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SPECIAL REPORT

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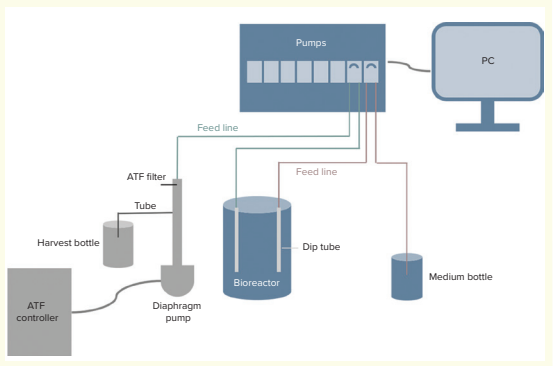
Upstream bioprocessing in perfusion mode holds great promise for industrial production of cells and biologics. In perfusion, fresh medium is added constantly to the bioreactor, and used medium is harvested while the cells are retained in the bioreactor. As a result, the composition of the cell culture medium stays quite constant during the process. This offers several advantages. In perfusion, higher cell densities can be reached than in batch and fed-batch processes, therefore enhancing volumetric productivity. Because medium composition can influence cell metabolism and therefore product characteristics, the more constant the process conditions, the more consistent may be the product quality. Products that pass through the cell retention device are constantly harvested. The time they reside in the culture medium is reduced from that of a fed-batch culture, which is advantageous for production of less-stable products.

Apart from being used for production of secreted proteins or viral vectors, perfusion may be used for the earlier substeps of some bioprocesses, as in production of high-density seed trains or to achieve high cell densities before transfection/infection of cells for viral vector production. In all cases, perfusion bioprocessing requires a cell retention device within or attached to the bioreactor. The choice of retention technology depends on whether adherent or suspension cells are cultivated; whether a soluble protein, a virus, or the cells themselves are the product of interest; whether the product needs to be harvested continuously; whether the process needs to be scalable; and other factors.

In proof-of-concept studies, we have connected Eppendorf bioreactors to different cell retention devices. We equipped Eppendorf bioprocess systems with an alternating–tangential-flow (ATF) filtration device, a packed-bed basket impeller, or a microcarrier spin filter. We assessed the suitability of each system for mammalian cell culture in perfusion and evaluated its scalability.

Figure 1: Connection of bioprocess system with ATF cell retention device



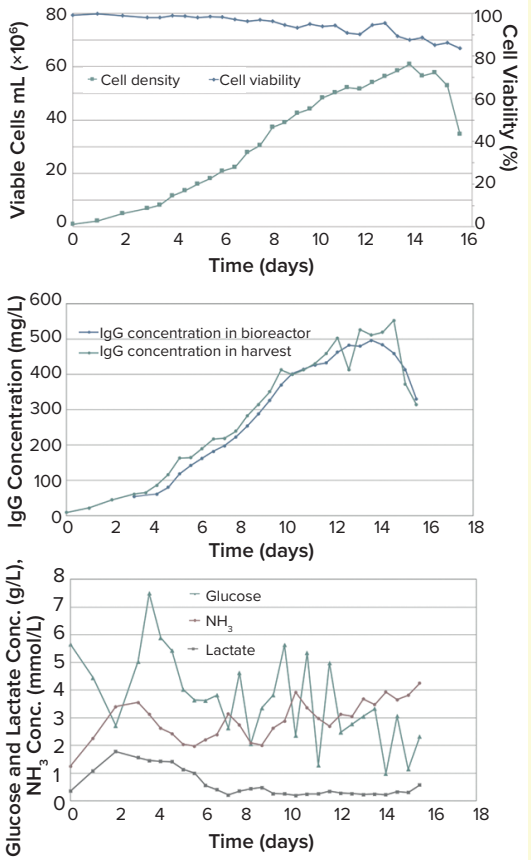
Case Study: ATF Filtration

Cell retention devices based on ATF are filters that hold back the cells and let liquid and small molecules pass. In tangential-flow filtration (TFF) the liquid flows past the pores of hollow-fiber filters tangentially rather than being forced through them orthogonally, thus reducing the likelihood of clogging. ATF devices use the same principle of tangential flow, but they reverse the direction of flow regularly to minimize fouling and reduce shear forces on the cells.

Experimentation at small scale is crucial for the cost-efficient development of bioprocesses, which then can be transferred to larger production volumes. To grow Chinese hamster ovary (CHO) cells in perfusion at small scale, we connected an ATF-2 filtration device from Repligen with a DASGIP parallel bioreactor system.

ATF Perfusion at the 1 L Scale: The bioprocess run was conducted at a working volume of 1 L. We cultivated an antibody-producing CHO cell line from TPG Biologics Inc. in Dynamis AGT medium (Thermo Fisher Scientific, USA) supplemented with

Figure 2: Analysis of perfusion process at the 1 L scale; (TOP) cell growth and viability, (CENTER) IgG concentration in bioreactor and harvest, (BOTTOM) metabolite concentrations



8 mM L-glutamine and 1% Gibco anticlumping agent (Thermo Fisher Scientific). Figure 1 schematically represents the system setup.

Bioprocess System Characteristics and Setpoints:

• **Dissolved oxygen (DO):** Perfusion cultures can reach high cell densities, which is why the oxygen demand of the culture may become high. The DASGIP parallel bioreactor system supports a broad range of gas flows from 0.5 to 250 SL/h. We set the DO to 50% and controlled it by sparging air and/or O_2 using a macrosparger.

• **Temperature, pH, agitation:** The pH was set to 7.0 (deadband = 0.2) and controlled with CO_2 and sodium bicarbonate. The culture was agitated at 309 rpm with a pitched-blade impeller.

• **Offline analysis:** Twice daily we took samples through a dip tube with a swabable valve. We analyzed cell growth and viability offline as well as product and metabolite concentrations.

• **Perfusion rate:** The bioprocess system was equipped with a DASGIP MP8 multipump module. Depending on the pump head tube diameter, the pumps support feed rates of 0.3 to 9.5 mL/h or 13 to 420 mL/h. The broad feed rate range allows for adjustment of the perfusion rate during the run.

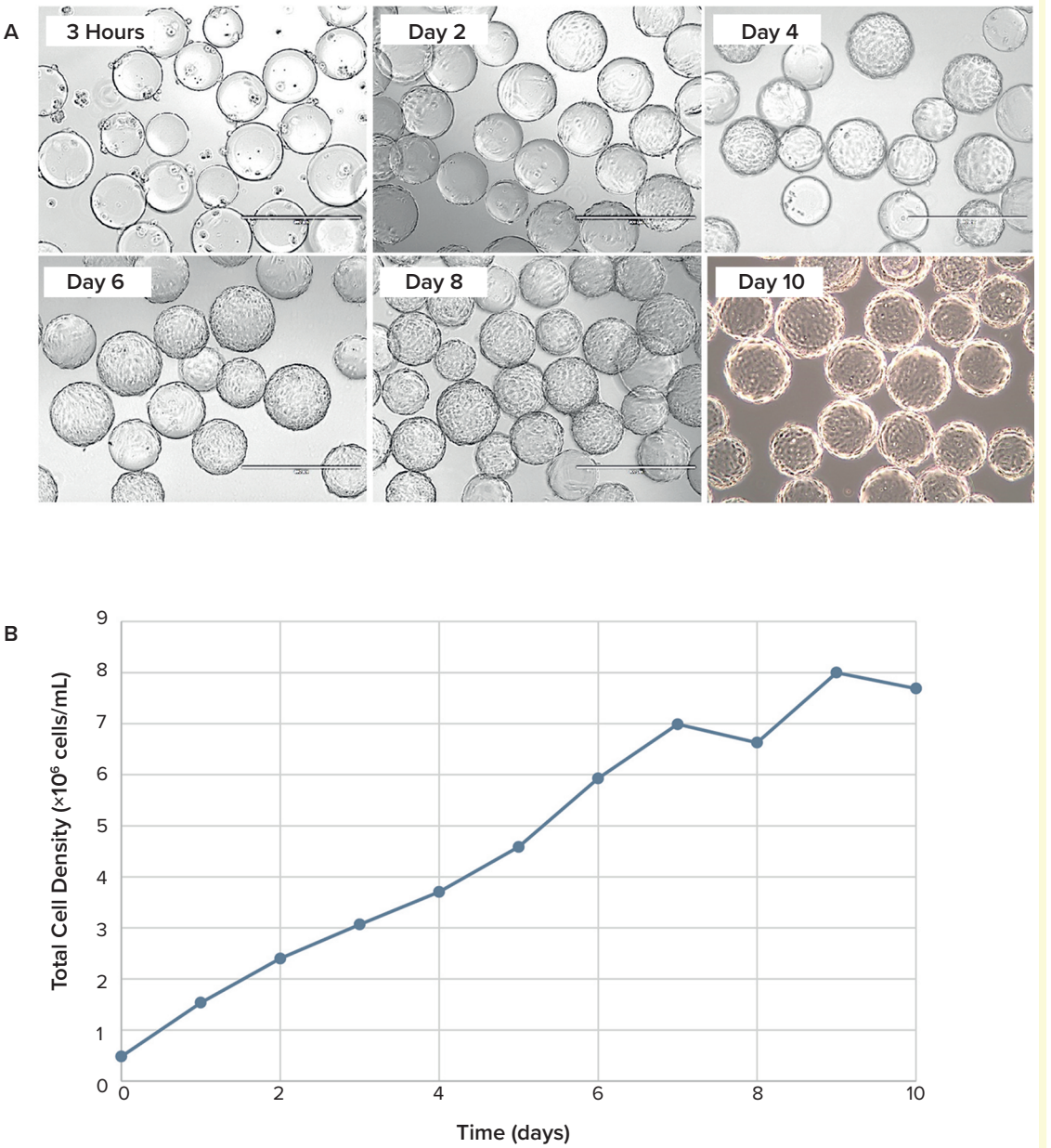
Figure 3: Microcarrier spin filter

Our perfusion target was to keep the ammonium concentration below 4 mM. At day 2, the ammonium concentration exceeded 3 mmol/L, so we started perfusion at 0.2 vessel volumes per day (VVD). We gradually increased the perfusion rate over the course of the run, based on ammonium concentrations determined offline. We also aimed to keep the glucose concentration above 3 g/L. In addition to replenishing glucose throughout the perfusion process, we bolus-fed the culture if the glucose concentration dropped below 3 g/L. The lactate concentration increased up to 1.9 g/L on day 2. With the startup of perfusion on day 2, lactate concentration decreased and was kept below 1 g/L from day 6. The IgG concentrations in the bioreactor and harvest increased up to about 500 mg/L. We had inoculated the culture at a density of 0.9 × 10^6 cells/mL. On day 13, the culture reached a peak cell density of 60.88 × 10^6 cells/mL. At that time point, 90% of the cells were viable (Figure 2).

ATF Perfusion at the 3.75-L Scale: The results described above demonstrated the feasibility of cultivating CHO cells using perfusion in a DASGIP parallel bioreactor system equipped with an ATF-2 cell retention device. In a previous study (**1**) we had cultivated the same cell line in perfusion using a

The results described above demonstrated the FEASIBILITY of cultivating CHO cells using perfusion in a DASGIP parallel bioreactor system equipped with an ATF-2 cell retention device.

Figure 4: (A) Vero cell cultured on microcarriers 3 hours after inoculation and on day 2, 4, 6, 8, and 10; (B) cell growth curve on Cytodex 3 microcarriers

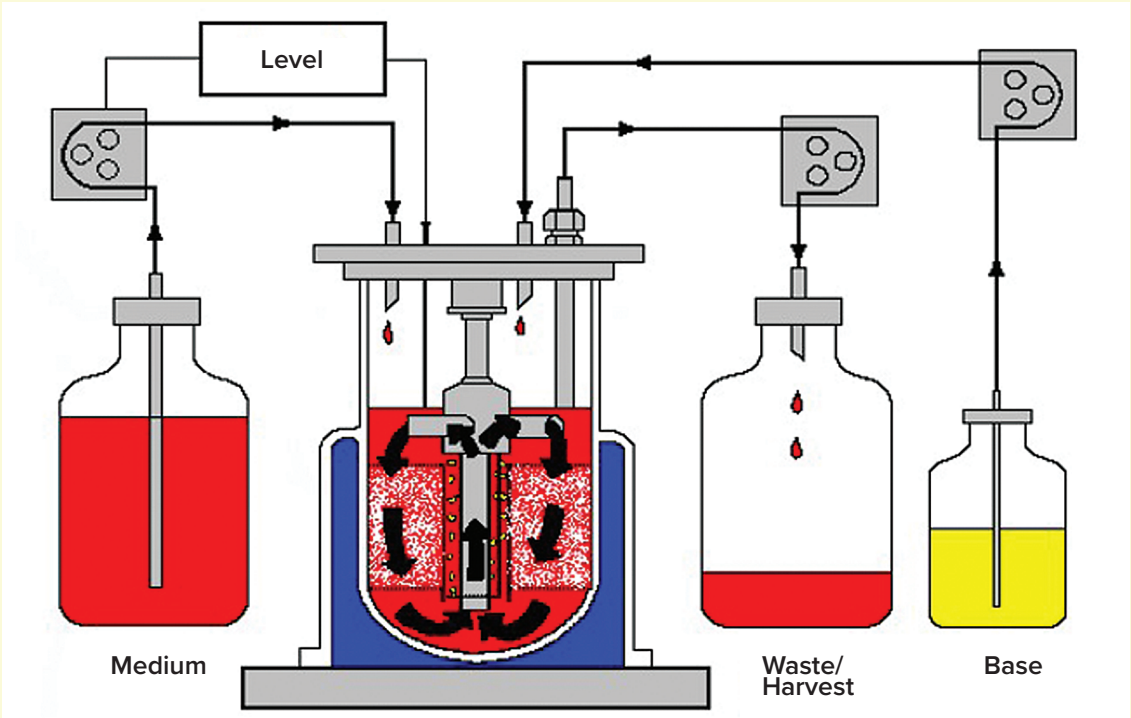


custom BioBLU c single-use vessel (maximum working volume of 3.75 L) equipped with an ATF-2 device and controlled with a BioFlo 320 bioprocess control station. Similarly, an ammonium concentration of ‹4 mM and a glucose concentration of ›3 g/L were targeted. The peak viable cell density was 74 × 10^6 cells/mL at day 15. The fact that we achieved similar cell densities in CHO cell perfusion processes with working volumes of 1 L and 3.75 L indicates the scalability of the system. Available larger ATF filtration devices in principle support perfusion cultures with working volumes of ›1,000 L.

Case Study: Microcarrier Spin Filters

To cultivate adherent cells in stirred-tank bioreactors, microcarriers often are used as a growth matrix. Spin filters are a fairly simple filter variant that can be used to harvest medium while retaining the microcarriers in a bioreactor. The spin filter is a cylinder-shaped cage surrounding and spinning with the impeller shaft (Figure 3).

Figure 5: Perfusion culture setup using a packed-bed vessel



Eppendorf microcarrier spin filters with a pore size of 75 µm keep microcarriers isolated outside the cage while microcarrier-free medium passes the filter and can be harvested from within the filter through a dip tube. Suspension cells can be retained in the bioreactor by spin filters with smaller pore sizes. Spin filters are relatively inexpensive but have some limitations, such as a tendency to clog.

We tested the suitability of a microcarrier spin filter as the cell retention device for perfusion cultivation of Vero cells in a working volume of 3 L. The aim was to produce enough cells for inoculation of a culture with a working volume of 32 L.

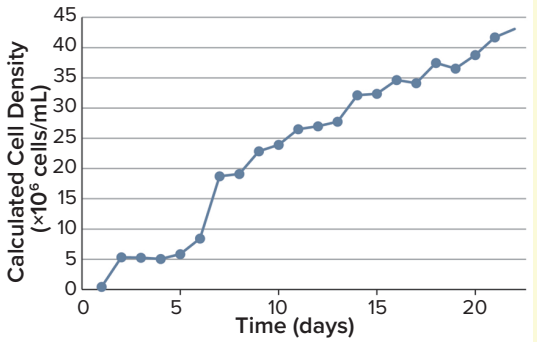
Perfusion Culture Setup: We used a BioFlo 320 bioprocess control station equipped with a 3-L glass vessel and a microcarrier spin filter. The culture was agitated with a pitched-blade impeller. We controlled the pH at 7.1 (deadband = 0.1) using a cascade of CO_2 (acid) and 0.45 M sodium bicarbonate (base). The temperature was set to 37 °C.

We cultivated the adherent Vero cell line ATCC CCL-81 in DMEM + antibiotic-antimycotic + 5 % heat-inactivated FBS (all from Thermo Fisher Scientific, USA). Cytodex 3 microcarriers (GE Healthcare, USA) were added to the medium at a concentration of 10 g/L before inoculating with

Figure 6: BioBLU 5p single-use vessel

4.6×10^5 cells/mL. The inoculum was added into the vessel while the medium was agitated at 40 rpm. To increase the chance for cells to attach to the microcarriers, the agitation was stopped two minutes after inoculation. The culture was agitated

Figure. 7: Calculated cell growth curve in packed-bed bioreactor



Unlike perfusion systems with ATF or spin filters, packed-bed bioreactors allow the separation of cells and culture medium WITHOUT needing FILTRATION.

intermittently (40 rpm agitation for two minutes followed by 28 minutes of incubation without agitation) for the next three hours. Then the culture was continuously agitated at 40 rpm.

We started perfusion at day 3. The strategy was to maintain the glucose concentration between 3 and 5 g/L and the ammonium concentration below 4 mM. The perfusion rate was increased gradually up to 1.2 VVD on day 10. In addition to perfusion, we bolus-fed the culture with glucose.

To measure the cell density, we extracted nuclei daily from a 1-mL sample of the microcarrier culture and counted using a VI-CELL cell analyzer (Beckman Coulter, USA). The cell density increased up to 8 × 10^6 cells/mL at day 10 (Figure 4).

By cultivating Vero cells on microcarriers in perfusion, we produced 2 × 10^{10} cells in a working volume of 3 L. This cell number is sufficient for the inoculation of a 32-L packed-bed bioreactor. The packed-bed bioreactor technology is described below.

If more or fewer cells are needed, perfusion processes using microcarrier spin filters can be performed in different-sized vessels. Eppendorf offers microcarrier spin filters for several vessels, the smallest being the 1-L glass vessel of the BioFlo 120 bench-scale bioprocess controller and the largest the 32-L stainless-steel vessel of the CelliGen 510 bioprocess controller.

Case Study: Packed-Bed Bioreactors with Fibra-Cel Disks As Growth Matrix

Unlike perfusion systems with ATF or spin filters, packed-bed bioreactors allow the separation of cells and culture medium without needing filtration. Eppendorf offers packed-bed bioreactors that are equipped with a basket impeller filled with Fibra-Cel disks as a growth matrix (Figure 5). Culture medium is circulated through the bed. Fibra-Cel disks have a diameter of 6 mm and consist of a fibrous matrix made of polyester and polypropylene. The matrix provides a three-dimensional growth environment, which protects cells from damaging shear forces. The disks are electrostatically pretreated to support cell attachment. Cell-free medium and soluble products can be harvested from the bioreactor without the need for a filtration step. Packed-bed bioreactors therefore are ideally suited for the production of secreted products such as recombinant proteins and viruses.

We used a BioBLU 5p single-use vessel equipped with a basket impeller, which was preloaded with Fibra-Cel disks, for cultivating the adherent Vero cell line ATCC CCL-81 in perfusion mode (Figure 6). The working volume was 3.75 L, and the process was controlled with a BioFlo 320 bioprocess control station. The vessel contained 150 g of Fibra-Cel disks. The total growth surface is 180,000 cm^2, which corresponds to the growth surface of 1028 T-175 flasks and 212 roller bottles. We inoculated the BioBLU 5p single-use vessel at a density of 4.6 × 10^5 cells/mL. Thirty minutes after inoculation, we could not detect suspension cells in a medium sample, indicating that the cells had adhered to the Fibra-Cel disks rapidly.

Perfusion started at day 3. We increased the perfusion rate during the run and bolus-fed the culture to keep the ammonium concentration below 4 mM and the glucose concentration greater than 3 g/L, as described above. When we ended the process at day 21, the cell density had increased to 43 × 10^6 cells/mL. This cell density is about fourfold higher than when cultivating Vero cells on microcarriers in perfusion. One explanation may be that the growth surface provided by 10 g/L Cytodex 3 microcarriers is smaller than the surface provided in a packed-bed bioreactor of the same working volume. Furthermore, the cells cultivated in the packed-bed bioreactor may be better protected from damaging shear forces (**2**).

Analysis of Cell Growth in Packed-Bed Bioreactors: Because cells attach to the growth

matrix and Fibra-Cel disks cannot be removed from the vessel while the process is running, the cell number cannot be determined directly during the process. Nevertheless, it is possible to analyze the cell growth.

After the Vero cell perfusion process had ended, we measured the cell density. We cut the vessel open and collected Fibra-Cel disks from different locations of the basket. We extracted cell nuclei from the disks and counted them in a VI-Cell analyzer (**2**). By calculating the glucose consumption rate on the last day of the process and the cell density at the end of the run, we obtained the conversion ratio of glucose consumption to cell density. Assuming that this conversion ratio remained relatively unchanged over the duration of the culture, we converted the daily glucose consumption rate into daily cell density (Figure 7).

Scalability: Packed-bed bioreactors from Eppendorf cover working volumes of 1 L to 32 L. To support scale-up, we have experimentally determined the impeller power numbers of packed-bed vessels with working volumes of 3.75 L and 32 L, representing an approximately ninefold scale-up. By knowing the power number, bioprocess engineers can calculate the power input per liquid volume. Keeping that ratio constant across scales is one of the most widely used strategies for scale-up.

Enabling Efficient Process Development

Cell culture bioprocessing in perfusion requires equipping the bioreactor with a cell retention device. In proof-of-concept studies we cultured suspension CHO and adherent Vero cells in Eppendorf bioreactors equipped variously with an ATF-filtration device, a microcarrier spin filter, or a packed-bed basket impeller. The technologies differ in their suitability for use not only with adherent or suspension cells and in ease of cell recovery, but also in investment and scalability.

ATF-perfusion is well-suited for cultivation of suspension cells and production of secreted proteins. High cell densities can be reached, and cells can be recovered from the culture easily. Perfusion using packed-bed bioreactors loaded with Fibra-Cel disks is ideally suited for production of secreted products such as proteins or viruses by adherent cells. The system is easy to set up and comparatively inexpensive because no perfusion device is required except for the vessel itself. High cell densities can be reached, but cell recovery can be challenging. Perfusion culture of adherent cells on microcarriers using a microcarrier spin filter also is relatively inexpensive and easy to set up. We reached lower cell densities with that strategy than in the packed-bed bioreactor, but cell recovery from the microcarriers is easier. The system is suitable for production of inoculum for larger attachment cell culture vessels as well as for production of secreted products.

ATF-perfusion is **WELL-SUITED** for cultivation of suspension cells and production of secreted proteins. High cell densities can be reached, and cells can be recovered from the culture easily.

The choice for a cell retention system needs to be made case by case. For efficient process development, techniques compatible with small-scale bioreactors that can be scaled-up to larger production volumes are appropriate.

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