyme 1998) as shown in Figure 2. This type of settling cone requires microcarriers for cell attachment to assist with the gravity based settling. The settling cone device sits on the top of the bioreactor, and it includes: i) a body for allowing the settling of the particles whereby the particles separate from a portion of the liquid; and ii) an outlet for allowing the removal of liquid; and iii) a vacuum source for drawing a portion of the particle suspension into the settling vessel and for periodically removing a portion of the liquid out of the settling vessel, whereby a continuous flow of the liquid periodically exits the apparatus wherein the liquid exiting the apparatus is particle free.

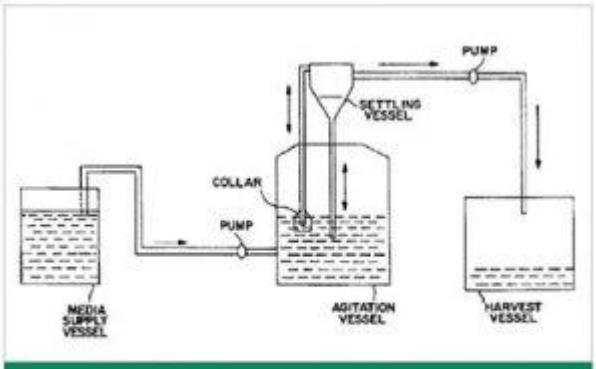


Figure 2. Genzyme patented settling cone cell separation for perfusion process.

Filter based separation can be divided into tangential flow filter, cross-flow filter, hollow fiber filter, vortex-flow filter (Mercille S 1994), spin-filter, ceramic filter (Dong H 2005), and floating membrane cell-retention filter (Tang YJ 2007). The most commonly used in commercial production is the hollow fiber filter system: tangential flow filtration (TFF) and alternating tangential flow (ATF) system. For tangential flow filtration system, the cell suspension is flowing tangentially to the membrane potentially preventing membrane fouling compared to the cross flow filter. The ATF as shown in Figure 3 uses TFF but with the cell broth flow direction alternated using a diaphragm pump, creating less shear stress than the TFF peristaltic pump, and with a cycle time around 1 min. The alternated movement creates a back flush in the filter membrane, which can help prevent fouling.

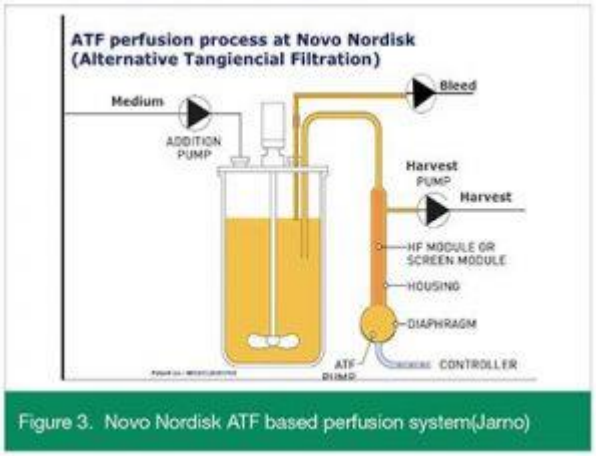


Figure 3. Novo Nordisk ATF based perfusion system(Jarno)

ATF is one of the most common choices for a perfusion system without microcarriers in current industry practice. It can be easily used to retrofit the existing batch or fed batch commercial bioreactor to a perfusion system, and also work with a single unit bioreactor system easily. The disadvantage of ATF is that the filter tends to foul over time which could result in the retention of the secreted protein leading to the loss of active product.

Acoustic Separation System

Acoustic separation system uses high frequency resonant ultrasonic waves to separate cells from product instead of a physical mesh or membrane as shown in Figure 4. It can sit on the top of the bioreactor similar to the settling cone system but does not need to have microcarriers to facilitate the gravity settling. It overcomes the filter system for non fouling/non clogging retention system, and can be easily scaled up. This technology was first reported in nature (Felix 1994) with hybridoma cells with 700 hours operation, 50E6 cells/ml and 99% viable cell retention. The same group later published an optimized system with the

air back flush system. The new system allows 110 days operation at the cell density at 100E6 cells/ml (Gorenflo, Angepat et al. 2003). This system is currently commercially available with an operating range of 1L/day to 1000L/day.

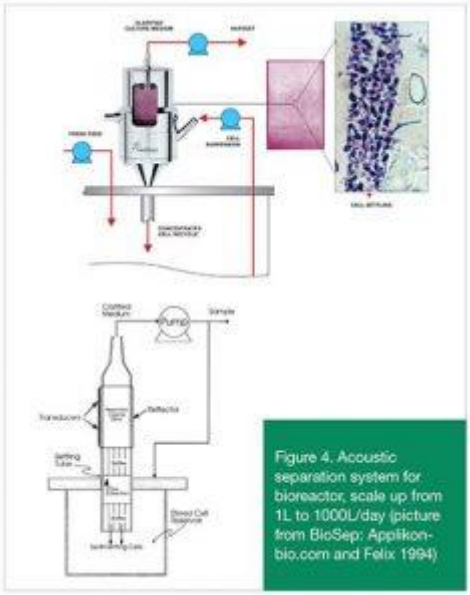


Figure 4. Acoustic separation system for bioreactor, scale up from 1L to 1000L/day (picture from BioSep: Applikon-bio.com and Felix 1994)

Centrifuge separation system

Ishimaru et al reported in 1989 (Hamamoto, Ishimaru et al. 1989) using a continuous cell separation at low centrifuge rate 300 g to 500 g, 30 minutes, 2 times/day for a perfusion system with no negative impact on the cell growth as spinner. The experimental set up is shown in Figure 5. Centrifuge perfusion with optimized revolution speed, discharge and instantaneous feed rate can achieve equivalent productivity as filter based technology in the bioreactor. A less optimized condition might stress the cell and compromise the specific productivity as compared to the filter based system, but with minimum product loss (Johnson, Lanthier et al. 1996).

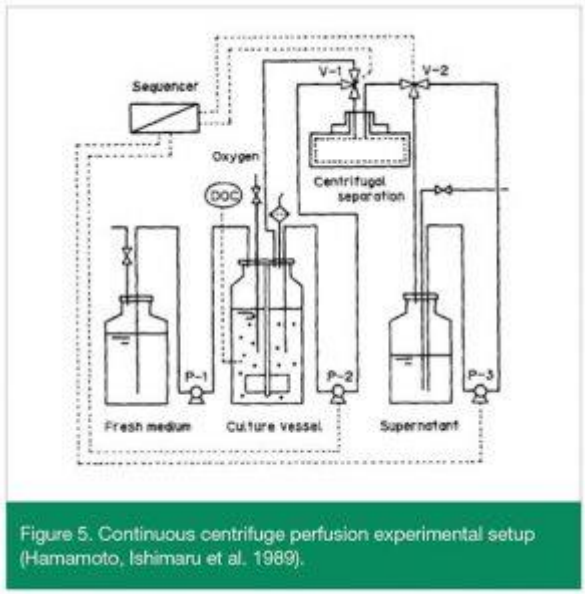


Figure 5. Continuous centrifuge perfusion experimental setup (Hamamoto, Ishimaru et al. 1989).

Advantage/Disadvantage of Perfusion Process

Perfusion processes has been well established at industrial scale since the 1990s. Advantages of perfusion process include: 1. Higher volumetric throughput as a result of higher cell density and shorter product exposure to cultivation conditions. Typical perfusion cultures can reach cell densities from 30 to 100E6cells/ml and maintain these higher cell densities for an

extended period of time. The fed batch process typically reaches between 5 E6 to 25 E6 cells/ml with a typical exponential growth profile. The short product residence time makes the perfusion technology particularly attractive to less stable product; 2. Reduced turnaround time and cost. A typical fed batch process lasts 14 to 20 days, while a typical perfusion lasts for a couple of months, the operation cost of turn-around can be doubled or tripled. The turn-around operation cost includes labor and materials involved in cleaning in place, steam in place, seed train expansion, equipment checkup/replacement, and waste treatment; 3. More consistent product quality; and 4. Smaller initial capital cost for the same output than fed-batch/batch.

The disadvantages of the perfusion process include: 1. Longer development cycle and commercial launch. Compared to the fed batch process, the perfusion process has a longer duration. The design space development can be difficult; additionally as parameters are changed the effects may not be detected until much later in the perfusion stage. Similar to development cycle, the process validation can take months instead of couple of weeks when compared to a fed batch process; 2. Expensive medium cost. Depending on the titer, perfusion rate, and type of medium used in the perfusion process, the medium cost can be double of that compared to a similar fed batch process production; 3. More complex operating system. As the complexity increases it is easier to have an operation failure and have a higher requirement for technical skills for operation; 4. It is harder to obtain a consistent business output projection for biological perfusion process. The impact on the overall manufacturing process output with even one failed run or lower productivity run can be big.

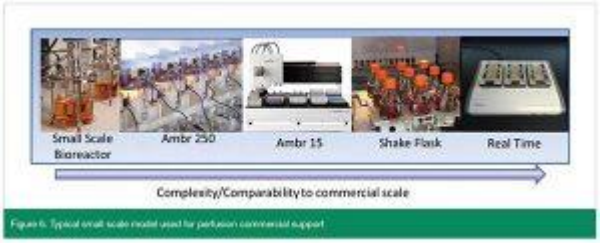
Choice of the type of the process will be dependent on the projected market size, specific product, and required development timeline.

Commercial Perfusion Process Operation and Process Improvement

After a perfusion process has been commercially launched, continuous process improvements are expected to be made during the product lifecycle. To be successful the following needs must be met:

Establishment of Representative Small Scale Models

It is critical to establish small scale models that can predict the commercial production which can be used for further process improvement initiatives. Similar to the fed batch process, multiple types of small scale cell culture systems are typically used to support commercial perfusion production. These may include bench-top bioreactors, Ambr®250, Ambr®15, shake flask, and real time growth monitoring device such as Xcelligence®. As shown in Figure 6, the complexity of the operation and setup is reduced and throughput is increased from left to right. However the comparability to the manufacturing scale is also reduced.



In the ideal case, a small scale model should be able to predict the exact performance of that at large scale; however, in most cases it is difficult to exactly match the perfusion culture, especially when employing the high throughput systems such as shake flask and plates.

These are not able to operate easily in a true continuous perfusion mode. Those systems can only do batch refeed at a reasonable frequency and lack of the proper control for pH and dissolved oxygen. Ambr®15 and Ambr®250 from Sartorius have improved process control for pH and dissolved oxygen as the real bioreactor system, however, the continuous perfusion design was not built into this system yet. The bench-top bioreactor is the only system that can fully mimic the commercial operation; however, the throughput is low which makes it difficult to do process improvement that requires using the design of the experiment approach. Regardless of the type of model, it is important to verify a trend can be obtained in both the small scale model and in the manufacturing scale. For example, if testing the impact of a temperature shift at small scale, similar product and metabolism shifts should be observed at the commercial scale.

Establish a Reliable and Consistent Raw Material Supply

To ensure a consistent raw material supply with reliable quality for commercial production, it needs to establish at a minimum two independent suppliers for each raw material. A qualified second supplier will allow the manufacturing to handle well the shortage of the raw material resulting from unexpected nature disasters and undesired change notification from the existing supplier. To access the quality of the raw material, it is important to establish both a reliable analytical tool and a cell culture model. Small changes such as the source of one component may contain some trace contaminants under the detection levels using the analytical method but may have a significant impact on the cell performance. A reliable small scale model is a key to control the raw material feed into the perfusion process with reasonable throughput. Successful routine screening should be done before the batch of the material used in commercial production. This will reduce the risk of the process variation resulting from the impact of the raw materials.

Process Control Monitoring

Fast turn-around of the test result is important to make a business decision for the continuous process, for example, a quick method to detect contamination. Typical colony counting method for bacterial contamination requires an incubation time of 3 days minimum. It is critical for n-1 operation, as the long lead time may allow the contaminated n-1 seed to proceed to the production bioreactor leading to a bigger loss. A PCR method or flow cytometry based method may be developed to detect the low level of bacterial contamination in a shorter time frame than the convention plate method.

Additionally, further development of the process real time monitoring system can improve the process understanding and control. New technology with a good potential should be evaluated and implemented to modernize the commercial facility to allow supplementary process parameter monitoring. An example is the addition of a Raman probe, which can be used for monitoring process metabolism, product titer, cell count, and nutrient usage. The existing process monitoring tools are also continuously evolving, such as 1) pCO_2 using a more sensitive Severinghaus electrode instead of the chemical dye method, 2) optical DO to replace Polarographic membrane based DO system, and 3) add intelligence sensor management system (ISM) to the probe to reduce the transmitter failure, etc. The continuous trend collected from the commercial reactor will be much more insightful than the daily offline sample testing results, and it will be extremely useful to predict the process output and understand the impact of the process deviations such as agitation, temperature, dissolved gas, and the other process variables.

Due to the long duration of the perfusion process, it is important to have a reliable system

that will allow monitoring the key process parameter process control charts against the approved process limits and typical process limits. Periodic review of the data against the historical trends is required by regulatory department, and it is also important for the process consistency monitoring. For example, a significant shift over certain period, or a slower up/down trend will need to be understood to ensure consistent manufacturing.

Improving Process Output from the Perfusion Bioreactor

Perfusion processes need an extended timeline to allow for development and validation. Perfusion processes need an extended timeline to allow for development and validation, a strategy of developing shorter perfusion first should be considered before submitting a commercial application. A broader design space should be understood at the development stage to allow for additional flexibility when pursuing improvement initiatives. For example, once commercial approval has been obtained additional development could be pursued to extend the perfusion process. For these process improvements one would need to demonstrate the process is still in control while the product would require supporting characterization data commensurate with the change being made. For this example, extending the process duration will likely require a regulatory filing update. An active communication with regulatory authorities can be helpful when determining the scope of the data package. Significant process changes that are outside of the ranges previous filed would typically require process validation with subsequent product characterization. Small scale studies should be used to determine which process improvements should be pursued. Also the data collected from the small scale work can be used to supplement the change made at scale but should not be used in place of at scale data.

Small process improvements within the filing range are always desired since they can be implemented in a relatively short period of time. These improvements for a fed batch process, can be very straightforward as the small scale evaluation can be assessed for the entire duration of the run and multiple batches can be verified side by side and sequentially to increase the confidence prior to the commercial implementation. For the perfusion process, due to the long duration, some of the incremental improvements can only evaluated in small scale partial runs. The long duration of the perfusion process also increases the difficulty for the reproducibility of the process control as any spikes of DO probes can potentially shift the process and make the small scale reactor less reproducible and representable for the same change.

Small well understood changes can also lead to an unintended shift. For example, as shown in Figure 7, this small scale study focused on the early period of the change only and identified that a change in the reactor volume within the filling range can improve the product output. Later, it was identified the plateau of the process output in the late phase diminished the early improvement (purple trace). Since the bioreactor is not homogeneous, changing the reactor volume within the filing range can potentially cause the DO and pCO_2 control to shift at various points in the harvest stages. Reliable small scale models allowed further development of the DO and pCO_2 control strategy within the filing range, and demonstrated the ability of recreation and removing the process output plateau. This minor change achieved consistent overall improvement.

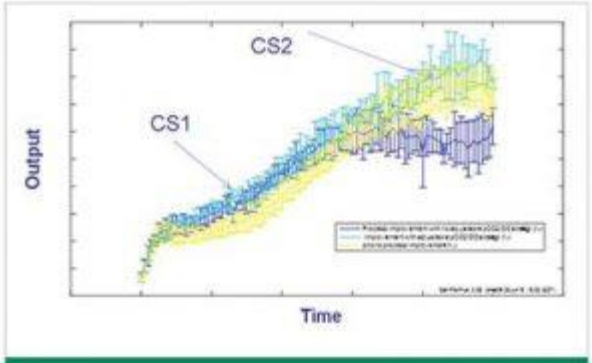


Figure 7. Perfusion bioreactor process output improvement within the process filed range. Yellow, process output in the original process; Dark blue, process output after reactor volume change; light blue, process output profile with reactor volume change, and two stage pCO2/DO optimization.

Conclusion

The majority of biotherapeutics today are manufactured using suspension CHO cells grown in fed-batch culture due to the long development cycle and technical challenge for perfusion process development. However, companies are beginning to explore the idea of continuous processing and use of perfusion bioreactors are a key component of this strategy. To make a successful commercial perfusion process, it is important to continue the process improvements after reaching the commercial stage, and to focus on improving process monitoring, developing a reliable small scale model to ensure reliable raw material supplies and process deviation, and to further the process understanding. More technically competent people need to review and interpret the process control data as well as the key metabolism data on a routine basis. This additional oversight will allow for correct actions to be implemented in time to reduce the risk of process failure.

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