

ABSTRACT

ROBINSON, DAREN FRANCIS. Development of a Perfusion System for Microcarrier Based Cell Culture in a Single Use Bioreactor. (Under the direction of Dr. Balaji Rao).

Perfusion feeding has shown promise in improving culture consistency, parameter control, and cell growth. One of the critical components of perfusion feeding is the need to retain cells in the culture. While there are several commercially available technologies for retaining cells in suspension cultures, this is not the case for applications where cells must be grown on microcarriers. The objective of this thesis was to develop a system that allowed for perfusion feeding while retaining cells on microcarriers. This system needed to work with a single-use bioreactor while being simple and inexpensive to implement and operate. To achieve this goal two different cell retention devices are tested: 1) a microsparger system that retains cells by filtration 2) a settling column that retains cells by sedimentation. The systems were evaluated on their retention ability as well as effects on the culture conditions and cells produced. While the microsparger system successfully retained cells on microcarriers in the initial experiment, it clogged when outflow rates were increased in the second experiment. The settling column system was able to provide retention of cells on microcarriers for three 10 L cultures. This retention allowed for comparison of perfusion feeding to the standard half-batch fed cultures. Overall trends did show an improvement in cell growth, culture consistency, and parameter control in the perfusion fed culture compared to the half-batch fed culture. Due to its success, the settling column system can be used in microcarrier based perfusion cultures. Proposed future work would be to automate and dynamic perfusion feeding strategy using a single-use settling column retention system.

Development of a Perfusion System for Microcarrier Based Cell Culture in a Single Use
Bioreactor

by
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LIST OF ABBREVIATIONS

BSC	Biosafety cabinet
DPBS	Dulbecco's phosphate buffered saline
ID	Inner diameter
PDT	Population doubling time
RPM	Revolutions per minute
SLPM	Standard liters per minute
VVD	Vessel volumes per day
WFI	Water for injection

CHAPTER 1: INTRODUCTION

Regenerative medicine is relatively new but is becoming a promising field for producing life-saving therapies. One of the main challenges of this field is the efficient production of large quantities (billions to tens of billions) of human cells. To address this challenge, focus has been put on developing a process that produces this large number of cells, quickly, constantly, and at a low-cost. To achieve this, two commonly used bioproduction methods are being investigated: first, culturing cells in large bioreactors and second, perfusion style feeding. The following thesis will describe the efforts to develop a perfusion system for bioreactor culture of adherent cells.

1.1 Microcarrier Culture

For many regenerative medicine applications, primary adherent human cells are needed. The cell's adherent nature makes traditional bioreactor culture, where cells are suspended in medium, not feasible. So, to allow for use of bioreactor technology the cells needed to be suspended while maintaining adherence. To achieve this, microcarrier technology is being used.

Microcarriers were first introduced by A. L. van Wezel (1967) when he found that primary mammalian cells would attach to DEAE-Sephadex beads after 20 hours. He was then able to take these beads and suspend them by adding gentle agitation. After several days, he observed growth rates in line with traditional monolayer culture. He was also able to successfully infect these cells and produce a viral titer. Since 1967, many different types of microcarriers have been commercialized. These microcarriers vary in size, shape, composition, and culture surface (Microcarrier Cell Culture: Principles and Methods). Table 1 (adapted from Chen et al., 2020) presents different commercially available microcarriers, their properties, and some general

applications. Several of these microcarriers have been shown to support culture endothelial cells (Schrimpf and Friedl, 1993), which will be the cell type used in this thesis.

Table 1: Commonly used commercially available microcarriers. (Adapted from Chen et al, 2020)

Name	Type	Diameter (µm)	Density (g/mL)	Internal composition	Surface modification	Applications
Cytodex 1	Solid	190 ± 58	1.03	Dextran matrix	Positively charged diethylaminoethyl groups	Substrates for the Propagation of articular bovine chondrocytes
Cytodex 2	Solid	135-200	1.04	Dextran matrix	N, N, N-trimethyl-2-hydroxyaminopropyl groups	Injectable for cartilage regeneration
Cytodex 3	Solid	175 ± 36	1.04	Dextran matrix	Denatured porcine collagen	Prolonged and rapid cells growth
Hillex II-170	Solid	170 ± 10	1.12	Polystyrene	Treated	Used in cell culture systems for bioreactor scale-up
ProNectin F	Solid	169 ± 44	1.02	Polystyrene	Recombinant proteins	3D culture analysis
FACT III	Solid	169 ± 44	1.02	Charged polystyrene	Collagen	Cell expansion
CGEN 102-L	Solid	169 ± 44	1.02	Polystyrene	Type I porcine collagen	Tissue-healing processes
Cytopore 1,2	Macroporous	240 ± 40	1.03	Cross-linked cellulose	Positively charged diethylaminoethyl groups	Stirred tank cultures
CultiSpher G, S, GL	Macroporous	255 ± 125	1.04	Cross-linked type I porcine collagen	None	All types of cell culture

By allowing adherent cells to grow on microcarriers, the benefits of a bioreactor culture system can be employed. In the handbook “Microcarrier Cell Culture: Principles and Methods” the benefits of this system are discussed. They include increasing the yield of the adherent cells, increasing the control of environmental conditions such as pH and dissolved oxygen, more efficient use of media, and lower risk of contamination. However, there are some challenges that need to be considered when using microcarriers such as avoiding excess shear on the cells while suspending the microcarriers and separating cells from microcarriers when cells are the primary product.

1.2 Perfusion

Cell culture perfusion, or the constant exchange of fresh medium, was first successfully demonstrated by Kruse et al. (1963). In this paper, rat sarcoma cells were cultured in eight T-60 flasks with constant medium exchange for 8 days. The researchers found that the environmental conditions (pH and glucose) were well maintained throughout the culture, unlike environment conditions seen with typical periodic feeding. They also found more cells were produced using perfusion culture compared to periodic feeding.

More recently there have been several publications that show an increase in the number of cells produced for therapeutic applications by adapting perfusion feed strategies. Several of these publications (Bauwens et al., 2004, Serra et al., 2010, and Fernandes-Platzgummer et al., 2013) describe reasons for the increase in cell number. The first reason is an improved supply of nutrients and growth factors provided to the culture. The second reason cell number increases is the constant removal of waste products and potential cell-secreted growth inhibitors, which have been found to build up in standard feeding regimes. Finally, there is a decrease in metabolite and oxygen fluctuations that can negatively affect cell growth and metabolism. Weegman et al. (2013) explain that because of these reasons, perfusion feeding creates an environment that is more like the one experienced by cells *in vivo* and this leads to the improved health and growth of the cells.

In perfusion culture of suspended cells, the bulk number of cells must be retained to either increase the viable cell density of the culture or to continuously produce the product of interest. Su (2009) described multiple retention devices that he grouped into two categories based on how they retain cells. The first category was systems that retained cells via filtration, such as spin filters and

alternating tangential flow filters. The second category was systems that retained cells based on sedimentation, such as vertical settlers, inclined settlers, and acoustic resonators. A graphical representation of these two principles is shown in figure 1.

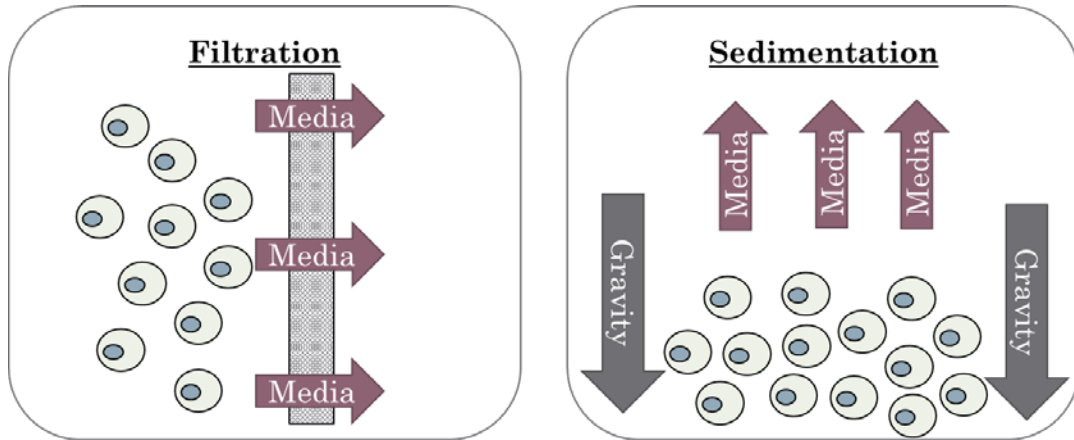


Figure 1: Graphical representation of the two different retention principles.

Two commonly used filtration-based retention systems that have shown promise in literature are spin filter systems and alternating tangential flow filtration systems (Voisard et al, 2003, Bielser et al. 2018). A typical spin filter consists of a cylindrical mesh that rotates either inside the bioreactor or externally. The porosity of the mesh allows for media to flow through while the cells are retained due to the forces imparted by the rotating filter. This system has been used for microcarrier culture (Han and Sha, 2020). Alternating tangential flow (ATF) systems use a hollow-fiber module to separate cells and media. The pore size of these hollow fibers allows for cells to be retained inside while media flows out. The additional feature of the ATF is a pump that allows for the filter to be back-flushed regularly (Bonham-Carter and Shevitz, 2011), returning the retained cells to the culture vessel. The media and cells enter and exit the ATF system using the same path, but the flow direction is regulated by the pump. The system supports low-shear flow

while enabling quick return of cells back to the culture for continued expansion without imparting excess stress.

Sedimentation-based retention systems offer advantages over filtration as they are more robust and impart less shear on cells (Voisard et al, 2003). However, these systems tend to be less common in industrial applications as the cells have high residence times (1-2 hours in some applications) in the sedimentation vessel. This residence is not favorable as the conditions in these vessels are not controlled and therefore suboptimal compared to the culture vessel. In general, these systems work by exploiting density differences between cells and surrounding medium. Because cells are denser, they will settle out of the culture medium under static condition. Because perfusion requires the movement of medium out of the culture, either the flow of the medium needs to be less than that of the settling velocity of the cells or other principles need to be exploited to ensure proper cell retention. For vertical settlers, the first condition is true. By using a settler with a large enough volume, appropriate fluid outflow rates are achieved, and cells are able to settle back into the culture vessel. Acrivos and Herbolzheimer (1979) developed the basis for an alternative system where the settler was put at an incline, which allowed for improved settling efficiency. Another type of system, acoustic resonators, have also been shown to improve settling efficiency by inducing temporary aggregation of cells, increasing the particle size and ultimately the g-force. This increase in g-force allows for higher outflow rates compared to a vertical settler.

1.3 Thesis Objective

In an effort to decrease culture variability and increase the number of cells produced, a perfusion feeding system for use in microcarrier culture was investigated. This system would have two main

requirements. First, the perfusion system would need to be compatible with a single-use bioreactor system. Second, the perfusion system would need to be simple to operate and inexpensive.

The first requirement was the most important, as a redesign of the existing processes was to be avoided. Therefore, the system needed to fit the single-use bioreactor system already in use. To achieve this, the system would need to easily fit into one of the two PG 13.5 ports on the bioreactor headplate. This eliminated use of perfusion systems like the previously mentioned spin filter, which is only compatible with glass and stainless-steel bioreactors.

The second requirement was less important but needed to be considered because a large capital investment (and/or high operational cost) and complicated set-up would make wide-spread usage more difficult. Therefore, the perfusion system would have to be inexpensive to purchase and run, while also being simple for operators to handle. This requirement eliminated systems like the previously mentioned ATF system, as it requires a high capital investment (estimated \$10,000 – \$20,000 per bioreactor) and requires continued purchase of a consumable (estimated \$200-\$300 per run).

Keeping both requirements in mind, two perfusion systems were investigated, one that retained cells via filtration and the other via sedimentation. Both systems were evaluated on the systems' ability to support perfusion feeding of cells on microcarriers. Improvements in total cell yield as well as nutrient, pH, and dissolved oxygen stability would be expected due to results from literature. The perfused cultures will be compared to a typical half-batch feed strategy control condition to investigate improvements that perfusion feeding may offer. In this typical feed

strategy, 50% of the media is removed and replaced daily. Finally, a decision was made on which system could be used for perfusion of microcarrier cultures with some potential future improvements for that system.

CHAPTER 2: CELL AND MICROCARRIER RETENTION VIA FILTRATION USING A MICROSPARGER

2.1 Introduction

One of the main challenges of bioreactor perfusion feeding is the ability to retain cells and microcarriers in the culture vessel while effectively removing media. One avenue of addressing this challenge was to use filtration. By imparting a filter with a pore size smaller than the size of the microcarrier, media could be removed while the sieving action of the filter would allow for microcarriers to be retained. One complicating issue is that the filter needs to be submerged into the bioreactor for an extended amount of time (> 6 days) without breaking down or fouling. Roldao et al. (2018) overcame this issue by using a stainless-steel 20 μm pore size microsparger system, while perfusing media in a 3D aggregate culture of H157 cells. Using this system, the authors were able to perfuse media for up to 15 days while retaining aggregates with sizes from 80 – 180 μm . Because the retained aggregates were smaller than the microcarriers used in the predefined process, the system should be feasible for this application.

The retention system used by Roldao and colleagues was an inexpensive (<\$200) off-the-shelf stainless steel microsparger (Eppendorf). This system can be inserted into any pg 13.5 port which is commonly found on many different types of bioreactors. Because it fit the two criteria laid out in Chapter 1, it was determined that it should be tested as a retention method in the current application. The general concept of this system is displayed in figure 2.

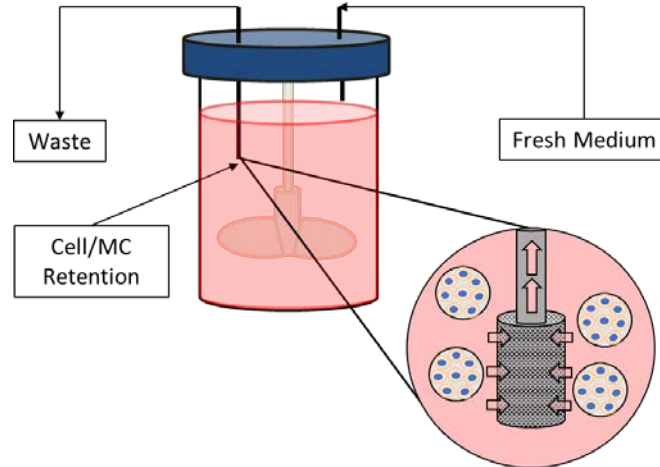


Figure 2: General microsparger-based retention perfusion system overview.

This chapter will describe two experiments performed using the microsparger retention system. The first experiment was using a small-scale (1 L) bioreactor system and the other was using a 1 L glass spinner flask. In both experiments, cells were cultured for several days before perfusion was initiated. The first culture used a perfusion rate that was matched to the daily half-batch feeding (0.5 VVD) while the second focused on maintaining a set glucose level. Cell growth, nutrient levels, and the ability for perfusion to be continuously run following initiation was evaluated.

2.2 Methods

2.2.1 Small Scale (1 L) Bioreactor Culture

2.2.1.1 Culture Set-up

A 1 impeller BioBLU 1c bioreactor (Eppendorf) was set-up by aseptically inserting a glass pH probe (Mettler Toledo) and the microsparger system outfitted with a weldable 0.125" ID tubing. The vessel was connected to a DASGIP parallel bioreactor system (Eppendorf) for monitoring and control of dissolved oxygen, temperature (both using probes that do not come in contact with the

culture), pH, and agitation. A control bioreactor was set-up in an identical fashion without the addition of the microsparger system.

Commercially available vascular cell growth media was made according to the manufacturer's instructions and 0.5% Penicillin-Streptomycin (Lonza) was added. Microcarriers were hydrated according to manufacturer's instructions. The microcarriers were resuspended in media and added via gravity to the bioreactors. The media and microcarriers were equilibrated for 2-4 hours by heating media to 37°C, overlaying air at 0.1 SLPM, and agitating at 40 RPM.

2.2.1.2 Metabolic Sampling

Throughout the culture, media samples were taken to collect data on the concentration of metabolites and gasses as well as pH. This sample was taken by stopping agitation and allowing microcarriers to settle away from the vessel sample port. The sample line was primed with 10 mL of media. This primed media was discarded. A 5 mL sample was taken and run on a NOVA Flex 2 metabolic analyzer (NOVA Biomedical). The metabolic values (glucose, lactate, glutamine, and ammonium) given by the system were recorded and the pH and dissolved oxygen probes were calibrated via a 1-point calibration. Depending on the day in culture and bioreactor condition, agitation was restarted.

2.2.1.3 Culture

After equilibration, previously expanded primary human vascular cells were thawed in a 37°C water bath for 2.5 minutes. The cells were resuspended in media and counted using a Cellometer K2 cell counter (Nexcelom). Prior to seeding, a baseline metabolic sample was taken, as previously

described. Cells were added to each bioreactor via gravity over the course of approximately 5 minutes. A “seeding” program that was previously set-up on the DASGIP controller (Eppendorf) was turned on. This program instructed the system to turn agitation on and off at certain time intervals for a determined number of cycles. After these cycles were completed, the agitation stayed at a constant rate.

On culture day 2, a metabolic sample was taken. 500 mL of media was added to each bioreactor to bring the culture to its final volume of 1 L. A 3 mL sample of microcarriers and media was taken via the sample port and visually inspected via microscopy. After allowing the system to reach a temperature of 37 ± 0.5 °C a final metabolic sample was taken.

On culture days 3 – 4 metabolic samples and phase images of microcarriers were taken for each condition as previously described.

On culture days 5 – 7 metabolic samples and phase images of microcarriers were taken for each condition as previously described. The control condition was fed by turning off the agitation, tilting the vessel, and allowing the microcarriers to settle away (approximately 5 minutes) from the “harvest” line. 500 mL of media was removed via gravity into an aseptically connected waste bottle. 500 mL of fresh media was added onto the vessel and was added via gravity. Agitation was restarted and after allowing the system to reach a temperature of 37 ± 0.5 °C, a final metabolic sample was taken. Perfusion was started in the perfusion condition starting on day 5.

2.2.1.4 Perfusion Set-up and Operation

The perfusion system was set up by welding on a bottle containing 1.5 L of media to the inlet line of the bioreactor. This 0.125" ID tubing line ("feed line") was inserted into one head of a dual head perfusion pump with the rotational direction set to direct media into the bioreactor. The tubing line on the previously inserted microsparger system was welded to an 0.125" ID tubing line connected to an empty 2 L bottle. This "waste line" was inserted into the other head of the dual head perfusion pump with the rotational direction moving liquid out of the bioreactor and into the waste bottle. An image and schematic of the set-up is shown in figure 3. A dual head perfusion pump was chosen to eliminate any variability between two separate pumps.

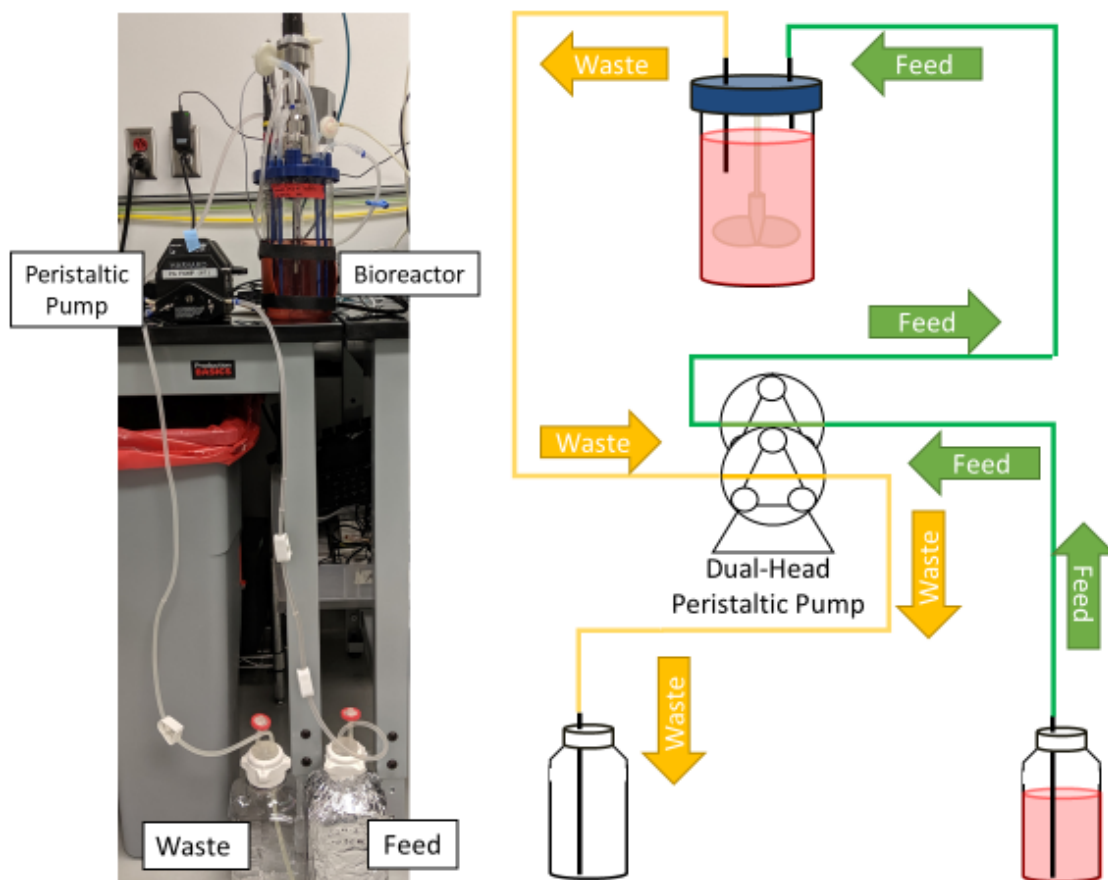


Figure 3: Picture (left) and schematic (right) of the perfusion bioreactor.

Perfusion was initiated by turning on the dual pump at a speed of 0.347 mL/min (0.5 VVD). This rate was chosen to match the control media exchange rate of 500 mL every 24 hours. The perfusion was set to run from culture day 5 to culture day 8 and stopped prior to harvest. The system was set to pump fresh media into the headspace of the bioreactor and pump out media from bioreactor through the microsparger.

2.2.1.5 Harvest

On culture day 8, a 3 mL sample of microcarriers and media was taken via the sample port for visual inspection via microscopy. A final metabolic sample was taken just prior to harvest.

The harvest of the cells was the same in both the perfusion and control conditions. The harvest was performed by turning off the agitation, tilting the vessel, and allowing the microcarriers to settle away from the “harvest” line. All but ~125 mL of the media was removed via gravity. 500 mL of phosphate buffered saline was added to the bioreactors via gravity. Each vessel was placed upright and agitated for about 5 minutes to ensure even mixing of the DPBS. After the 5-minute mixing period, agitation was turned off, the vessels were tilted, and microcarriers were allowed to settle away from the “harvest” line. All but about 125 mL of the DPBS wash was removed from the bioreactors via gravity. A microcarrier dissolving solution described by the manufacturer was prepared for each condition. This dissolving solution was added to each vessel via gravity, the vessel was set up right and agitation was turned on. This cell solution was then collected via gravity into an aseptic collection bottle. Each vessel was rinsed. The bottle was sealed off from the vessel and placed into a biological safety cabinet (BSC). The cell solution from each vessel was added to centrifuge tubes and centrifuged at 300 x g for 15 minutes. After centrifugation, the supernatant

was aspirated, and the cell pellet was resuspended in cryopreservation reagent. A count was taken of the cell solution before transferring the cells to cryovials. These cells were cryopreserved using a control rate freezer and stored in a cryopreservation tank for future cell assessment.

2.2.1.6 Cell Analysis

Cells were tested to ensure they met previously established quality control specifications. Cells were taken from cryopreservation tank and thawed at 37°C for 2.5 minutes. They were then resuspended in media to a final 1:5 dilution. The cells were counted to establish recovery and viability.

2.2.2 1 L Glass Spinner Flask Culture

2.2.2.1 Set-up

A 1 L glass spinner flask (Corning) was adapted to accept the microsparger system via a hole that was drilled in the cap. The microsparger was used exactly as previously described with a weldable 0.125" ID tubing set attached. An addition line was added to the side arm to allow for media to be added aseptically to the spinner flask. A sample line was added to the opposite side arm to allow for sampling throughout the culture. A control spinner was also set-up without any additional items. Both systems were autoclaved prior to use.

Airway epithelial cell medium was made according to the manufacturer's instructions and 0.5% Penicillin-Streptomycin was added. Microcarriers were hydrated according to manufacturer's instructions. The microcarriers were resuspended in 500 mL of media and added via gravity to the

bioreactors. The media and microcarriers were equilibrated for multiple hours in an incubator set to 37°C and 5% CO₂ and agitating at a constant rate.

2.2.2.2 Metabolic Sampling

Throughout the culture media samples were taken to collect data on the concentration of metabolites and gasses as well as pH for the cultures. This sample was taken by placing the spinner in the BSC (control condition) or stopping agitation (perfusion condition) and allowing microcarriers to settle. A 5 mL sample was taken and run on a NOVA Flex 2 metabolic analyzer. The values given by the system were recorded. Depending on the day in culture and vessel condition, the spinner was then returned to the incubator and/or agitation was restarted.

2.2.2.3 Culture

After equilibration, previously expanded primary human epithelial cells were thawed in a 37°C water bath for 2.5 minutes. The cells were resuspended in media and counted using a Cellometer K2 cell counter (Nexcelom). Prior to seeding a baseline metabolic sample was taken as previously described. Cells were added to each spinner flask. The spinner plate was programmed to turn agitation on and off at certain time intervals for a determined number of cycles. After these cycles were completed, the agitation stayed constant.

On culture day 2, a metabolic sample was taken. 500 mL of media was added to each spinner to bring the culture to its final volume of 1 L. A 3 mL sample of microcarriers and media was taken via the sample port for visual inspection of via microscopy. Before returning the spinners to the incubator, a final metabolic sample was taken.

On culture days 3 – 4 metabolic samples and microcarrier phase images were taken for each condition as previously described.

On culture days 5 – 6 metabolic samples and microcarrier phase images were taken for each condition as previously described. The control condition was fed by placing the spinner in the BSC and allowing microcarriers to settle. Half of the media (500 mL) was aspirated from the culture and 500 mL of fresh media was poured into the vessel. Before returning the spinners to the incubator a final metabolic sample was taken.

2.2.2.4 Perfusion Set-up and Operation

The perfusion system was set-up by welding on a bottle containing 1.5 L of media to the side arm inlet line of the spinner. This 0.125” ID tubing line (“feed line”) was inserted into one head of a dual head perfusion pump with the rotational direction set to direct media into the spinner. The tubing line on the previously inserted microsparger system was welded to a 0.125” ID tubing line connected to an empty 2 L bottle. This “waste line” was inserted into the other head of the dual head perfusion pump with the rotational direction moving liquid out of the spinner and into the waste bottle. An image of a bench top food coloring test and schematic of the set-up is shown in figure 4. A dual head perfusion pump was chosen to eliminate any innate variability between two separate pumps.

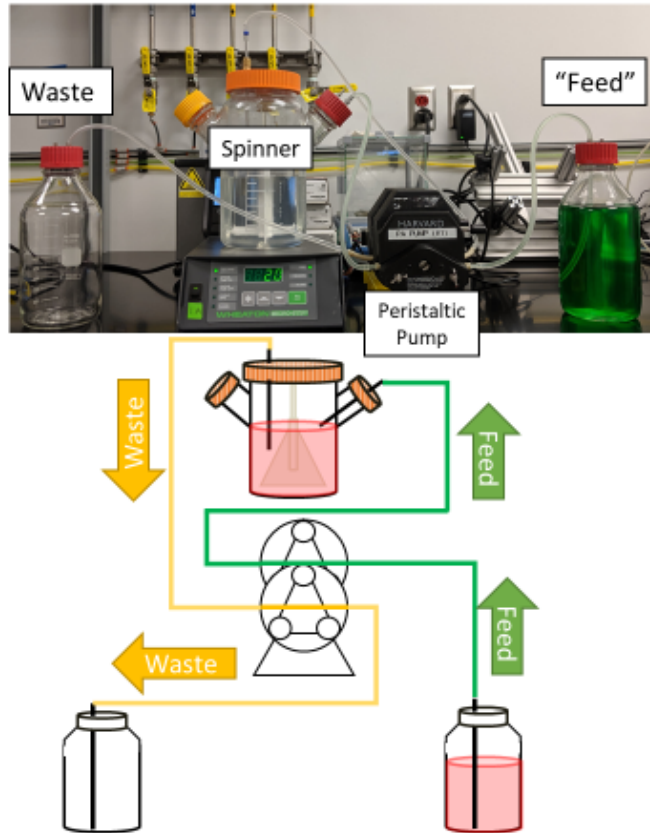


Figure 4: Picture (top) of a food coloring test and schematic (bottom) of the perfusion spinner flask setup.

The system was set to pump fresh media into the headspace of the bioreactor and pump out media from bioreactor through the microsparer. The perfusion strategy was different in this test in that the perfusion rate was dynamic and driven by glucose consumption to keep the glucose value above 0.8 g/L. Because of this dynamic feed strategy, perfusion was manually initiated when the glucose dropped below 0.8 g/L (on culture day 3) by turning on the dual pump at a speed of 0.2 mL/min (0.288VVD). The perfusion rate was increased on culture day 4 and 5 to maintain glucose levels. On culture day 6 the level of media in the system increased rapidly and the culture was terminated. This outcome will be discussed in the results section.

2.3 Results and Discussion

2.3.1 Small Scale Bioreactor Culture

In this experiment, the microsparger-based retention system was tested in a small scale (1 L) bioreactor. Perfusion was initiated at 0.5 VVD on culture day 5 and continued until the end of the culture (culture day 8). The microsparger system retained microcarriers and cells while allowing for outflow of medium. There were no signs of filter fouling throughout the culture.

As previously stated, samples of microcarriers were taken throughout the culture and were visually inspected to assess coverage and cell morphology. Phase images were taken pre-harvest and are presented in figure 5. Coverage appears to be higher in the half-batch fed condition, which is consistent with the harvest count. The cell morphology in both conditions was observed to be of cobblestone nature which is typical of endothelial cells.

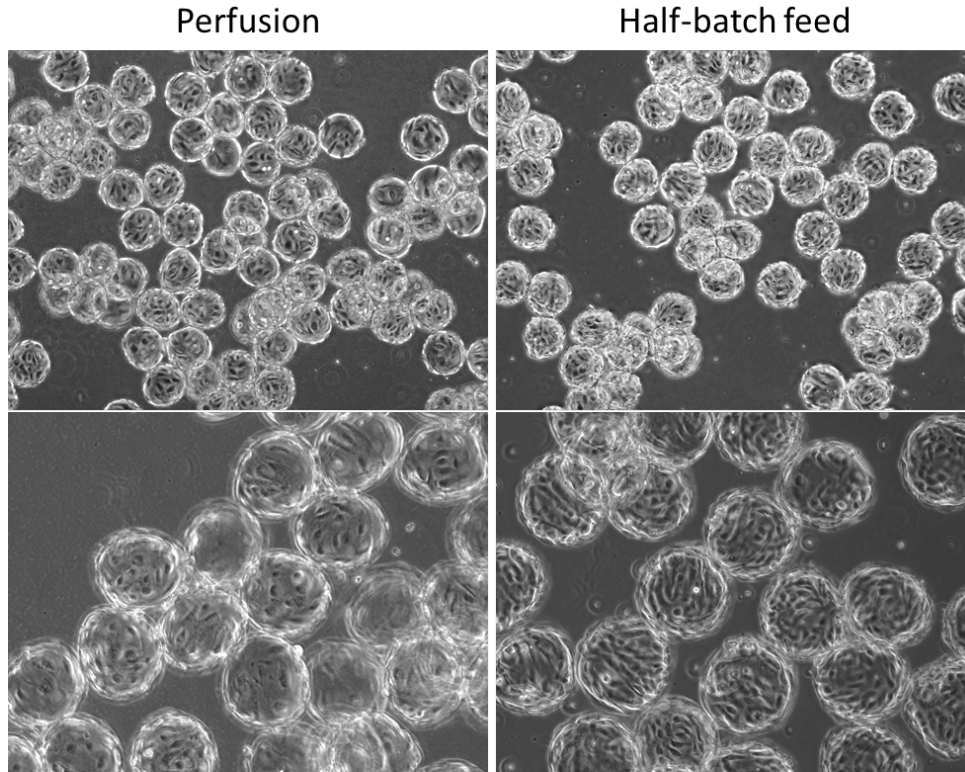


Figure 5: Pre-harvest phase images of cells on microcarriers. Top row images are at 5x magnification and bottom row images are at 10x magnification.

After harvesting the culture, the cells were counted, as previously described. The number of cells produced under each condition and the viability of the cells were assessed by acridine orange and propidium iodide staining and automated counting on a Cellometer K2. These results are displayed in Figure 6. Viability was shown to be higher in the perfusion condition, which could potentially be attributed to the consistent growth environment. The number of cells produced was lower in the perfusion condition. This result is inconsistent with literature that suggests improved growth rate under perfusion conditions. Because this was only one experiment, it is not clear if this result was due to variability in the cell counts or due to differences in testing conditions. An increased number of experiments will need to be run to confirm or deny these results and provide conclusions on the effects of perfusion.

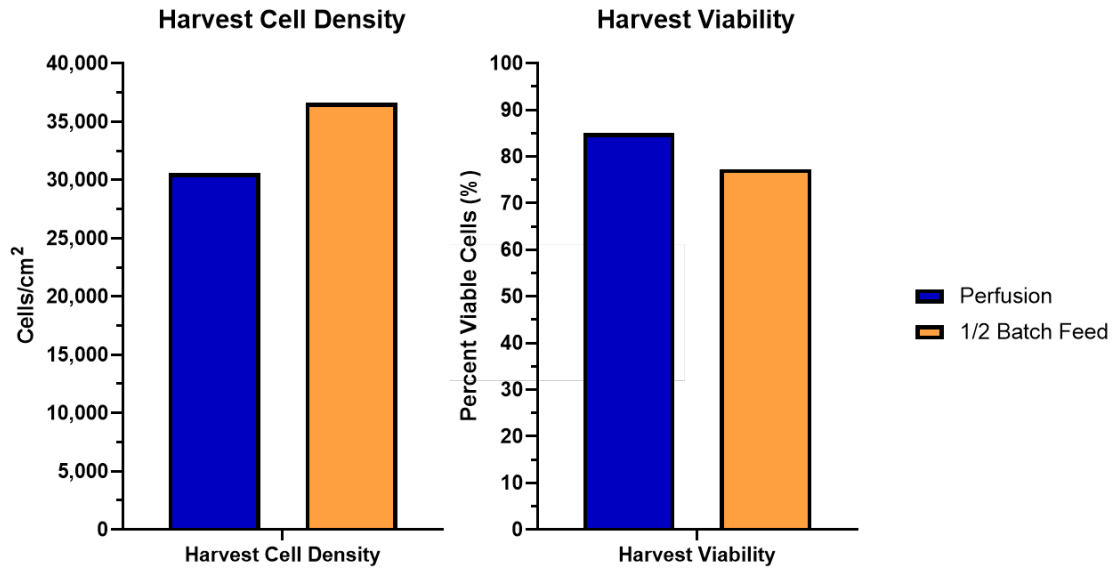


Figure 6: Graphs comparing viable cells per growth area and viability for each condition.

Using the counts taken at harvest, two growth metrics were calculated, fold increase and population doubling time (PDT) in days. Fold increase was calculated according to equation 1 and PDT was calculated according to equation 2. These two results are graphed in figure 5. These results indicated that the cells in the half-batch feed condition had faster growth than in the perfusion fed (PDT of 2.1 days vs 2.2 days) condition and therefore resulted in a higher increase in cell number. As with the results presented in figure 6, this data is contrary to results in the literature and will be reinvestigated in the future experiments.

$$\text{Fold Increase} = \frac{(\text{Number of Cells Harvested} - \text{Number of Cells Seeded})}{\text{Number of Cells Seeded}} \quad (1)$$

$$\text{PDT} = \frac{\ln(2)}{\left[\frac{\ln\left(\frac{\text{Number of Cells Harvested}}{\text{Number of Cells Seeded}}\right)}{(\text{Day of Seed} - \text{Day of Harvest})} \right]} \quad (2)$$

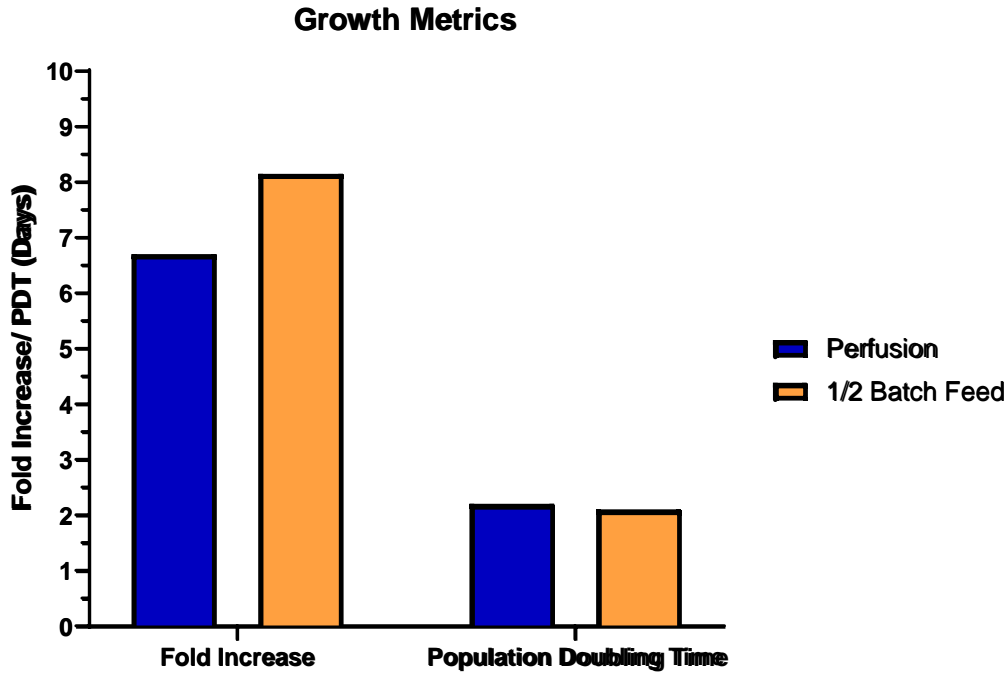


Figure 7: Graph of growth metrics for each condition.

During the culture, metabolic samples were taken of both the control and perfusion conditions. The metabolite values for each condition were graphed and are presented in figures 8 and 9. This metabolic data shows that perfusion provided a more consistent culture environment than the ½ batch feed condition with regards to metabolite concentration. The variability of nutrients in the half-batch feeding is due to the bolus addition of nutrients to the culture as opposed to the constant supply of nutrients imparted by perfusion feeding. As mentioned in Chapter 1, this environmental consistency is one of the benefits of perfusion.

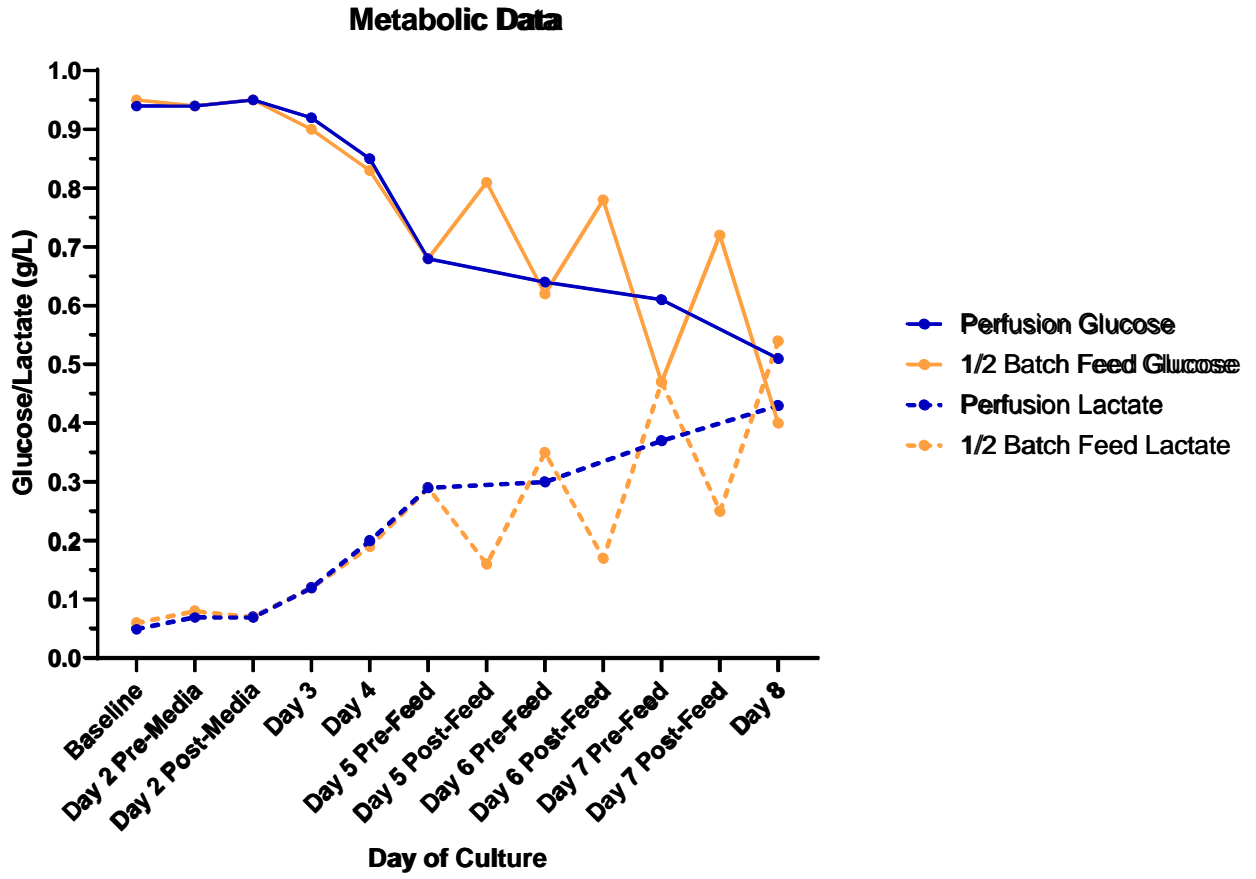


Figure 8: Graph of glucose and lactate throughout the small-scale bioreactor culture.

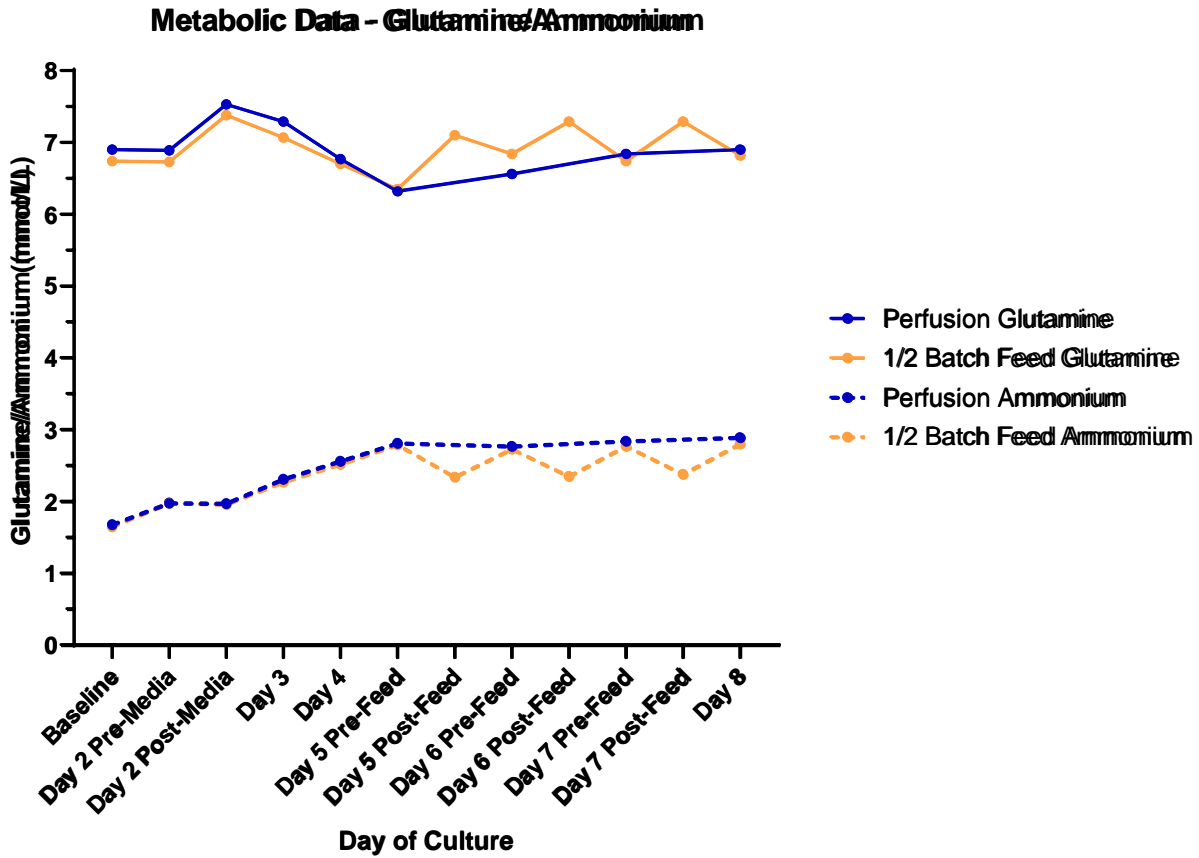


Figure 9: Graph of glutamine and ammonium throughout the small-scale bioreactor culture.

To assess the cells produced by the two conditions, cells were thawed and counted as previously described. Using the counts from this process, the percent of cells recovered, and viability were assessed. These metrics are compared for each condition and the data are displayed in figure 10. More cells were recovered from the perfusion condition and the viability was better maintained during the cryopreservation process. This could be due to the consistent environment improving the health of the cells and making them more amenable to cryopreservation, however more experiments and testing (discussed in chapter 4) will need to be run to confirm this.

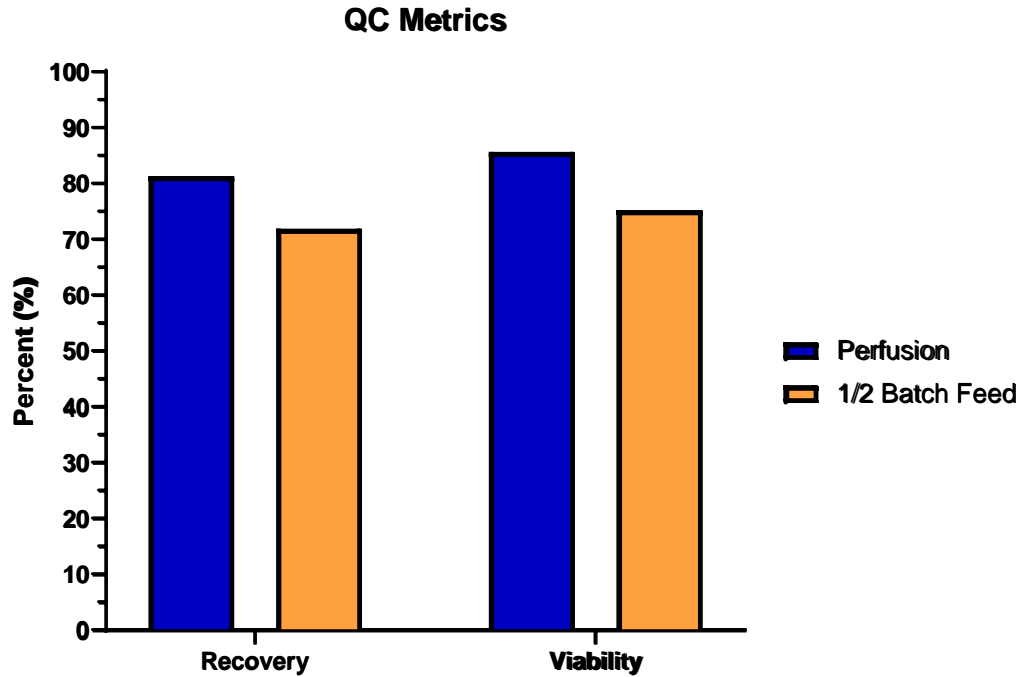


Figure 10: Graph of percent of cells recovered and viable cells after cryopreservation.

Overall, it was found that a more consistent nutrient environment could be provided by the perfusion system compared to the normal 1/2 batch feed process. While there was no indication that this consistent environment improved the growth of the cells, it may have improved the health and therefore viability of the cells at harvest and after cryopreservation. One flaw of this study was that only one run was performed with each condition and the assays performed have inherent variability in them. Because of this limitation, more work will need to be done to show these results were repeatable.

2.3.2 Spinner Flask Culture

As mentioned in 2.2.1.4 the spinner flask culture was ended on culture day 6. This early termination was due to the culture volume increasing rapidly and almost overflowing (figure 11).

The increasing volume was determined to be due to a lack of media waste removal from clogging of the microsparger system. This clogging was confirmed with visual inspection of the microsparger. It is apparent that microcarriers were lodged inside the pores of the microsparger, shown in figure 12. Because of the early termination of the culture, no culture harvest was performed and therefore no data was collected in this experiment.

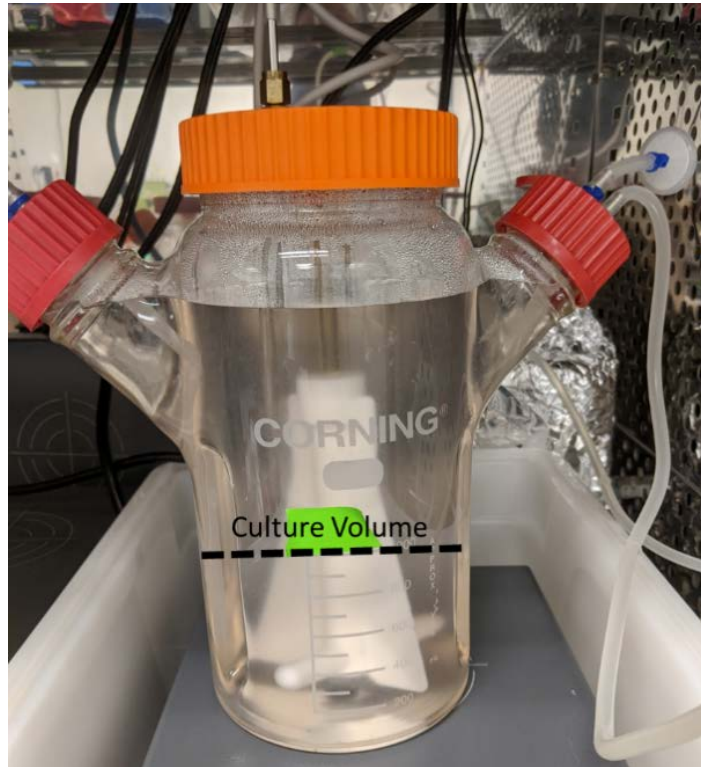


Figure 11: An image of the culture volume prior to ending the culture.

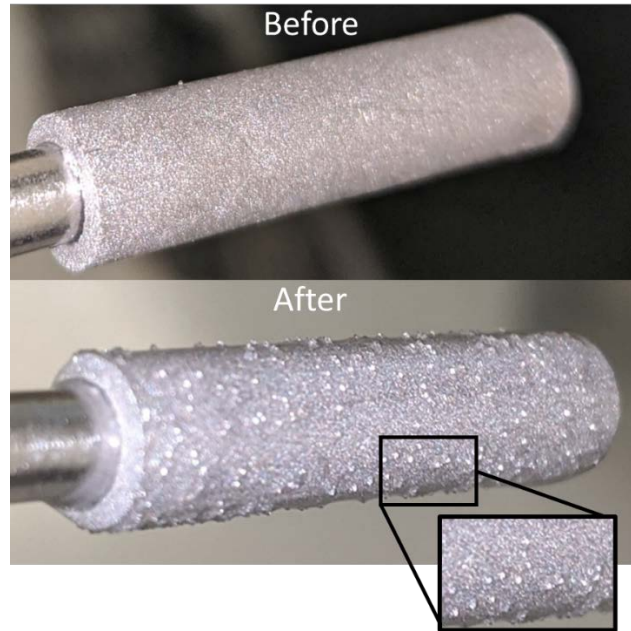


Figure 12: An image of the microsparger before and after the spinner test depicting microcarriers lodged in the pores.

It is suspected that this clogging happened due to the increase in perfusion rate. While the system worked well at the initial rate of 0.347 mL/min, increasing past this point caused the microcarriers to become lodged in the microsparger pores. This obstruction likely inhibited media from entering through most of the pores of the microsparger and therefore prevented it from leaving the system. The ability to monitor and control the weight of the system, would allow for automatic stopping of media addition from the inlet pump to avoid any culture overflowing. This weight management system is a future investigation that will be discussed in Chapter 4.

2.3.3 Further Investigations and Conclusion

After clogging was observed using the 20-micron pore size microsparger, a system with increased pore size was investigated. By increasing the pore size to a point below the microcarrier diameter, the amount of outflow obstruction should be reduced while allowing the microcarriers to be

retained. A 100-micron sparger tip (Chemglass) was found and outfitted to a 0.125" stainless steel tube. This system was placed in to a 10 L bioreactor mock culture containing only microcarriers and media. The outflow was set to 3.47 mL/min (0.5 VVD) to test the system under typical, large-scale culture conditions. During this test, it was found that the larger pores clogged, and outflow was again impeded.

One shortcoming of the larger pore size microsparger is that it had a smaller filtration surface area than the original microsparger. It was hypothesized that a long tube with larger (100-150 micron) pores would fix the clogging issue as it would provide more filtration surface area. Unfortunately, no commercial product of this nature was found. One product, the Sephara (SecureCell), was identified as a possible alternative, as it has a longer tube (120 – 425 mm) with 3 silicon micropore arrays running up the tube. Unfortunately, according to the company, the pores of these arrays are small (0.2 – 2.2 micron) and only allow for filtration of up to 3.0 mL/min.

In this chapter, two different experiments were run to test the microsparger-based retention system. Initially, using a feed approach matching the existing $\frac{1}{2}$ batch feed volume exchange, the system was able to retain cells and microcarriers without clogging and allowed for perfusion feeding in the 1 L bioreactor. However, in the second experiment, when the perfusion rate was increased beyond the 0.347 mL/min rate in order to maintain glucose at a constant level, clogging was observed, and the culture was not able to be further perfused. This second experiment showed that this system would not be applicable for large scale (10 L) culture as the perfusion rate would need to be 10 times higher just to match the $\frac{1}{2}$ batch feed strategy. A larger pore size system with high filtration surface area may be able to overcome these shortcomings. However, a system of this

design was not found at the time of investigation. Because of this limitation, the microsparger system was abandoned and a new system was investigated in Chapter 3.

CHAPTER 3: CELL AND MICROCARRIER RETENTION VIA SEDIMENTATION USING A SETTLING COLUMN

3.1 Introduction

3.1.1 Background

The microsparger filtration system discussed in Chapter 2 was an inexpensive, simple system for perfusion culture of bioreactors. However, it was not a feasible option for large-scale perfusion due to the pores clogging as the perfusion rate increased. This outcome prompted the investigation into a new system that did not rely on cells and microcarriers being retained via filtration.

In this investigation, a system described by Butler et al. (1983) was found. Butler described the system as a “column separator”, in which the cell-laden microcarriers were pumped into a silicone tube barely submerged in a microcarrier culture. A drawing of the system from Butler et al. (1983) is presented in figure 13. The maximum rate used by researchers in this paper was 1 mL/min, which was higher than what was achievable using the microsparger system. The theory behind this system is presented in section 3.1.2.

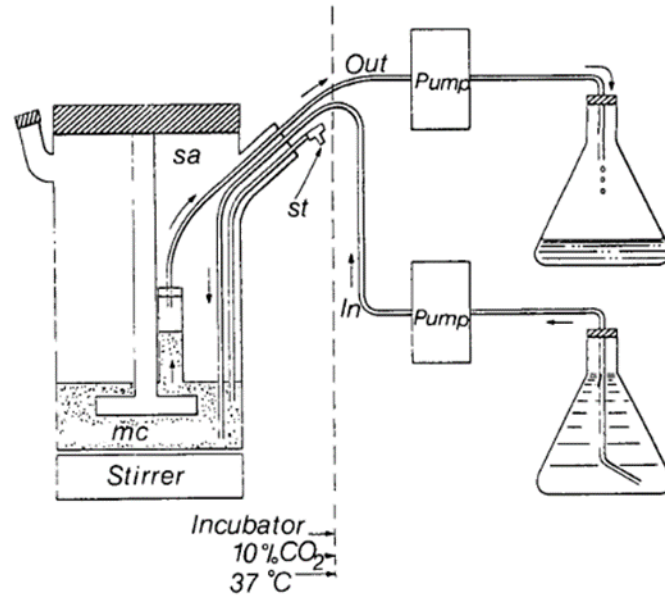


Figure 13: Perfusion set-up for microcarrier culture. sa, separation apparatus; mc, microcarrier culture; out, outflow; in, inflow; st, sampling tube. (Butler et al., 1983)

3.1.2 Settling Column Theory

This system operates by taking two factors into account, particle settling and fluid flow. To achieve appropriate retention of cells on microcarriers, the velocity of the fluid flow from the system must be lower than the settling velocity of the microcarriers.

The velocity at which a particle settles is dependent on three forces. The first is the force of gravity which acts in the downward direction. The second is the force of particle buoyancy which acts in the upward direction. The third is the force of drag which also acts in the upward direction. By taking all these forces into account, Su (2009) states that in a dilute suspension Stokes' law can be used to determine the velocity of the settling particle (v). Equation 3 states Stokes' law where d is the diameter of the particle, μ is the viscosity of the liquid, ρ_s is the density of the particle, ρ is the density of the liquid, and g is the gravitational acceleration.

$$v = \frac{d^2}{18\mu} (\rho_s - \rho)g \quad (3)$$

In this system, media flowing out of the vessel opposes the settling of the microcarrier. This fluid velocity (Q) can be calculated using equation 4, where r is the radius of the tube, h is the height, and t is the time.

$$Q = \frac{\pi r^2 h}{t} \quad (4)$$

Using these two equations the theory behind retention in this system is graphically represented in figure 14.

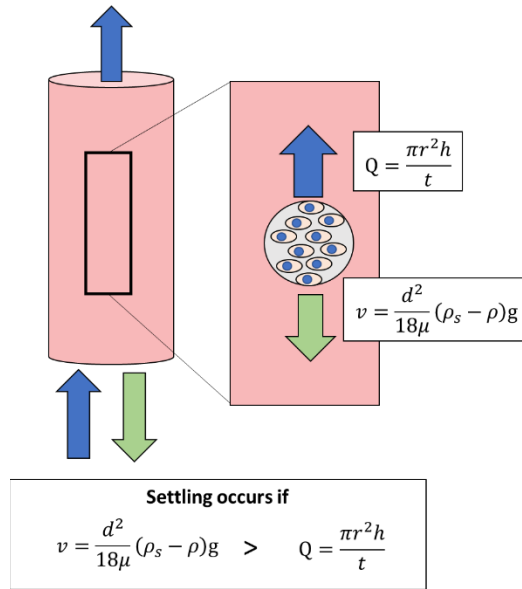


Figure 14: Diagram of theory behind microcarrier retention using the column separator.

3.1.3 Application

While the Butler system worked well for the researchers in a spinner flask culture, there were shortcomings that needed to be addressed before it could be used in the proposed bioreactor cultures. One of these shortcomings is that the size of the port on the bioreactor would restrict the maximum diameter of the column. Another shortcoming was that the originally designed system

connected the settling column to the external pump using small bore silicone tubing. This design would make the system non-rigid and difficult to aseptically insert into the bioreactor as well as stay vertical in the flow from the agitation. To address these shortcomings, a decanter column (Eppendorf) was used. It featured an external column separator system that was made of rigid material (stainless steel and glass). While this system is much more expensive than the microsparger (approx. \$4,400), it is less expensive than other systems, such as the previously mentioned ATF, and does not come with repeated consumable cost. Figure 15 depicts a schematic of this system, which will be referred to as the “settling column”.

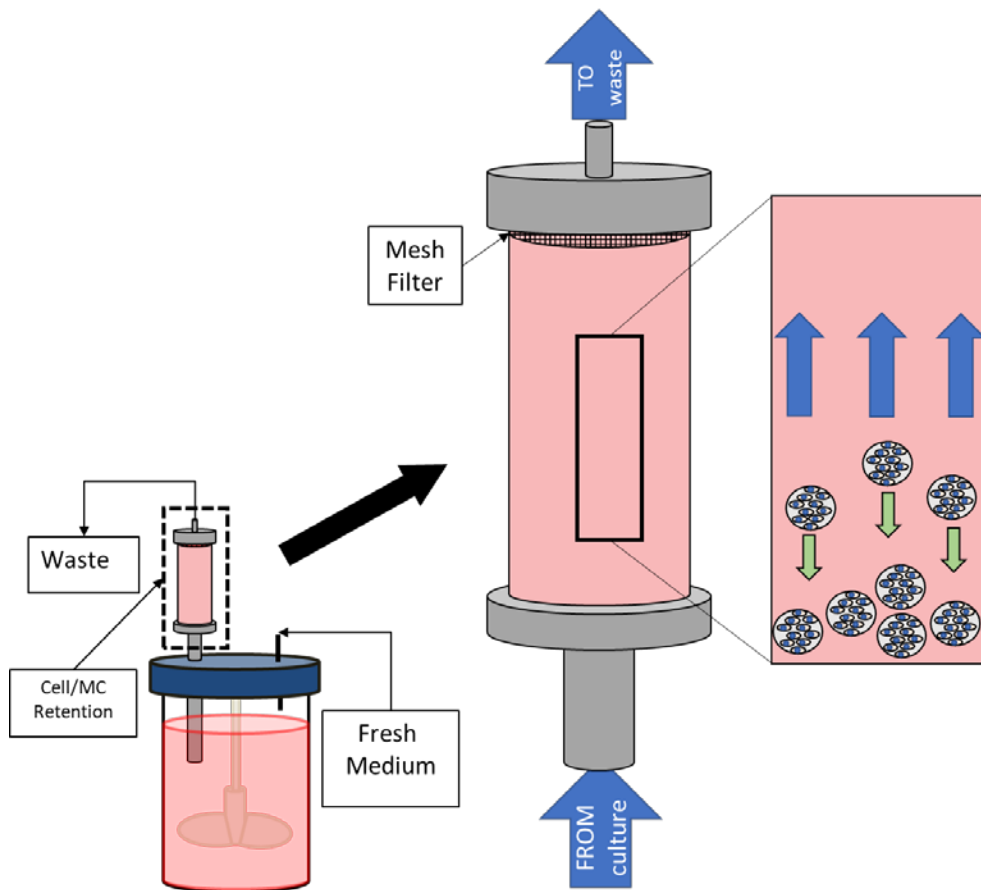


Figure 15: Schematic of the settling column principle.

This settling column was evaluated mathematically using equations 3 and 4 to determine the maximum flow rate. Using the microcarrier property values provided by the manufacturer and (listed in table 2) and assuming the media properties were similar enough to water, the settling velocity of the microcarrier was calculated to be 6.76 cm/min. Using this value as the maximum velocity (or height/time) and the settling column property values in table 2, the maximum flow rate was calculated at 26.5 mL/min. This value was over seven times higher than the anticipated flow rate of 3.47 mL/min and therefore could be used in this application.

Table 2: Property values of the microcarrier and settling column.

Property	Value
Microcarrier Diameter (average)	0.0175 cm
Microcarrier Density	1.04 g/cm ³
Settling Column Diameter	3.2 cm

In this chapter, testing of the settling column in three 10 L bioreactor cultures is presented. These cultures were compared to control cultures where half-batch feeding was used. Cell yield, cell phenotype, cell growth profile, nutrient concentrations, and in-process culture data is compared between the two feeding regimes. Overall, the perfusion system was evaluated on its ability to provide continuous media outflow, retain cells and microcarriers, and provide similar or improved results compared to the half-batch fed cultures.

3.2 Methods

3.2.1 Culture Set-up

Due to lack of availability of the microcarriers used in Chapter 2, the microcarrier type used in this chapter was changed to a different microcarrier with similar properties. 20g of microcarriers were hydrated and sterilized according to manufacturer's instructions.

Prior to the start of culture, the settling column was assembled by attaching 0.125" ID tubing to the barbed fitting on the top of the system. The terminus of the tubing set allowed for future connection of the system to a single-use bag for collection. This entire set-up was sterilized in an autoclave.

A BioBLU 10c with macrosparger (Eppendorf) was set-up in a biosafety cabinet by inserting the settling column in one of the available spare ports. 2 x 20 L single-use bags (ThermoFisher) were connected to each other and then connected to the end of the settling column line. This vessel was connected to the Hyperforma G3 Lab Universal controller (ThermoFisher) for monitoring and controlling dissolved oxygen, temperature, pH (using non-invasive probes) and agitation. When applicable, a control bioreactor was set-up in a similar fashion without the settling column.

The previously hydrated and autoclaved microcarriers were rinsed with DPBS. This DPBS was then exchanged for 4.5 L of commercially available vascular growth medium (prepared as previously described in section 2.1.1.1). This microcarrier and media mixture was transferred to each bioreactor. The media and microcarriers were equilibrated for 2-4 hours by heating media to 37°C, overlaying air at 1 SLPM, and constant agitation.

3.2.2 In-Process Counting

Throughout the culture, microcarriers were sampled and the cells were counted. In order to count, microcarriers were removed from the culture via the sample port and added to a tube. These microcarriers were allowed to settle and the supernatant was removed until 1 mL of microcarriers in media remained. This supernatant was put into a separate tube. The 1 mL sample of microcarriers was mixed 1:1: with Reagent A100 and Reagent B (Chemometec), which lysed the cells and stabilized the nuclei. The nuclei were counted using either a Nucleocounter NC-200 or NC-202 (Chemometec) using a manufacturer-developed program. This program counts the number of nuclei as “total” cells and any nuclei in non-lysed sample (the supernatant in this case) as “dead” cells. The number of live cells is determined as the difference of total cells and dead cells while the viability is the quotient of live cells over total cells.

3.2.3 Culture

After equilibration, previously expanded primary human vascular cells were thawed in a 37°C water bath for 2.5 minutes. The cells were resuspended in media and counted using either a Nucleocounter NC-200 or NC-202. Cells were added to 500 mL of media for each bioreactor being seeded. Prior to seeding, a baseline metabolic sample was taken, as previously described. The cell solution was pumped into each bioreactor. A “seeding” program that was previously set-up on the controller was turned on. This program instructed the system to turn agitation on and off at certain time intervals for a determined number of cycles. After these cycles were completed, the agitation stayed at a constant agitation rate.

On culture day 2, a metabolic sample was taken as previously described. 5 L of media was added to each bioreactor to bring the culture to its final volume of 10 L. A 33 mL sample of microcarriers and media was taken via the sample port. 2 x 15 mL samples were used for in-process counting. The remaining 3 mL were used for visual inspection via microscopy. After allowing the system to reach a temperature of 37 ± 0.5 °C a final metabolic sample was taken.

On culture days 3 – 4 metabolic samples, in-process counts of 2 x 5 mL microcarrier samples, and phase images of microcarriers were taken for each condition as previously described.

On culture days 5 – 8 metabolic samples, in-process counts of 2 x 5 mL microcarrier samples, and phase images of microcarriers were taken for each condition as previously described. The control (half-batch) condition was fed by turning off the agitation, tilting the vessel, and allowing the microcarriers to settle away from the “harvest” line. 5 L of media was removed by pumping out into an aseptically connected waste bag. A bag containing 5 mL of fresh media was welded onto the vessel and pumped in. Agitation was restarted and after allowing the system to reach a temperature of 37 ± 0.5 °C, a final metabolic sample was taken. Perfusion was started in the perfusion condition starting on day 5.

3.2.4 Perfusion Set-up and Operation

The perfusion system was set up by welding on a bottle containing 10 L of media to the inlet line of the bioreactor. This 0.125” ID tubing line (“feed line”) was inserted into “pump 1” of the bioreactor controller. The tubing of the settling column (“waste line”) was inserted into “pump 2” of the bioreactor controller. A schematic of the set-up is shown in figure 16. These bioreactor

pumps were previously calibrated according to the manufacturer's instructions and using the calibration prompts in the software.

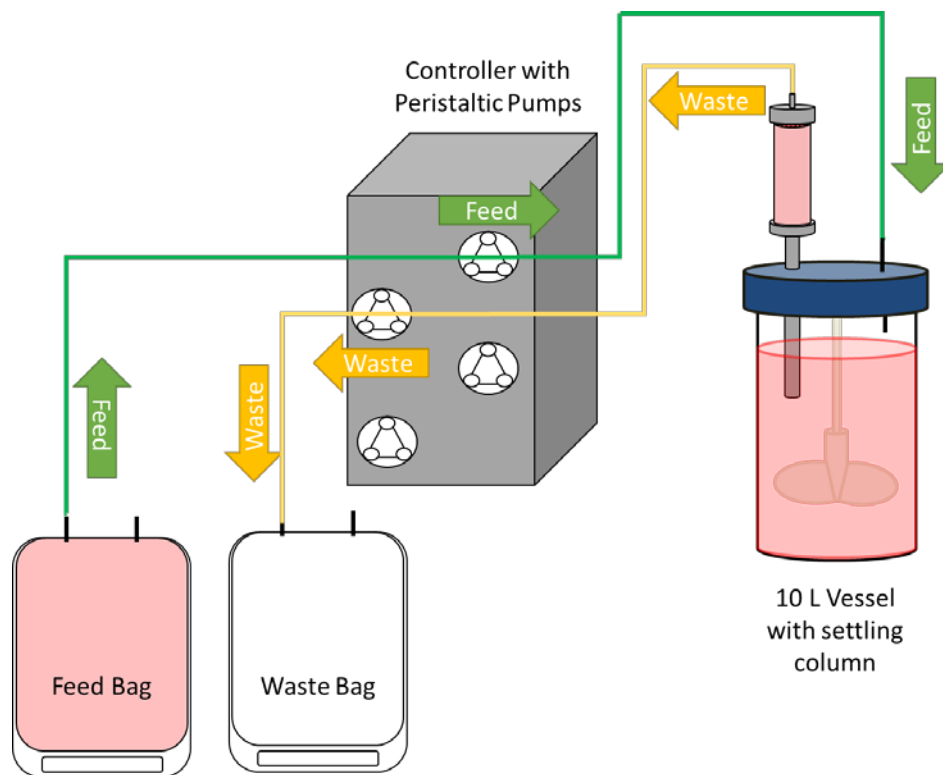


Figure 16: Schematic of the settling column perfusion system.

Perfusion was initiated by setting pump 1 and pump 2 setpoints to 3.47 mL/min (0.5 VVD). This rate was chosen to match the control media exchange rate of 5 L every 24 hours. This rate was also found to be less than settling rate of the microcarriers in the column using a mock test of microcarriers in water. The perfusion was set to run from culture day 5 to culture day 9 and stopped prior to harvest. The system was set to pump fresh media into the headspace of the bioreactor and pump out media from bioreactor through the settling column.

3.2.5 Harvest

On culture day 9, a 13 mL sample of microcarriers and media was taken via the sample port. 2 x 5 mL samples were used for in-process counting. The remaining 3 mL were used for visual inspection via microscopy. A final metabolic sample was taken just prior to harvest.

The harvest of the cells was the same in both the perfusion and control conditions. The harvest was performed by turning off the agitation, tilting the vessel, and allowing the microcarriers to settle away from the “harvest” line. Most of the media was pumped out and replaced with 5 L of DPBS. After rinsing the cells on microcarriers, most of the DPBS rinse was pumped out and replaced with pre-warmed TrypLE (Gibco). The vessels were agitated for a set amount of time. During this period, the “harvest” line was connected to a Harvestainer bioprocess container (ThermoFisher). This system consists of a 90-micron mesh inside a bag and allows for flow through separation of microcarriers from cells. The cell-microcarrier solution was pumped through the Harvestainer and into a collection bag. Each vessel was rinsed by pumping in a quench solution and swirling. This rinse was pumped through the Harvestainer and into the collection bag with the cell solution. The collection bag was sealed off from the vessel. The cell solution from each vessel was transferred to centrifuge tubes in a BSC and centrifuged. After centrifugation, the supernatant was aspirated, and the cell pellet was resuspended in cryopreservation reagent. A count was taken of the cell solution before transferring the cells cryobags and cryovials. These cells were cryopreserved using a control rate freezer and stored in a liquid nitrogen tank.

3.2.6 Cell Analysis

Cells were analyzed according to the process outlined in section 2.2.1.6. Identity testing was performed by flow cytometry. Cells were centrifuged, supernatant was removed, and cells were resuspended in DPBS. Cells were exposed to fluorophore conjugated primary antibodies for 30 minutes and run on a FACSVerse (Becton Dickinson) flow cytometer the results were analyzed via FCSEXPRESS (De Novo Software) and cells were assessed for positivity for CD31 and CD144 and negativity for CD140b and CD90.

Cell function was assessed using two different tests. The first was performed by plating cells onto Matrigel (Corning) and qualitatively evaluating tube formation after four hours. The second was performed by plating cells in a 6-well plate for 2 days, adding a fluorescently labeled acetylated low-density-lipoprotein for four hours, and harvesting the cells from the plate. These harvested cells are analyzed via flow cytometry to assess uptake of the molecule.

Attachment of cells after cryopreservation was also assessed. This was done by plating cells in a collagen-coated 24 well plate. After 24 hours, the cells were fixed for 10-15 minutes using 4% PFA. After removing the fixative, Hoechst (ThermoFisher) was applied for 10 minutes. The plate was run on the Celigo (Nexcelom) using a nuclear counting program. This program calculates the number of nuclei in each well of the plate. The attachment efficiency was calculated as the percentage of nuclei (cells) on the plate vs the number of seeded cells.

3.2.7 Culture Data Handling and Analysis

During the culture, online data was continuously collected by the TruBio control platform (ThermoFisher). This data was stored locally and extracted into an excel file after each run. These excel files were manually cleaned to remove extraneous data points that either did not apply to the culture or were taken before the culture was seeded. This data was visualized and compared using Tableau Desktop (Tableau).

Where applicable, statistical analysis of results was performed using Prism 9 software (GraphPad). This analysis was performed using the software's internal Student's t-test or Welch's T test functions. The p-value threshold for significance was set to < 0.05 . Any significant or non-significance of data is indicated in the figure caption. If no significance is indicated, there was no analysis performed.

3.3 Results and Discussion

3.3.1 Growth Comparison

As previously stated, samples of microcarriers were taken throughout the culture and were visually inspected to assess coverage and cell morphology. Phase images were taken pre-harvest and are presented in figure 17. While run-to-run variability in coverage was observed, there was no observable difference between perfusion and half-batch feeding. Cells were observed to have cobblestone morphology, which is typical of vascular cells.

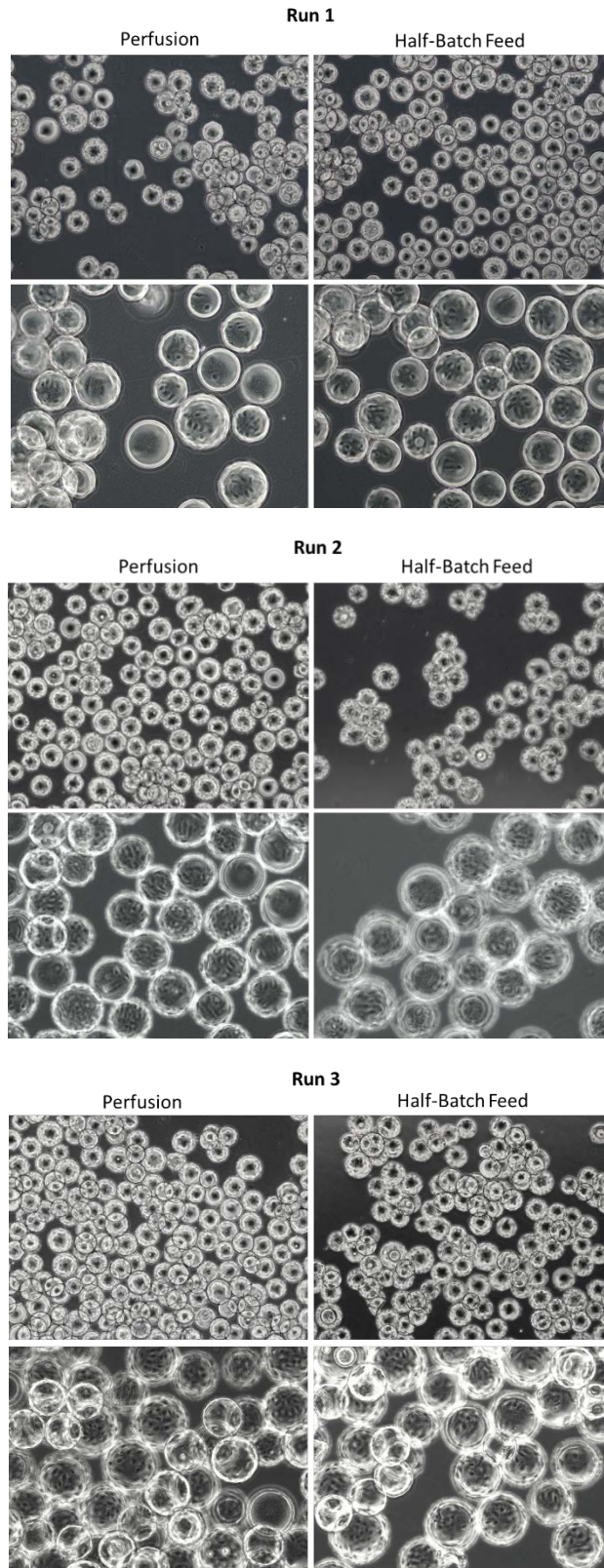


Figure 17: Pre-harvest phase images of cells on microcarrier. Top images are 5x magnification, bottom images are 10x magnification.

In-process counting using the Nuclecounter was used to assess cell growth during the culture, as previously described. The data from each run is displayed separately in figure 18. Overall, the general trend that was observed in these counts is that the perfusion cultures had faster or equal growth to that of the half-batch feed condition after feeding was initiated at culture day 5.

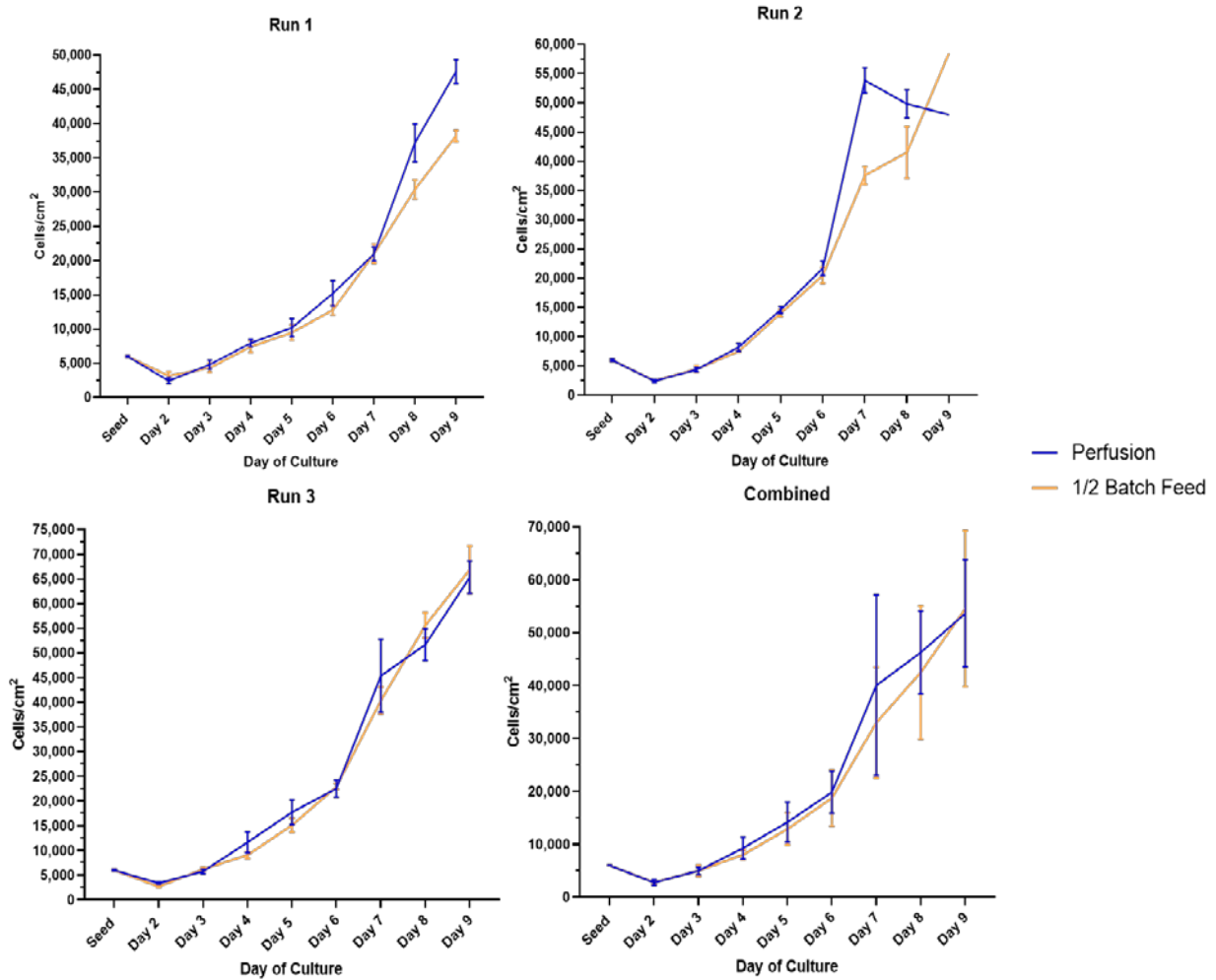


Figure 18: Graphs of in-process counts. Individual runs are displayed with the error bars representing N=4 counts. The combined graph shows error bars representing the N=3 runs.

During the harvest of the cultures, cell number and viability was assessed. The data from the three runs were compiled into graphs presented in figure 19. It is important to note that the cell density

for half-batch feed only includes two of the three runs because following the harvest, run 2 was concentrated using a different method than the other cultures. This downstream change caused a loss of cells and therefore was excluded from the dataset as to avoid skewing the results. Overall, the perfusion cultures yielded a slightly higher number of viable cells with less variability than that of the half-batch feed cultures. However, these differences were not found to be significantly different. While this result does not match the increased cell yield described in the literature, it does make sense as the media (nutrient) supply rate is not different between the two methods.

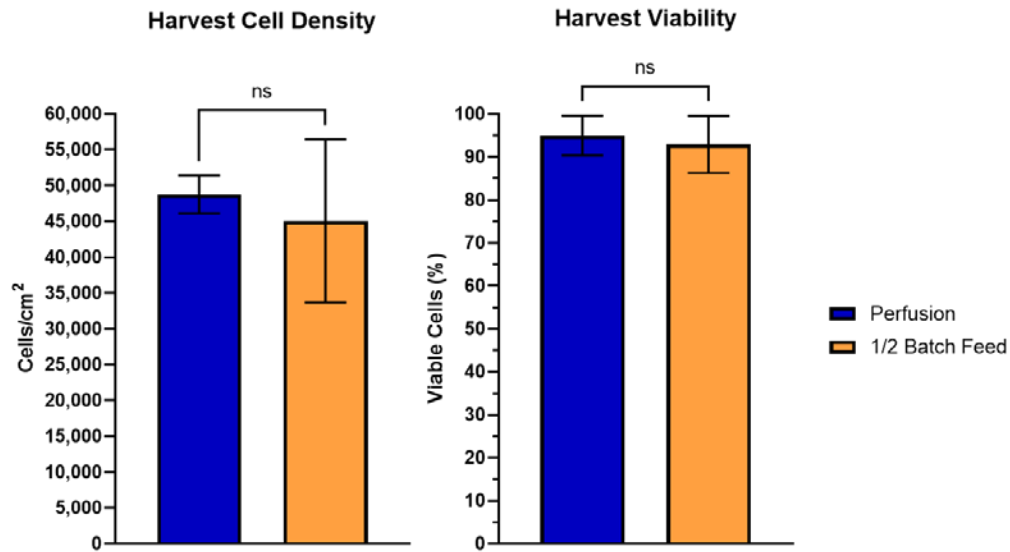


Figure 19: Graphs of harvest metric comparison between the two conditions. Error bars represent standard deviation between N=3 cultures for perfusion and N=2 cultures for ½ batch feed. “ns” indicates the differences are not significant ($p = 0.60$ and $p = 0.22$) according to Welch’s t test.

Finally, the two previously described growth metrics, fold increase and PDT, were calculated for the three runs. These metrics were compared and graphed in figure 20. As with the previously stated results, it appears that perfusion more consistently grew faster than the half-batch fed culture however these findings are not significant.

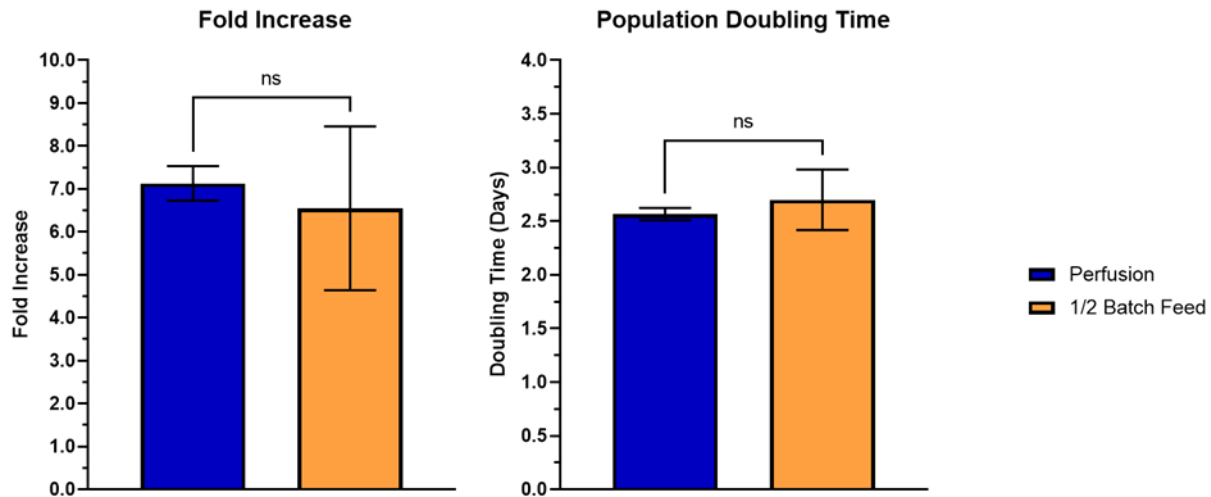


Figure 20: Graphs of the growth metric comparison between the two conditions. Error bars represent standard deviation between N=3 cultures for perfusion and N=2 cultures for ½ batch feed. “ns” indicates the differences are not significant according to Welch’s t test.

3.3.2 Culture Condition Consistency

As previously stated, one of the advantages of perfusion is imparting consistency into the culture conditions. This culture consistency was observed in the metabolic profile of the runs. The glucose and lactate values of each run are presented in figure 21, with the amount of glucose being consumed per day presented in figure 22. This consumption data was calculated on the assumption that the media leaving the system had the same metabolite levels as the media inside of the reactor as metabolic samples of the outflow were not taken. Glutamine and ammonium values were also graphed and are presented in figure 23. As was seen using the microsparger system, the metabolite values were more consistent in the perfusion process as compared to the half-batch feed. However, the concentration of these metabolites did change over time in the perfusion culture which is an area for improvement that will be discussed in chapter 4. The total amount of glucose consumed during the culture trended higher for perfusion feeding compared to half batch feeding (9.94 ± 0.98 vs 9.40 ± 1.16), however this difference was not found to be significant.

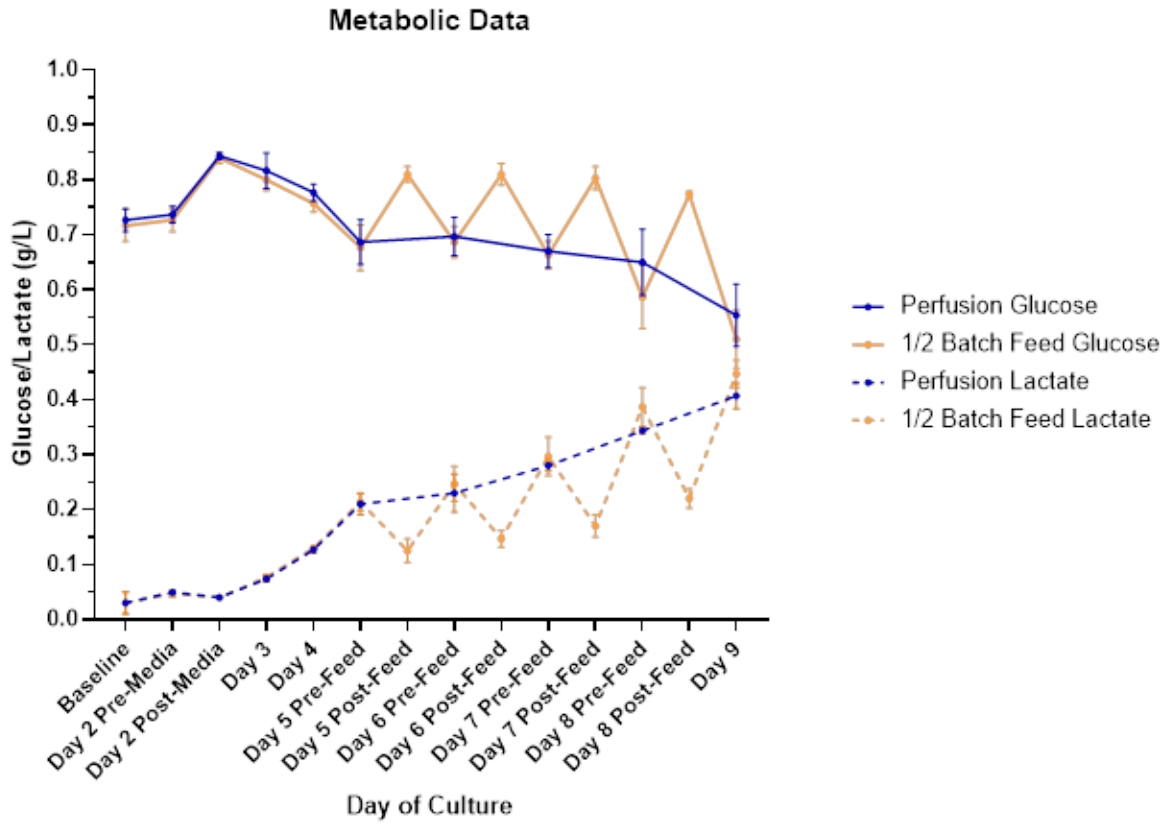


Figure 21: Graph of glucose and lactate profiles for each condition. The error bars represent the standard deviation between N = 3 cultures.



Figure 22: Graph of amount of glucose consumed per culture day. The error bars represent the standard deviation between N = 3 cultures.

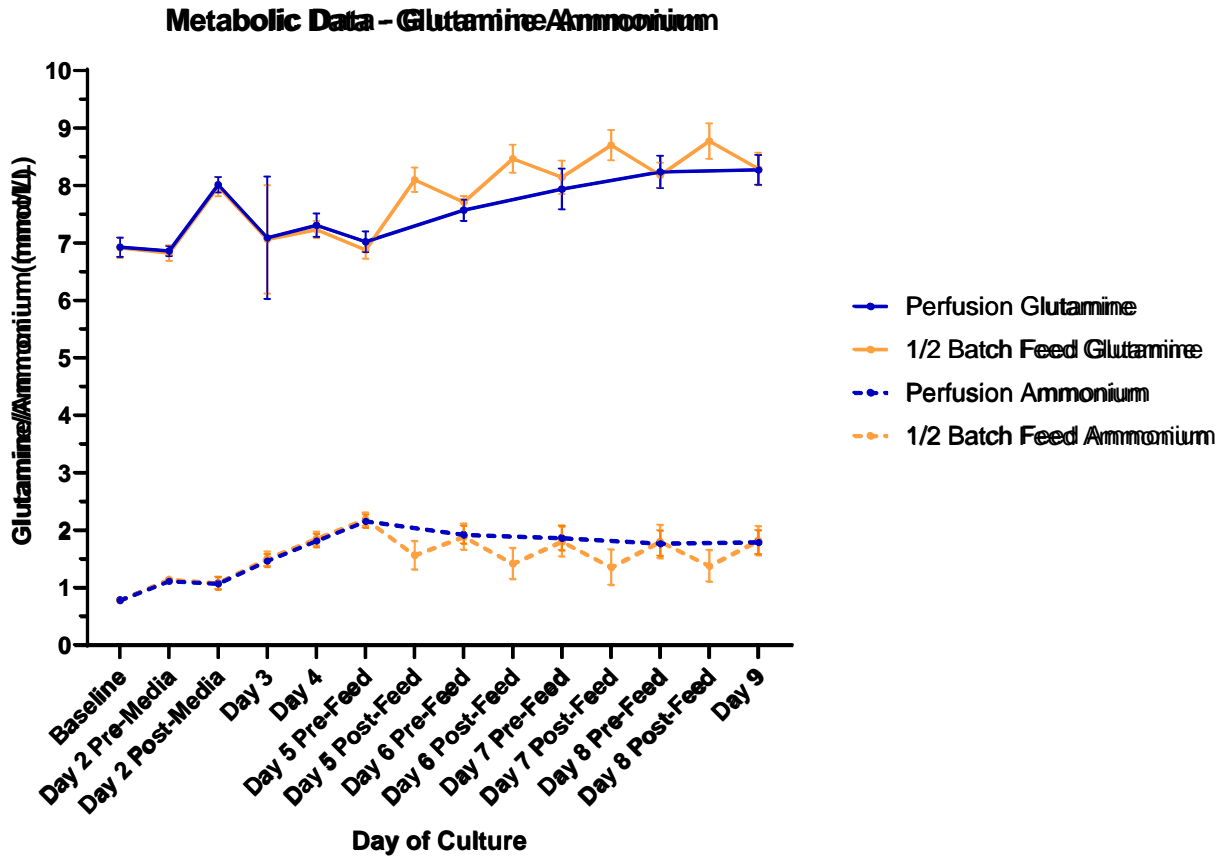
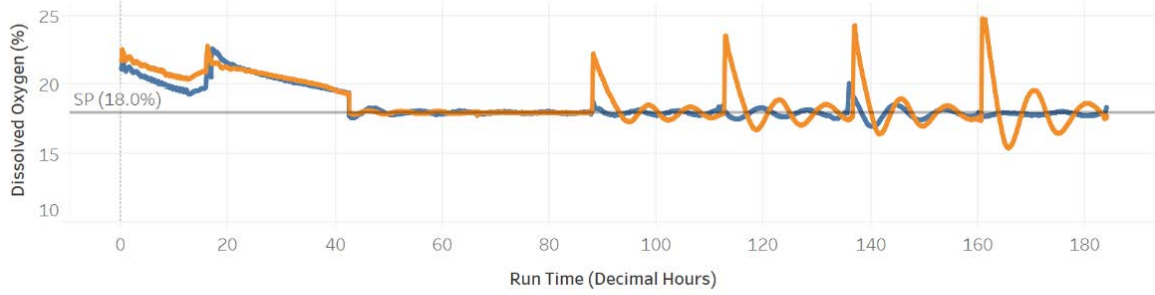


Figure 23: Graph of glutamine and ammonium profiles for each condition. The error bars represent the standard deviation between N = 3 cultures.

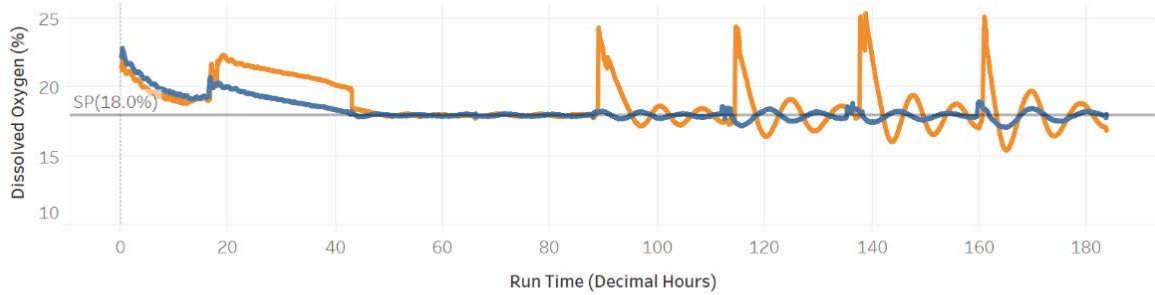
Culture consistency was also measured by the probes present in the culture bioreactor. The dissolved oxygen, pH, and temperature profiles of the conditions in each run are displayed in figures 24, 25, and 26 (respectively). It appeared that perfusion feeding led to better control of each parameter compared to 1/2 batch feeding. To further assess this, the average deviation from the setpoint was measured for each run. This was calculated by isolating the values from the feed period of each run and averaging the difference of the value and the setpoint. These average deviations were combined and compared in figure 27. There was a significant decrease ($p = 0.011$) in dissolved oxygen deviation when feeding via perfusion compared to half batch feeding. The

average deviations in both pH and temperature in perfusion feeding were not found to be statistically significant ($p = 0.25$ and $p = 0.14$, respectively).

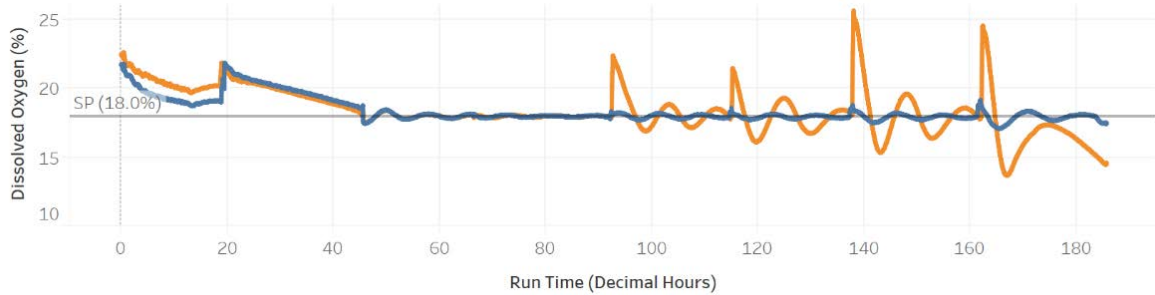
Run 1 - DO



Run 2 - DO



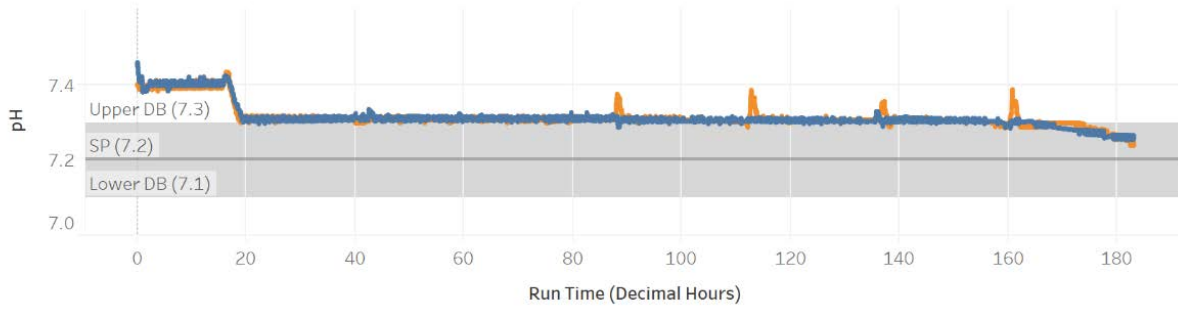
Run 3 - DO



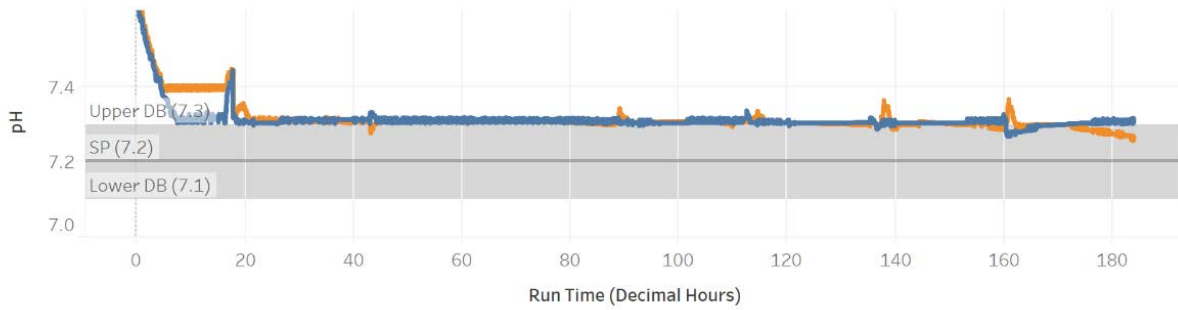
Key
■ 1/2 Batch Feed DO
■ Perfusion DO

Figure 24: Dissolved oxygen profiles for the three runs of both conditions.

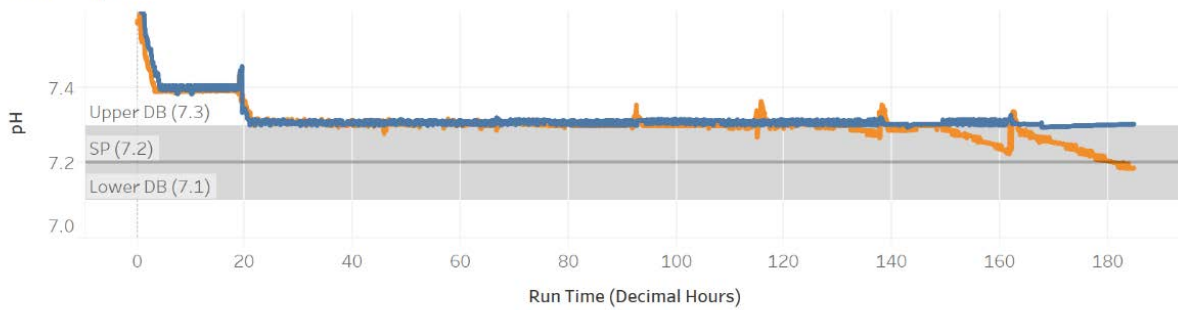
Run 1 - pH



Run 2 - pH



Run 3 - pH

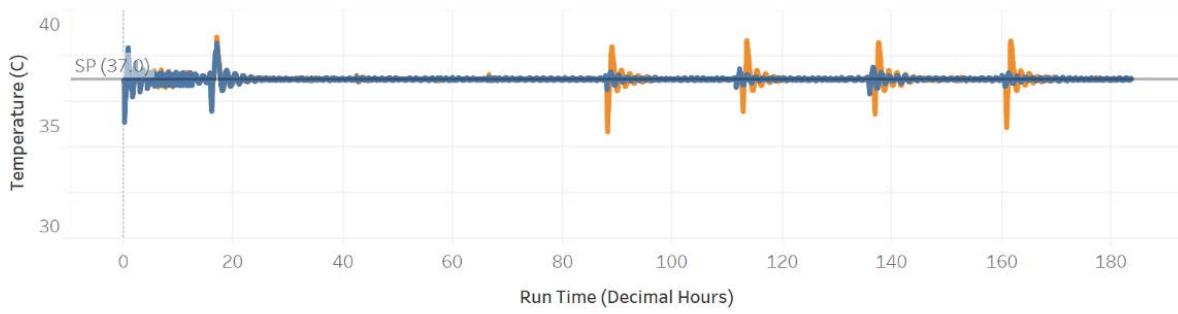


Measure Names

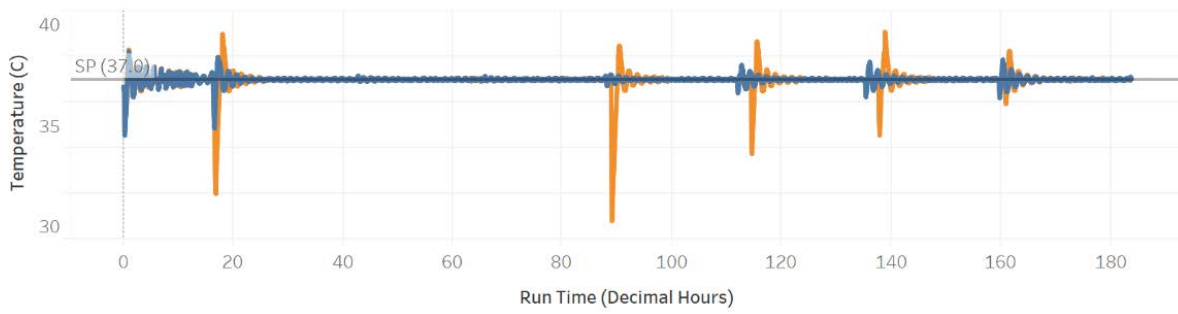
- Perfusion pH
- 1/2 Batch Feed pH

Figure 25: pH profiles for the three runs of both conditions.

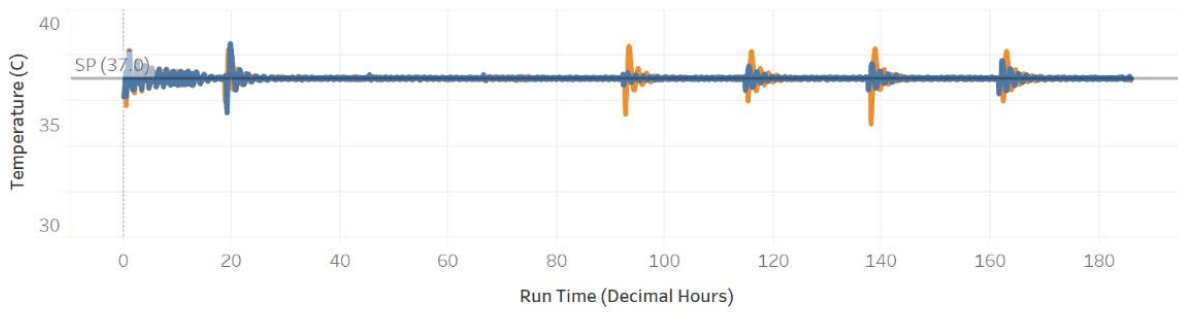
Run 1 - Temperature



Run 2 - Temperature



Run 3 - Temperature



Measure Names
■ Perfusion Temp
■ 1/2 Batch Feed Temp

Figure 26: Temperature profiles for the three runs of both conditions.

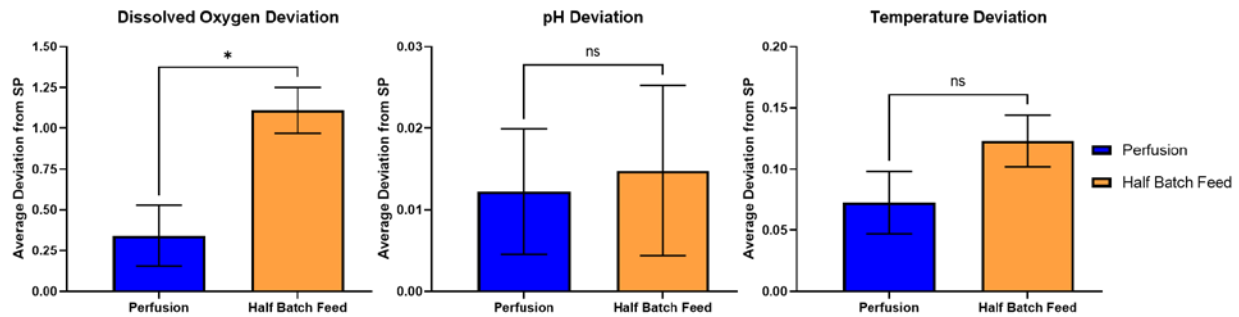


Figure 27: Graphs of DO, pH, and temperature average deviation for setpoint. Error bars represent standard deviation between N=3 cultures for perfusion and N=3 cultures for half batch feed. “*” indicates the differences are significant, whereas “ns” indicates the differences are not significant according to Welch’s t test.

3.3.3 Cell Analytics

After cryopreservation, the cells were thawed, counted, and assessed for identity and function metrics. These quality control metrics are graphed in figure 28. The green boxes in this graph indicate the acceptable ranges for these metrics. Due to acquisition issues, cell phenotype evaluation (CD31, CD144, CD140b, and CD90) was not performed on the first experiment. Phase images of the tube formation assay were also acquired and are presented in figure 29. There were no significant differences between the perfusion and half-batch fed cultures on any of the quality control metrics measured. This indicates that perfusion feeding does not have any negative effects on the phenotype or function of the cells being cultured as these metrics were in their acceptable ranges.

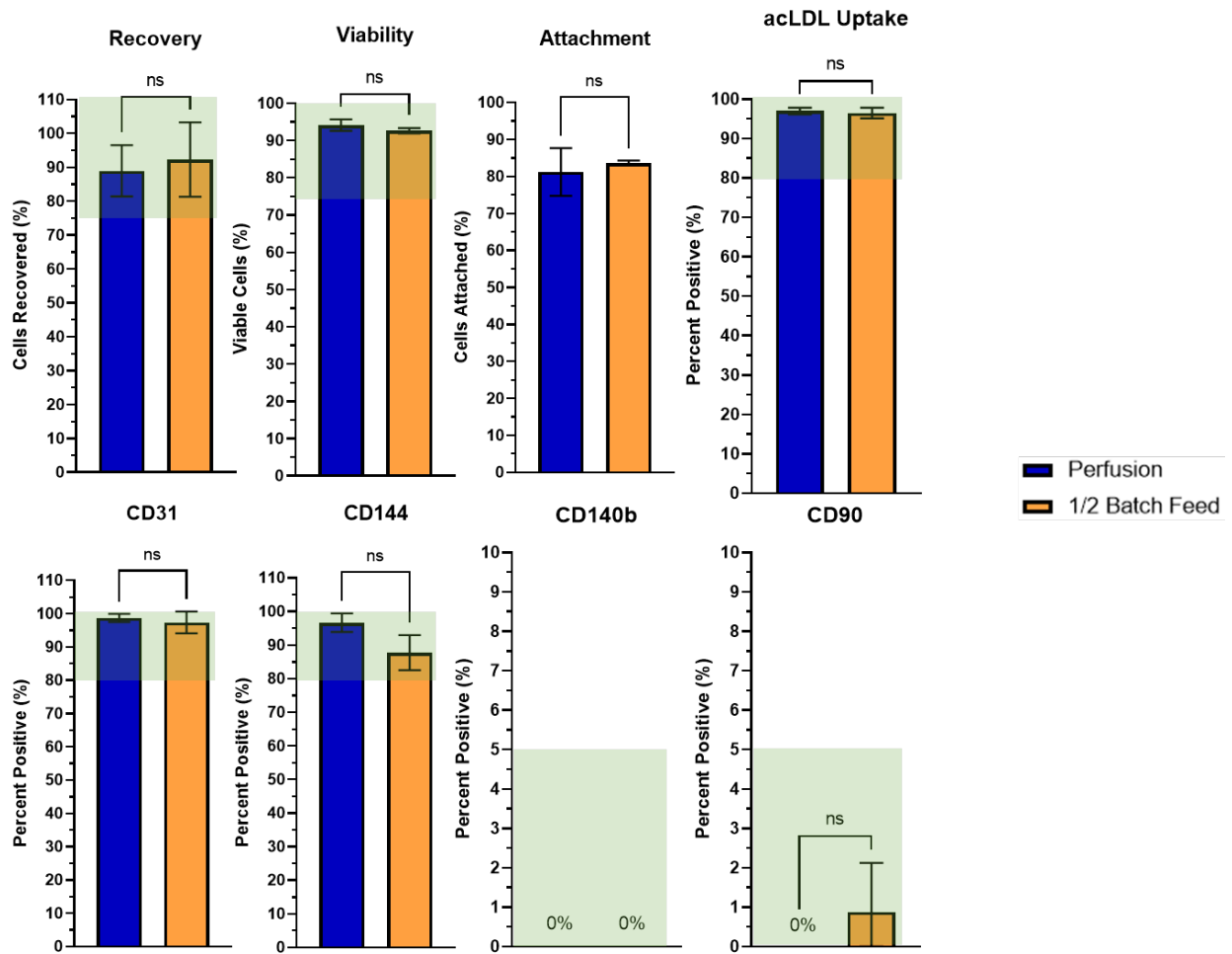


Figure 28: Graphs of quality control metrics comparison between conditions. Error bars represent standard deviation between N=3 cultures for top row metrics and N=2 cultures the bottom row of metrics. “ns” indicates the differences are not significant according to Student’s t test.

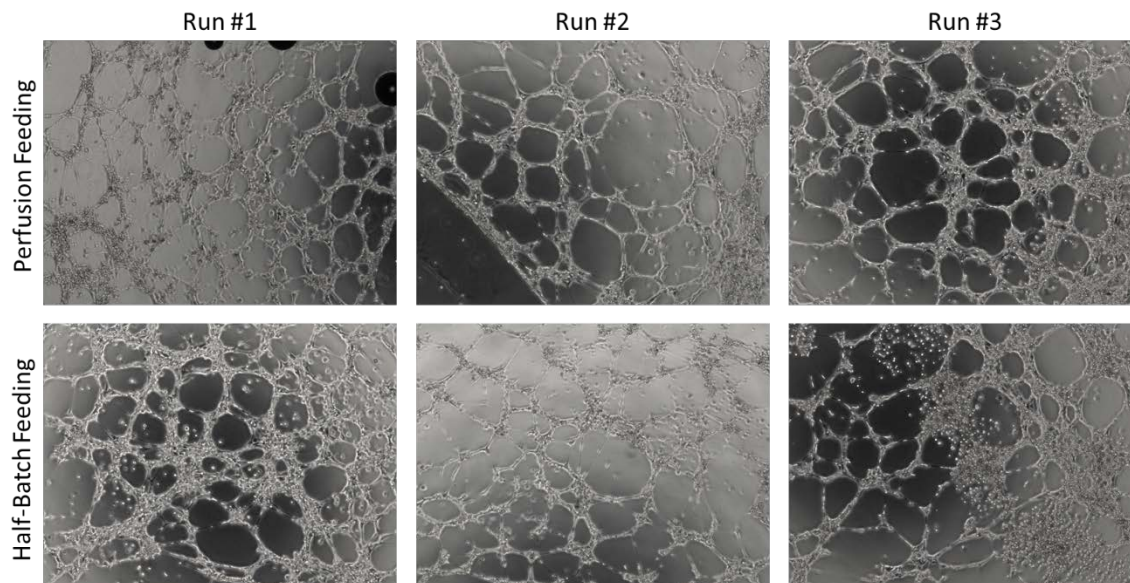


Figure 29: Phase images of tube formation assay results taken at 5x magnification.

In this Chapter, a sedimentation style retention system was presented. This system, referred to as the “settling column,” retains microcarriers with cells by pumping media out of the bioreactor at a fluid velocity lower than that of the microcarrier settling velocity. This system was able to retain cell-laden microcarriers while providing continuous media outflow for four days. Perfusion feeding of these cultures imparted improved control and consistency of in-process parameters, such as pH, dissolved oxygen, and temperature. Nutrient levels were also more controlled, but not maintained, in perfusion feeding when compared to half-batch feeding. Overall, cell yield did appear to be slightly higher in the cultures that were fed by perfusion. However, the difference observed was not statistically significant. Finally, perfusion feeding did not impart any negative effects on the function or identity of the cells produced. Overall, this system showed feasibility for perfusion feeding of cells on microcarriers.

CHAPTER 4: CONCLUSIONS AND FUTURE INVESTIGATIONS

4.1 Conclusions

The objective of this work was to develop a system that allowed for perfusion feeding of a microcarrier culture. This perfusion system needed to work with a single-use bioreactor while being simple to operate and inexpensive to adopt. In this thesis, filtration-based and sedimentation-based systems were tested. Both systems fit the established requirements but only the sedimentation-based system was able to perfuse without clogging.

4.1.1 System Decision

In Chapter 2, an inexpensive microsparger based filtration system was evaluated. While this system successfully retained cells and microcarriers in the bioreactor at a low perfusion rate, increasing the perfusion rate clogged the pores in the filter. This clogging caused the culture volume to increase well beyond the culture volume, which resulted in culture termination. The perfusion rate that led to the clogging was much lower than what would be required for large-scale (> 10L) cultures, rendering the system not feasible for future use.

The second perfusion system tested was an external settling column. This system successfully separated microcarriers from the perfusion outflow by enabling the microcarriers to settle back into the culture. This system showed no signs of clogging (such as the culture volume increasing) or loss of microcarriers and cells. While the capital investment is over 20 times higher for this system compared to the microsparger system, it is at least half of the cost of the commonly used

ATF system and requires no repeated consumable cost. because of these findings, the external settling column was established as the preferred method for perfusion feeding.

4.1.2 Summary of Advantages

Three 10 L perfusion runs were performed alongside three half-batch feed control runs. The perfusion culture demonstrated improved control of in-process parameters, such as pH, dissolved oxygen, and temperature, compared to the control half-batch feed strategy. The perfusion cultures also had more consistent nutrient profiles than that of the half-batch feed cultures. While the perfusion cultures did not yield a significant increase in cell number compared to the half-batch feed strategy, they showed more consistent number of cells produced between runs. The run-to-run consistency imparted by perfusion feeding would allow for a more controlled and reliable production process.

4.2 Future Investigations

4.2.1 Evaluation Metric Improvement

As previously stated, this work shows that perfusion feeding produces comparable results to half batch feeding. One future direction is to improve on evaluation metrics to gain a better understanding of the cells being produced. By incorporating new metrics into the evaluation, a more detailed assessment of perfusion can be provided.

While this work described viability as well as metabolic activity, more in-depth cell health metrics can be investigated. By investigating that amount of stress-related secreted factors such as reactive oxygen species (Karlsson and Möller, 2011) and lactate dehydrogenase (Legrand et al., 1992) a

more complete picture of cell health can be assessed using commercially available kits. Another avenue of assessing the effects of perfusion feeding on cells would be to look into mitochondrial function and health using the Seahorse system (Agilent). The vascular cells discussed in this work were assessed for common phenotype markers and functional tests. Other functional tests, such as assessments of barrier function via a FITC-dextran assay and response to shear forces using channel slides, may be used to identify functional improvements imparted by perfusion feeding.

4.2.2 System Improvements

The perfusion system described in this thesis showed promise for use in microcarrier-based cell production. Although, implementation of the system in its current state would be feasible, there are potential improvements that could be made. These improvements would focus on decreasing the culture cost and labor, increasing the culture yield, and making a single use system.

4.2.2.1 Dynamic Perfusion Rate

The first major system improvement would focus on developing a dynamic perfusion rate system. The perfusion cultures previously described were run at a specific, static rate (0.5 VVD) the entire culture. While this was shown to maintain nutrient (glucose and lactate) levels for the first day, glucose levels decreased, and lactate levels increased on subsequent days. Dynamic perfusion would allow for the perfusion rate to change based on set criteria. Some key variables to consider for rate determination would be nutrient changes (such as glucose consumption rates), capacitance, and nutrient level limits. The potential advantages of dynamic perfusion would be improved culture yield and decreased media usage, ultimately driving the cost of each culture down.

4.2.2.2 Automation

After developing a dynamic perfusion system, the next improvement would be to automate the perfusion system. The automated system would change the perfusion rate based on feedback from inline sensors. This would allow for real-time optimized nutrient delivery. Three major components would be needed for this automation. First, an inline nutrient sensor, such as the CITSense MeMo (C-Cit Sensors), would need to be integrated into the system to enable continuous nutrient (glucose and lactate) concentration readout. Second, a scale will need to be integrated into the system to determine the total amount of nutrients present based on culture weight. Scale integration would also ensure the vessel weight and culture volume is appropriately maintained. Finally, a feedback loop would need to be programmed into the system to determine the specific perfusion rate setpoint based on the two items previously listed. Automation of the system would decrease the labor required during the culture as well as decrease the cost of production, as previously noted.

4.2.2.3 Settling Column Redesign

Another potential improvement would be to redesign the settling column. This redesign would serve multiple purposes. While the current settling column is commercially available, it is expensive. By redesigning the system to only be made of cell culture safe plastic, the settling columns could be produced at a lower cost. Because of the lower cost and material change, the system could be single-use and discarded along with the culture vessel. Finally, by redesigning the system to have a larger column diameter and/or height, higher perfusion rates could be achieved. The higher perfusion rates would be needed for high microcarrier concentration or high-volume cultures, both of which would increase the number of cells produced by each run.

This thesis has shown the feasibility of a sedimentation-based system that allows for perfusion culture feeding of cells on microcarriers in a single-use bioreactor system.

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