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Scalability of a Disposable Bioreactor from 25L – 500L Run in Perfusion Mode with a CHO-Based Cell Line: A Tech Review

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Single-use, disposable components offer many advantages in the manufacturing of biologics. They are clean and ready to use when supplied, which obviates the need for sterilization and decreases the requirement for services such as water for irrigation (WFI) systems and steam generators. Disposable components are not used for subsequent operations, eliminating the chance of cross contamination between process runs. Long lead times for equipment installation can be avoided because the need for stainless steel equipment is reduced or eliminated. Systems are less complex, therefore engineering requirements are also reduced. There is no need for clean-in-place (CIP) or steam-in-place (SIP) operations, along with the associated piping, valves, controls, or pressure rating of vessels. Moreover, the use of disposable components reduces the complexity of validation. Because there are fewer reusable components, fewer items need to be tracked and extensive validation studies for sterilization and cleaning can be eliminated. Finally, by removing the limitations of hard piping and stationary tanks, disposable components allow for operations to be more rapidly reconfigured for a new process run.

The use of disposable components can lead to substantial cost savings in labor, equipment, and facility design as well as validation. Disposable components include: bioprocess bags, tubing, capsule filters, tangential flow capsules, bioreactors, chromatography capsules, and mixing systems.[2]

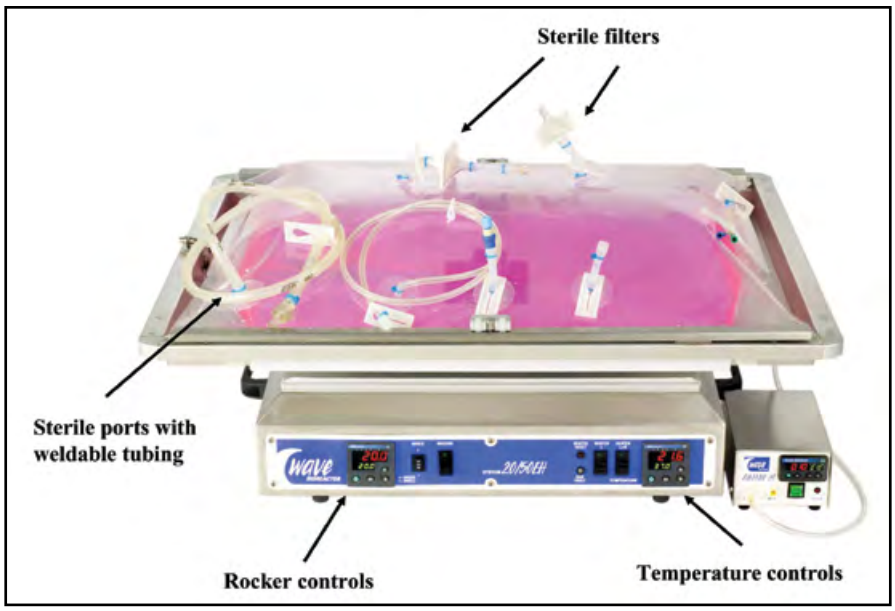

Figure 1. Wave Bioreactor. System20/50EH.

Use and Scale-Up of a Disposable Bioreactor

When used in certain applications, perfusion cell culture can offer advantages over batch and fed-batch processes. The chosen style of operation is based on a combination of requirements that are mandated by process specifications. Optimization of a process is often the only way to evaluate which type of production will work best. Batch is the most commonly used mode and relies on an endpoint. In batch mode, a bioreactor is inoculated and allowed to reach a determined cell density and product concentration at which point the cell supernatant is collected. With a fed-batch process, components are added to improve cell viability in the later stages of the process run to increase overall productivity. In perfusion mode, feed solutions are fed to the bioreactor continuously, and spent media is constantly removed. Running a process in perfusion mode can increase the longevity and cell density of the culture, which in turn increases the overall productivity of the run.[3,4,9]

Our protein of interest, AZ-IL2B, is a dimeric fusion protein consisting of human IL-2 linked to an scFv portion

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of a human immunoglobulin that is specific for pIgR.[6] This fusion protein is best suited to production in perfusion mode. The protein levels produced by the cells were low and AZ-IL2B is fragile, particularly at 37° C. The increased capacity offered by perfusion coupled with the short residence time in the reactor for the unstable protein allowed for better yield.

This article will summarize the results of scaling-up a disposable bioreactor manufactured by Wave Biotech, LLC, from a 25L working volume to a 500L working volume run in perfusion mode. We will compare three different systems with 25L, 100L, and 500L working volumes. Several parameters were measured for each bioreactor run. These include: cell count and viability, the amount of protein produced, and glucose and lactate levels. These parameters are compared for each system and the results demonstrate that the three different volume bioreactors are comparable.

Materials and Methods

Bioreactor Description

Each bioreactor consists of a presterilized plastic bag that is flexible and disposable called the Cellbag®. For an operation, the Cellbag is placed on a rocking platform and partially filled with media (Fig. 1). The remaining volume of the Cellbag is inflated with a process gas mixture composed of carbon dioxide (CO_2) and oxygen (O_2). These gasses are added using a sterile inlet filter that is pre-attached to the bag. This airflow provides oxygenation and gas exchange for pH control and CO_2 removal. Exhaust air passes through another sterile filter and a backpressure control valve. The backpressure control valve ensures that the Cellbag is always fully inflated at any airflow. The valve also prevents over-inflation and potential bursting of the bag. The gas-filled headspace accounts for half the volume in the Cellbag once the bag is filled. For example, a 50L Cellbag when full will contain 25L of cell suspension and 25L of air. Air is continuously passed through the headspace during cultivation. Process gasses, such as CO_2 and O_2, are mixed with air in a controlled manner. Liquid mixing and mass transfer of gasses are achieved by rocking the Cellbag back and forth. This rocking motion generates waves at the liquid-air interface. These waves greatly increase surface area to enhance gas transfer. The wave motion also promotes bulk mixing and off-bottom suspension of cells and particles without any damage to the cells. The rocking motion is controlled by setting the angle and number of rocks per minute. These parameters must be determined for each volume used in the Cellbag. Temperature in the Cellbag is controlled by a heater that sits in the baseplate of the unit and warms the underside of the bag. The heater is regulated by a non-invasive temperature sensor that is also in the baseplate of the unit.[8]

Special ports were developed to allow sterile additions and sample withdrawal without the need to place the bioreactor inside a laminar flow cabinet. A sterile tubing welder is used to attach media, buffer, and glucose stock solutions to the system. The bioreactors we used were the System20/50EH, System200, and System1000 (Wave Biotech, LLC).

Cell Thawing and Propagation

We used the cell line P6A2, which was a CHO-based suspension clone chosen for its protein production as well as growth characteristics. Cells were thawed into 120ml of filtered spent media and placed inside a 150ml spinner flask. The cells were expanded inside the spinner flask until there were enough cells to seed larger and larger spinner flasks, up to 6L. Once the culture was sufficiently expanded, it was transferred to the Cellbag for seeding. Cell densities were maintained at approximately 2 x 10^5 cells/ml in the spinner flasks. The medium used for expansion was serum free IS CHO-V (Irvine Scientific) with additives.

We used a hemacytometer to count cells and established viability using trypan blue dye exclusion. A Bioprofile® 300A (Nova Biomedical) was used to analyze cell culture chemistry. pH was determined either on-line using the Cellbag probe or off-line using a PHM220 pH meter (Meter Lab). Oxygen, CO_2, temperature, rocker speed, and angle were determined and controlled through the Wave Bioreactor® controller.

Cellbag Seeding

Cells were typically seeded into the Cellbag bioreactor at 1 x 10^5 – 4 x 10^5 cells/ml. We have seeded cells at lower densities (e.g., 6 x 10^4 cells/ml) with no deleterious effects on cell growth or production. Once the cells were sufficiently expanded inside the spinner culture(s), the Cellbag bioreactor was seeded. This was done by transferring

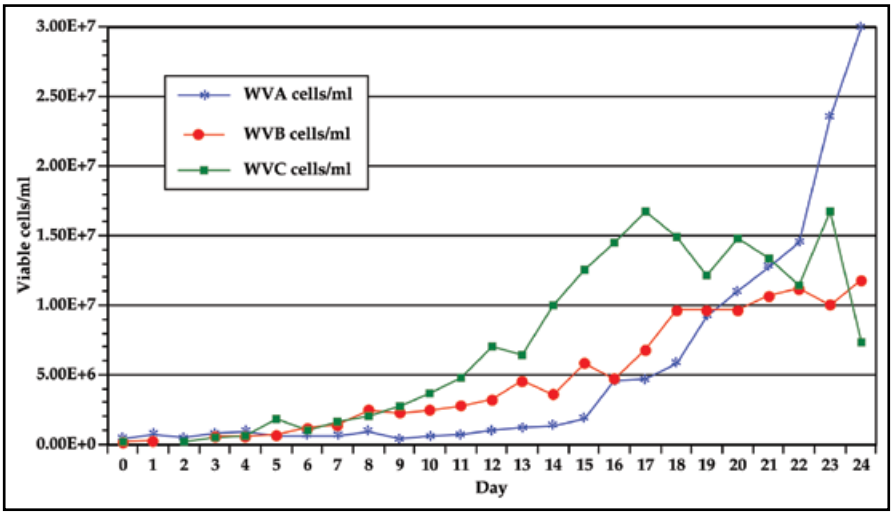

Figure 2. Comparison of daily viable cell densities. Comparison of cell densities from bioreactor runs WVA, WVB, and WVC. Perfusion was started for WVA on day 13. Perfusion was started for WVB and WVC on day eight.

the contents of the spinner flask(s) directly into the bioreactor. At this stage, the bioreactor had already been filled with CO_2 and O_2, and contained warmed media (37° C). The cell suspension was sterile welded onto the Cellbag and then pumped into the bioreactor through a sterile port.

Propagation in Bioreactor

The cells can reach much higher densities inside the Cellbag than in a spinner flask. This is most likely due to the increased oxygenation inside the Cellbag, as well as the ability to control pH. In a spinner flask, the density is maintained between $2 \times 10^5 - 6 \times 10^5$ cells/ml. This is increased to $8 \times 10^5 - 1 \times 10^6$ cells/ml in the Cellbag until perfusion is started, which allows for densities as high as 3×10^7 cells/ml with the P6A2 cell clone.

Perfusion

Once the cells reached $1 \times 10^6 - 2 \times 10^6$ cells/ml, perfusion was started. The spent media was harvested at the same rate that the bioreactor was fed fresh media, nutrients, and buffer for pH control. This was controlled directly through the bioreactor's weight-based perfusion controller. At this density, the volume exchange per day was approximately 70–75 percent. The reactor was perfused for as long as the cells were greater than 50% viable. Once the cells reached approximately 2×10^7 cells/ml, it became more difficult to control the accumulation of lactate and other toxic subproducts, which in turn rendered pH control very difficult. At this stage, cells were removed to maintain a density of $1 \times 10^7 - 2 \times 10^7$ cells/ml. The volume exchange per day was increased to approximately 100 percent; this included media, buffer, and other additives. Hollow fiber microfiltration cartridges with a 0.2 µm pore size cutoff (Amersham) were used for perfusion of the cell culture supernatant.

Protein Analysis by ELISA and Western Blot

Quantitation of the protein of interest, AZ-IL2B, was performed by ELISA. Cell culture supernatants were analyzed in a method designed to detect and quantitate the AZ-IL2B chimera. AZ-IL2B from cell culture supernatants was captured by binding to a microtiter plate coated with an antibody specific for the pIgR moiety. Captured AZ-IL2B was detected with a biotinylated goat anti-human IL-2 polyclonal antibody (R&D Systems, Inc.). Streptavidin-HRP conjugate (BD Biosciences, Pharmingen) was added as the final detection step. TMB substrate solution was added to the reactions and generated a color change in direct response to enzyme presence, which was directly proportional to the amount of AZ-IL2B in the sample. An AZ-IL2B standard curve and quality controls were used to measure the amount of AZ-IL2B present in cell culture supernatant samples.

The western blot was conducted according to standard protocols.[7] Proteins were size fractionated through an 8–16% Tris-glycine acrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated with rabbit anti-human interleukin-2 polyclonal antibody (Chemicon International, Inc.) as the primary antibody and donkey anti-rabbit IgG, alkaline phosphatase conjugated antibody (Pierce Biotechnology, Inc.) as the secondary antibody.

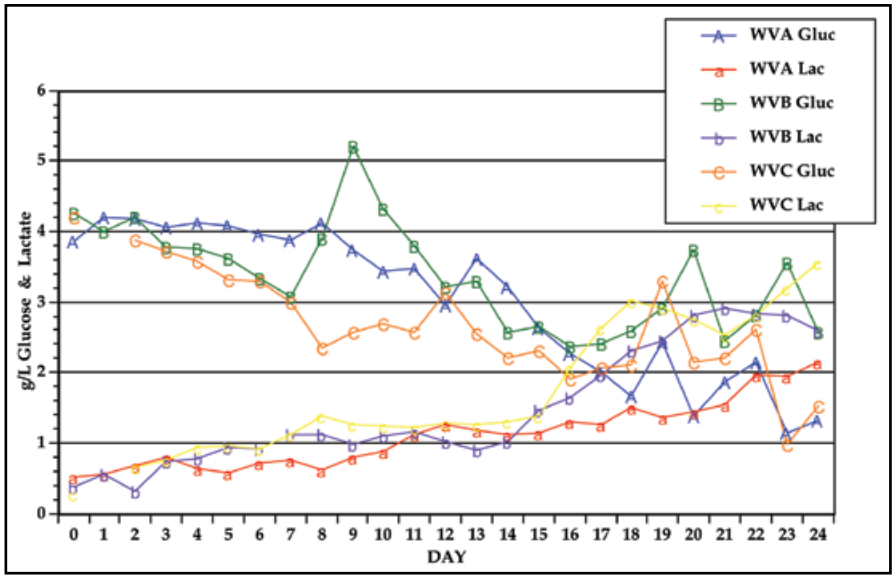

Figure 3. Chemistry analysis of daily glucose and lactate levels. Glucose and lactate levels for bioreactor runs WVA, WVB, and WVC.

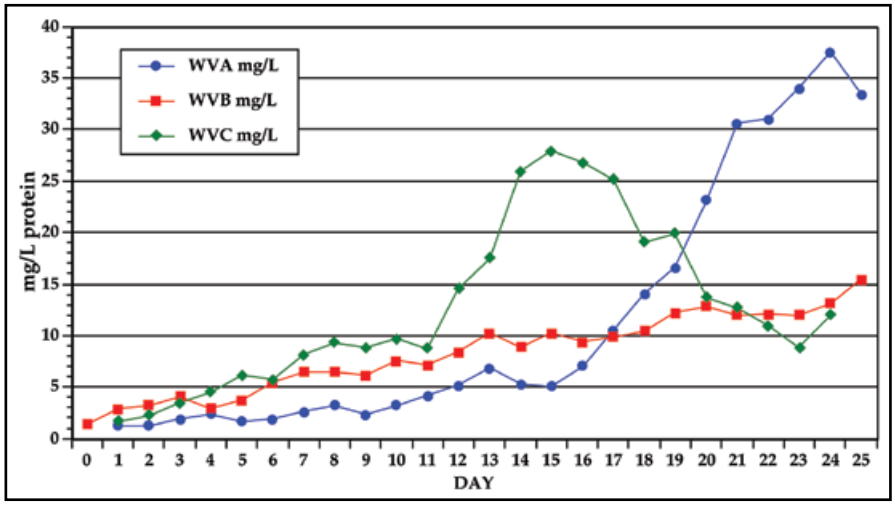

Figure 4. Daily protein concentrations. Comparison of protein levels for three bioreactor runs measured as mg/L. Protein levels were determined by ELISA.

Results

Bioreactor runs WVA, WVB, and WVC refer to Wave Biotech® bioreactors System20/50EH, System200, and System1000, respectively.

Cell Count and Viability

Figure 2 shows that cell growth and densities were comparable for each bioreactor. Bioreactor run WVB did not reach cell densities as high as runs WVA and WVC, but did reach 1.2 x 10^7 cells/ml. This was due to a spike in glucose after a high concentration was added on day eight (Fig. 3).

After running bioreactor WVA, we found that cell densities above 2 x 10^7 cells/ml were not supportable with the current media formulation or feed strategy. The culture's viability and cell growth in WVA dropped precipitously and was not recoverable after the cell density reached 3 x 10^7 cells/ml, presumably due to a lack of nutrients and build up of toxic substances, including high levels of lactate in the culture (Fig. 3).[5] Oxygenation was not an issue as we were able to regulate the amount of oxygen added to the culture. Dissolved oxygen was maintained in the range of 73–100 percent for each reactor run.

For subsequent reactors, cells were removed after reaching densities of 1.5 x 10^7 cells/ml or greater, and lactate levels of 2.2 g/L or greater. By removing cells and adjusting the perfusion volume per day, the bioreactor runs were extended, resulting in larger protein harvests. WVA took longer to reach perfusion density (1.2 x 10^6 cells/ml), which was most likely due to an accidental decrease in temperature at day six to 25° C. WVA perfused for 13 days at an average of 12.5 mg/L, while WVB perfused for 18 days at an average of 8.3 mg/L, five days longer than WVA, which resulted in a gain of 6.7 g of harvested protein. WVC perfused for 16 days at an average of 12.2 mg/L, three days longer than WVA, which resulted in a gain of 16.4 g of harvested protein. Any increase in the viability and longevity of a bioreactor run results in a higher yield.

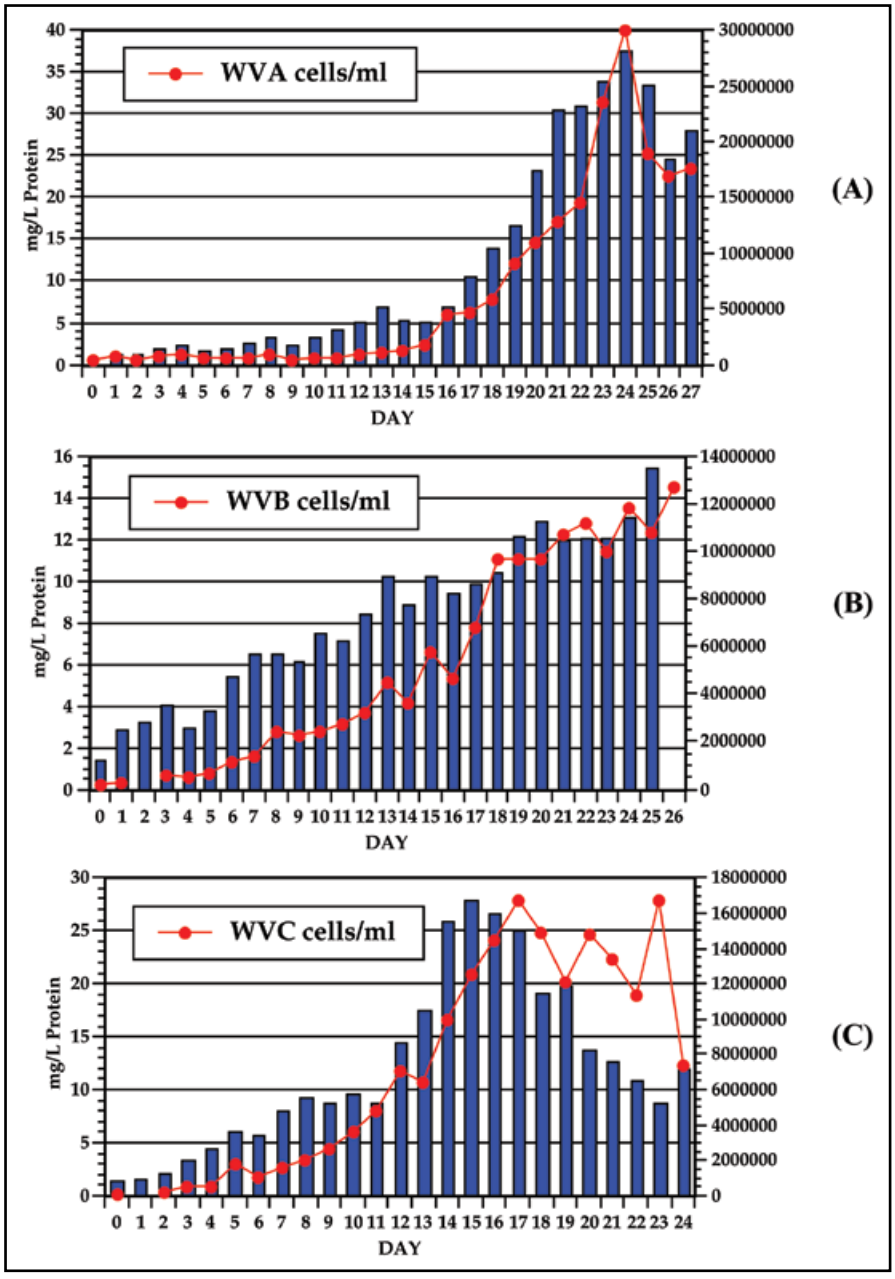

Figure 5. Protein level and viable cell density. Correlation of cell density and protein level. A. Bioreactor run WVA. B. Bioreactor run WVB. C. Bioreactor run WVC.

Protein Production

The protein concentrations for each run are compared in Figure 4. WVA reached a higher concentration of protein (37.5 mg/L) than WVB (15.46 mg/L) or WVC (27.91 mg/L), due to the higher cell density. The overall protein produced for each reactor was as follows: WVA 7.8 g; WVB 21.4 g, and WVC 149.2 g. Protein production correlates closely with cell density. This makes sense because when there are more cells present, there are more cells producing protein. WVB did not reach as high a protein concentration as WVA or WVC. This correlates well with the lower cell density observed.

In Figures 5a, 5b, and 5c, cell density is shown overlaid with protein concentration for each reactor run. The increase in protein titer correlates directly with cell density. The decrease in cell viability as well as a decrease in protein production was determined when each reactor run was terminated.

Glucose and Lactate Concentrations

The glucose and lactate concentrations for each of the runs are depicted in Figure 3. The levels are similar for

each run, with the exception of the glucose spike for WVB on day nine. As each bioreactor run progressed and the cell density increased, glucose was consumed and lactate increased. The spikes in glucose concentration in the latter stage of each run were caused by adding 50% glucose stock when the bioreactor glucose level fell below 2.0 g/L.

Protein Analysis

AZ-IL2B runs as a doublet between 36 and 50 kDa. Figure 6(a) is a Western blot of WVD, which is a reactor run with a 10 L working volume (Wave Biotech System20/50EH). Figure 6(b) is a Western blot of WVC, which has a 500L working volume. The illustration shows the comparison of days three through nine, and by Western blot, there is no difference between the protein produced in the smaller reactor and the protein produced in the larger reactor.

Discussion

Scaling up from 25L to 500L in the Wave Biotech disposable bioreactor had no negative effects on cell growth or protein production. Other factors that impacted viability and protein production included high cell density, improper additive concentrations, and temperature fluctuations. Not only were the protein levels similar in each system size, but the protein produced was equivalent when analyzed by Western blot and tested for biological activity (data not shown).[6] Cell growth and doubling time were comparable for each system. The average protein concentration correlated directly with cell density. Glucose consumption and lactate production were analogous for each size bioreactor. As each bioreactor run progressed, glucose levels dropped and lactate levels rose.

The choice of a disposable bioreactor allowed for quick turn-around time between bioreactor runs. The ease of set-up and seeding of the bioreactor, as well as disposal made it possible to terminate a bioreactor run and seed a new one in the same day. Ten liter, 25L and 100L bioreactors can be taken down and reseeded within a standard eight-hour workday. It takes longer to empty, fill, and warm the media for the 500L Cellbag, but the bioreactor can be taken down and reseeded within two eight-hour workdays. The procedure is simple: the Cellbag is emptied, decontaminated, and discarded. A new, presterilized Cellbag is placed on the platform and filled with CO_2 and O_2. Media is added and warmed and then the cells are inoculated. Typically 10L and 25L working volume bioreactors are inoculated from spinner flasks. One hundred liter and 500L bioreactors are inoculated from the smaller 25L bioreactor.

Scaling-up the system from 25L to 500L was straightforward and proportional, making adjustments for changes in volume. No changes were made with the media formulation or additives added to the bioreactor. The cell line propagated and produced similarly at 25L, 100L, and 500L.

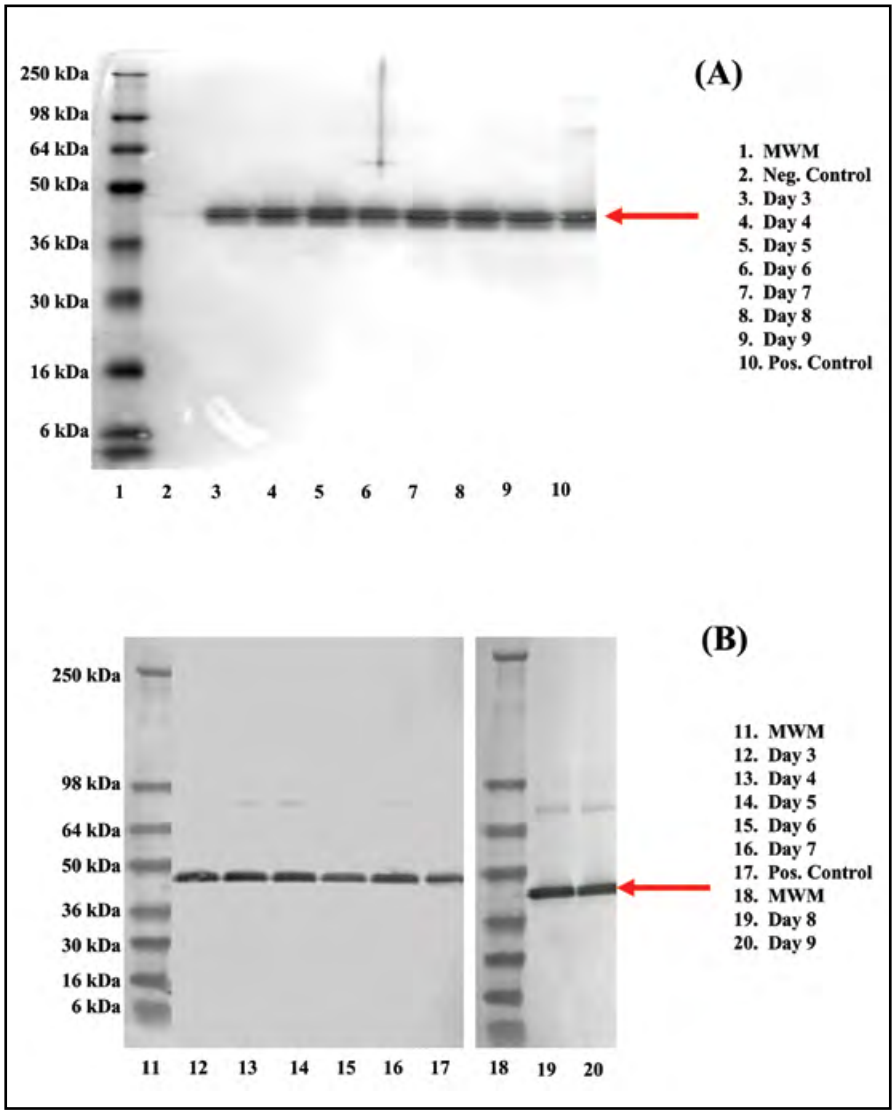

Figure 6. Western blot analysis comparing two bioreactor runs. A. Bioreactor run WVD has a 10L working volume. B. Bioreactor run WVC has a 500L working volume. MWM = molecular weight marker.

Conclusion

A successful perfusion process is a balance between the health of the culture and optimal production. What may be good for optimal cell growth and health could be detrimental to production. For example, increasing the perfusion rate to remove toxic substances could dilute the product to such a low titer that downstream processing would be burdensome, not to mention the increased cost of goods

associated with the increase in perfusion. Adjustments made to the media formulation and additives as well as feed strategies could increase the number of days of perfusion without diluting the protein's titer level. It has been reported that using two different media formulations to control cell metabolism during a bioreactor run can decrease cell growth while maintaining viability, thus lengthening the number of days of product collection.[1]

Removing cells from the bioreactor is an option that has been incorporated into our process to control cell density. This is difficult to engineer on a continual basis and increases biohazardous waste. A media formulation that would support rapid cell growth early on, and then control cell growth during production would be ideal.

A disposable bioreactor offers advantages over reusable bioreactors in the areas of cleaning, sterilization, validation, set-up, and turn-around time between runs. We have demonstrated that systems run with a 25L, 100L, or 500L working volume are comparable for cell growth, protein production, glucose consumption, and lactate production.

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