A Packed-Bed Bioreactor System for Enhancing Vero Cell Growth in a Semi-Continuous Mode of Operation

A Major Qualifying Project Report

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ABSTRACT

The utilization of Vero cells for vaccine production has contributed significantly to controlling viral diseases and improving the healthcare system in the US and around the world. In the United States, the adherent cell line Vero 76 has never been grown within a packed-bed bioreactor using Eppendorf's BioFlo 320 system, however they are typically grown on microcarrier beads in a pitched-blade vessel. In this investigation, I compare a glass vessel to a BioBLU® 5p single use vessel running in a semi-continuous mode of operation while taking daily glucose uptake and lactate production samples to track growth. The objective of this study was to determine if a packed-bed bioreactor can be seeded at a lower than typical density to ultimately decreases time, cost, and human risk during the seed train and scale-up process.

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INTRODUCTION

Infectious diseases, both bacterial and viral, represent a major global health burden. Vaccines are not only useful as a health precaution, but also have been proven vital in the fight against illness and the eradication of such diseases. Millions of people are vaccinated each year in the United States alone, even more across the globe (Williams et al., 2015). For example, the Center for Disease Control and Prevention (CDC) estimates that 171 to 179 million doses of Flu vaccine are produced each year (Seasonal Influenza Vaccine, 2016). A vaccine, in general, is an injection administered to a patient, oftentimes an inactivated form of a virus. The vaccine is intended to generate an immune response from the body, without danger of infection. Once the body develops antibodies against this virus in a safe and controlled way, it will be able to fight off the virus if it ever is introduced to the body again (Hussein et al., 2015).

Vaccine Production Systems

Vaccines are typically produced in chicken eggs or cell culture based systems (How Influenza Vaccines are Made, 2015). Cell culture is a major mode of production with both mammalian and insect systems have been utilized as factories for growing and producing the vaccine (Rappuoli, 2006). Given the global impact and demand for vaccines, cell culture techniques and growth are important steps in the production pipeline, both from a health and economic perspective. Thus, it is vital to be constantly improving current and developing new technologies to increase vaccine production in most efficient ways possible with respect to time and cost.

Vaccine production in cell culture based systems involves a number of basic steps including, cell growth, viral infection/amplification, and subsequent purification. Starting with a

single cell line cells are amplified through a seed train to prepare them for inoculation into a large-scale vessel called a bioreactor for high volume growth. Through this upstream process, the cells are scaled up to help create and propagate large quantities of vaccine.

When choosing how vaccine will be produced using cell-based systems, it is important to consider the types of cells available. As indicated earlier, both mammalian and insect cells are options. In addition, cells typically vary in being either adherent or non-adherent; meaning they grow by attaching to surface or by free-floating in media, respectively. Non-adherent cells, such as Chinese Hamster Ovary (CHO) cells, don't require surface attachment for growth and therefore are grown in flasks and bioreactors simply circulating in media.

In contrast adherent cells, such as Vero cells - epithelial cells that come from the kidneys of African Green monkeys, are also commonly used cell culture (Sheets, 2000). This and other adherent cell lines are most often grown in roller bottles, t-flasks, or on surfaces that will enhance adherence and growth. After a seed train of T-flasks or roller bottles has grown enough cells to inoculate a bioreactor, this inoculum can be added to a bioreactor with an attachment matrix. Within the reactor, the adherent cells are grown on disks or other material that are packed to create a bed (i.e. packed bed bioreactor). As the Vero (adherent) cells grow, they must be transplanted to a larger medium with more surface area. For this to occur, cells must be trypsinized and removed from the current surface, and moved to the new medium with greater surface area, along with fresh media and allowed to attach. Theoretically, this process may continue infinitely as long as the Vero cells are supplied with enough fresh media and ample surface area upon which to attach. This upstream process of amplifying or scale up of cells in large volumes involves the use of bioreactors, which can range from 1L to 20,000L.

Bioreactor systems

Two main categories of bioreactor are stirred-tank bioreactors and packed-bed bioreactors (Figure 1, Benninghoff, et al., 2012). Stirred-tank bioreactors are typically used for growing non-adherent cells by circulating media using a pitched blade, and allowing the cells to float and grow freely. The agitator is the pitch blade, swirling the media and circulating the cells. Media circulation is critical for cell growth because it will distribute nutrients, warmth, as well as gases and metabolites necessary for growth and survival. This pitch-blade is also used for bursting the bubbles of gases such as oxygen, air, and carbon dioxide. These gases are necessary for cell cycles to be completed, leading to growth, as wel as pH and environmental control maintenance within the vessel. These gases come from the sparge, so that they may be evenly mixed into solution. Within the biotechnology industry, a pitched-blade bioreactor (see Figure 1-left panel), and this vessel would be full of media and microcarrier bead circulating.

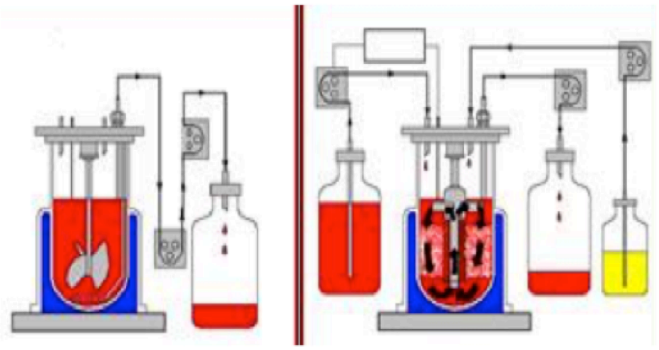


Figure 1. Graphic diagram of stirred tank (left panel) versus packed bed (right panel)

In contrast packed-bed bioreactors have the same tank shape as a pitch-blade bioreactor, but with different mechanics. The agitator brings media from the bottom of the bioreactor and brings it to the top of the tank, where three hollow tubes allow the media to flow out, and this media will then circulate down through a bed of disks. The disks are double-sided with one side

made of polyester and the other side of polypropylene, typically made of Fibra-Cel, upon which the adherent cells will grow (Cino et al., 2011). During processing, the disks are electrostatically treated in order to aid in cell adherence. Typically, adherent cells can take hours to attach to a support, slowing the growth process. However, adherent cells may begin attaching to the Fibra-Cel Disks in as little as 15 minutes, expediting the growth process.

There are advantages and disadvantages to different bioreactor systems (Benninghoff et al., 2012). For example, the "shear free" environment provided by a packed-bed system allows for healthier growth of cells, and easier attachment (Benninghoff et al., 2012). CHO cells grown in a packed-bed bioreactor consumed more glucose, and at a faster rate over the course of 5 days as compared to the CHO cells grown in a pitched-blade bioreactor, consistent with increased cell numbers and metabolic activity. In addition, these cells produced more of the protein of interest, alkaline phosphatase (ALKP) as opposed to the amount produced by the CHO cells grown in the pitched-blade bioreactor. These scientists concluded that "packed-bed bioreactors provide significant advantages for moderate-scale production of cells" when compared to growth in a pitched-blade bioreactor (Benninghoff et al., 2012).

It has also been argued that when using Fibra-Cel Disks as the packed-bed, "product yields can be increased by as much as tenfold over comparable processes" (Cino et al., 2011). This is possible because more surface area created by the bed of FibraCel disks is available for cell growth within a packed-bed bioreactor than in any other bioreactor of comparable size. Because of increased surface area, the bioreactor can run for longer periods of time, as opposed to other bioreactors because of the media exchanges and glucose additions. This will save time and money, thereby increasing product yield and profit, ultimately.

Another beneficial feature of the packed-bed bioreactor for growing adherent cell lines is the Cell Lift impeller technology. This impeller acts as the agitator as it circulates medium through the bioreactor's system. Medium travels from the bottom of the bioreactor up through the impeller, receiving gases, and the medium is released at the top of the bioreactor through three hollow tubes. This gassed medium will then circulate down through the bed of Fibra-Cel disks holding the cells. By using this process, the cells are never directly exposed to gases, which could be potentially harmful to their growth and overall health. This creates a more efficient upstream process system as opposed to a pitched-blade system using microcarriers upon which the cells attach. One additional advantage of a packed-bed bioreactor over a traditional stirred bioreactor is the ability to inoculate in just one step, because of the Fibra-Cel bed (Cino et al., 2011). It would require a higher seeding density and up to six hours for cell attachment to take place on microcarrier beads, which are typically used for adherent cells in pitched blade bioreactors.

Additional system comparisons have been performed on different impeller types: Pitched-Blade vs. Spin Filter vs. Packed-bed Basket (Kohlstrom et al., 2013). Over the course of 160 hours, CHO cells grown using the packed-bed basket impeller grew to a significantly higher density and for a longer growth period, as opposed to the other two types of impellers. Taken together results from the above studies indicated that using a packed bed system may offer the opportunity for high cell yield in a shorter amount of time, using less resources.

Reusable versus Single-Use Bioreactor systems

The aforementioned types of bioreactors are available in reusable glass models, as well as single-use disposables. This is an additional variable and it is important to determine what effects, if

any, this could have on cell growth. Comparison of the growth of CHO cells expressing alkaline phosphatase has been done using reusable versus single-use pitched-blade and packed-bed bioreactors (Barnett et al., 2013). Glucose consumption and lactate production in the media, results indicated that packed-bed bioreactors were the most efficient and produced highest cell yield in all materials (Barnett et al., 2013). In the case of both pitched-blade and packed-bed bioreactors, single-use systems yielded slightly higher alkaline phosphatase levels, consistent with notion that single-use reactors led to increased cell growth relative to reusable glass bioreactors (Barnett et al., 2013). Thus, prior research supports the notion that single use packed-bed bioreactors may be better suited for upstream cell culture at a moderate scale relative to reusable and stirred-tank systems. To date, however, no similar assessment has been carried out with Vero cells, which are more commonly utilized in vaccine production relative to CHO cells.

Objective

Results of the aforementioned research led to the hypothesis that seeding a packed-bed (Fibra Cel) bioreactor at lower density than a pitched blade bioreactor that uses microcarriers, will produce greater cell yield over a period of 21 days. Given the health and economic impact improvements in cell growth and ultimately vaccine production could result in, the objective of this work was to directly assess the efficacy of growing Vero cells on Fibra Cel discs in a packed bed bioreactor for the first time.

MATERIALS AND METHODS

Seed Train for WCB

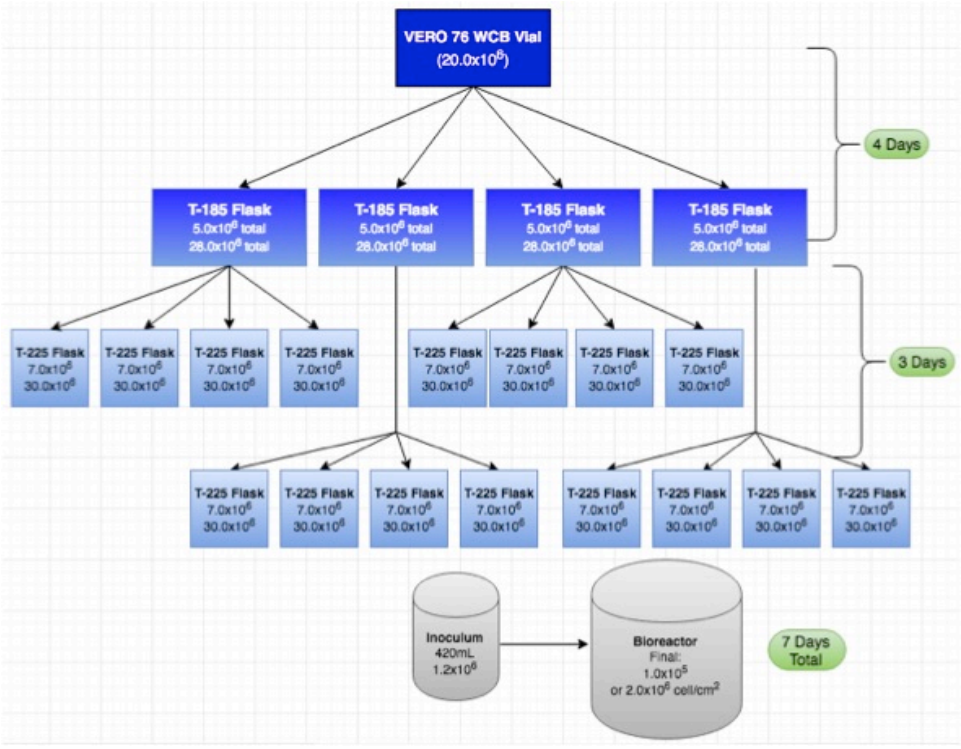
A seed train was created to produce a working cell bank (WCB) of 22 vials from 1 initial 1mL vial of $1.5x10^6$ cells that had been stored in liquid nitrogen [VERO 76 (ATCC 158); MCB vial #4; Batch: WPI-VERO-GREEN-MONSTA], each WCB cryovial containing $20.0x10^6$ cells. Initially, Hyperflasks were used initially for cells growth, however due to several failures, T-Flasks were used to reduce contamination risks. Enough cells were propagated, harvested, and spun down, and resuspended in 1.5mL freeze medium (DMEM/ 5% FBS/ 10% DMEM). Each cryovial was frozen with $1.5x10^6$ cells in each vial. The vials are frozen in a -40ºC freezer, moved to -80ºC, then stored in liquid nitrogen.

Vial Thaw and Inoculum Prep

The WCB of 22 vials was used to begin scale-up for inoculation of the glass 5L benchtop bioreactor. Cells were brought out of thaw by initial thawing in a water bath (90 seconds), 5 minutes in the centrifuge at 800rpm, resuspending the pellet in 10ml medium. Four (4) T182 flasks were inoculated with 2.5mL from WCB cryovial #9 resuspension with DMEM/ 5% FBS and incubated at 37ºC with 5% CO2 for 24 hours. These four flasks had a seeding density of $5.0x10^6$ cells total. After 24 hours, the old media was aspirated off each flask and replaced with pre-warmed DMEM/ 5% FBS and returned to the incubator. These flasks were incubated an additional 24 hours. After 48 hours from initial seeding, the four T182 flasks had the media aspirated off, a 10mL warm PBS wash which was then aspirated off, 7mL warm trypsin added, and incubated for 4 minutes until the cells were not longer attached to the bottom of the flasks. The cells in trypsin were then neutralized with 13mL warm DMEM / 5% FBS and pipetted

several times against side of T182 flask. A cell count indicated there were 28.0x10 cells in each of the four T182 flasks. 5mL from each of the original four T182 flasks was put into new T225 flasks, splitting 4 into 16 flasks. Warmed DMEM/ 5% FBS was added to the 5mL cellular material in each of the 16 flasks and incubated, the seeding density being $7.0x10^6$ cells per flask. These 16 flasks were seeded at incubated for 96 hours under the same conditions. Material from the 16 T-flasks was collected as inoculum using the same trypsinization techniques previously stated, totaling 420mL. Each of the 16 T225 flasks contained $30.0x10^6$ cells total. A sample from the inoculum indicated that the inoculum had a viable cell density of $1.2x10^6$ and a viability of 99%. The inoculum bottle was capped in the biosafety cabinet and aseptically welded onto the harvest line of the packed-bed bioreactor. Both bioreactors, single-use and glass, were seeded at a lower than typical density of $1.0x10^5$ cells/mL, or $2.0x10^6$ cells/cm^2. The day of inoculation is denoted as 'Day 0'. The seed train developed in order to develop enough cells to inoculate the bioreactors (glass and single-use) followed the following course for each run.

Figure 2- Seed Train to Inoculation



Glass Vessel

A 5L packed-bed glass bioreactor was assembled according to the Eppendorf Inc. manufacturer's standards (150g FibraCel Disks) and proper pH and DO probes were autoclaved outside the unit. Probes were calibrated 24 hours pre-inoculation, and an overnight hold was performed on the glass bioreactor for equilibration purposes. The glass unit was autoclaved with 2.5L phosphate buffered saline (to allow for steaming) on Cycle 3 Liquid Cycle with a 30 minute exposure time. 3.2L warm DMEM/ 5% FBS was pumped into the bioreactor. Bioreactor set points can be found below in Table 1.

Table 1- Bioreactor Setpoints

Temperature	**pH**	**DO**	**Agitation**	**GasFlo**
37.0ºC	7.1	40%	80rpm	3Gas: 0.002-1.00

Setpoints within the bioreactor and conditions (pre-inoculation) were recorded to act as a baseline. Metabolites (glucose, glutamate, lactate, LDH, ammonia, glutamine) were measured pre-inoculation, as well as daily during the cell culture process. Inoculum was added. These metabolites were also measure 30 minutes post-inoculation. A media sample was obtained 15 and 30 minutes post-inoculation in order to track cell adherence to the FibraCel disks, and after 30 minutes all cells had adhered to the packed-bed.

Six metabolites (glucose, glutamate, glutamine, LDH, ammonia, lactate) were monitored daily, in addition to bioreactor set points. The cells were grown within the packed-bed bioreactor for 21 days. Within the glass vessel, media exchanges were performed on days 6, 11, and 16 by pumping the contents of a sterile bag of media into the bioreactor. Half the working volume of old media pumped out, as this volume was replaced with new media. Thus, during a media exchange, half the working volume of media was replaced each time (not the entire volume as to not shock the cells). Glucose additions were performed by pumping 200g/L glucose into the bioreactor as needed on days 4, 8 13, and 18. Volumes of glucose to add were determined by measuring glucose levels, and calculating to bring those levels to roughly 5 g/L of 200g/L glucose.

BioBLU® 5p Single Use Vessel

A seed train was produced using the same procedures as with the glass vessel. The seed train for the single use bioreactor was created from vial #29 from the WCB. The Eppendorf single use vessel was opened and assembled aseptically within the biosafety cabinet, according to manufacturer's instructions. DO and pH probes were autoclaved separately from the unit and installed within the biosafety cabinet. A bag of 2900mL working volume of DMEM/ 5%FBS was welded aseptically onto the bioreactor's harvest line and pumped into the vessel. The single use bioreactor setpoints and all parameters remained identical to those used with the glass vessel. Sodium carbonate (base), antifoam, and glucose containers were welded onto the vessel and connected to the pump for use as needed by the system. The 16x T226 flasks produced 400mL inoculum (bringing the 5L vessel to a 3300mL or 3.3L working volume) at a density of 1 million cells/mL. With a total of 400 million cells in the inoculum, the bioreactor was seeded at a density

of 121,212 cells/mL with a 99.3% viability. Seeding density for both vessels was calculated using the following formula:

Concentration 1 x Volume 1= Total mass/ Volume 2= ***Seeding Density***

This growth period was run for 21 days, with media exchanges on days 6, 11, and 16. Glucose additions were performed as needed on days 5, 10, 15, and 20. Antifoam was also added in 1mL volumes as needed to prevent foaming into the head plate, which could ultimately cause exhaust clogging and failure. Note that on Day 6, the working volume of the single use vessels was changed from 3.3L to 3.5L to mirror that of the glass vessel.

Other Materials and Controls

- [Dulbecco's Modified Eagle Medium (1x) + GlutaMAX] : Lot# 1732408; Exp. 2016-09
- [Gibco Fetal Bovine Serum (FBS)] : Lot# 1723549; Exp. 2020-07
- [Eppendorf FibraCel Disks] : Lot# E304158H; Exp. 2017-02
- *Incubator Controls:* Temparature: 37C; CO2: 5% with humidification
- *Glass Bioreactor*: Autoclaved with 2.5L PBS; Cycle 3 Liquid cycle, 30min exposure. 150g FibraCel disks (Lot E304158H).
- *Eppendorf BioBlu Single-Use 5p Macrosparge Bioreactor:* Lot # E161006M, Exp. 2017-07. Serial #00001178. Setpoints: lmin: 55,43; lmax: 23,94; pHO: 6,76; dpH: 0,57; CalT: 37,0

RESULTS

To date the growth of Vero cells on disks in a packed bed bioreactor has not been characterized. To test the feasibility of this, Vero cells were grown in a packed bed bioreactor system (Eppendorf BioFlo 320) on Fibra Cel disks under continuous perfusion culture in both a disposable and reusable (glass) system. The only significant difference between the two vessels is the sparge feature, which introduces gases to the system. Within the single-use vessel, a macrosparger is used, producing smaller bubbles. However, in the glass vessel there is a ring sparger, which introduces slightly larger bubbles. Other than this slight difference, the vessels should perform nearly the same with culture.

Since Vero cells are anchorage dependent and will not float freely in media, cells cannot directly be sampled to count. As an indirect measure of cell growth and numbers daily samples were taken from the bioreactor and various metabolic activities were measured (Figures 3-6). To also help determine if media exchange or glucose addition was necessary, glucose consumption and ammonia production were recorded daily and charted to keep the cells in the exponential growth phase. To track cell health, glutamine, glutamate, LDH and lactate production were also measured daily.

Figure 3: Glucose Uptake and Lactate Production

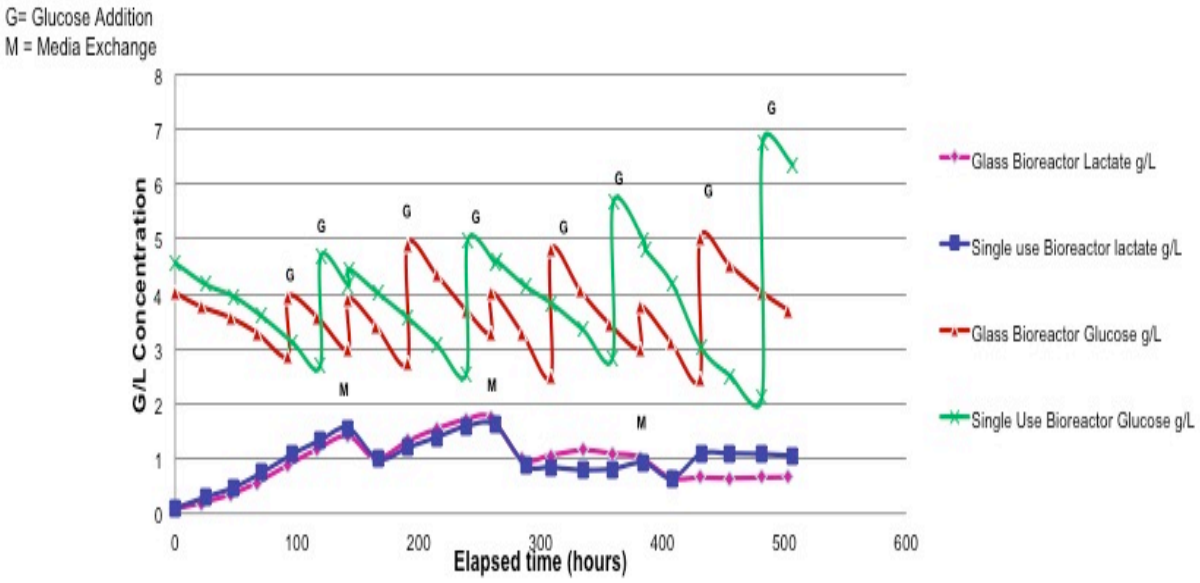


Figure 3 is a chart of the data from both the single-use bioreactor and the glass vessel. The red and green lines indicate glucose uptake in the glass and single-use vessels, respectively, over 21 days. The letters G and M are labeled above and below the lines to indicate when a media exchange was performed, or a glucose addition was made. The pink and blue lines chart lactate production over 21 days within the glass and single-use vessels, respectively. As glucose levels dropped below 5g per liter (typically around 2 g/L) glucose addition was necessary, and this was recorded by tracking metabolites. The small spikes in glucose levels within the glass vessel are because of the glucose present in the new media when an exchange was performed. These spikes are not as obvious with media additions in the single-use vessel because of more efficient sparging from the macrosparge system. The data within this graph indicates that glucose concentration within the vessel would decrease as lactate levels increased, indicating that the Vero cells remained in the exponential growth phase.

Figure 4: Total Glucose Consumption

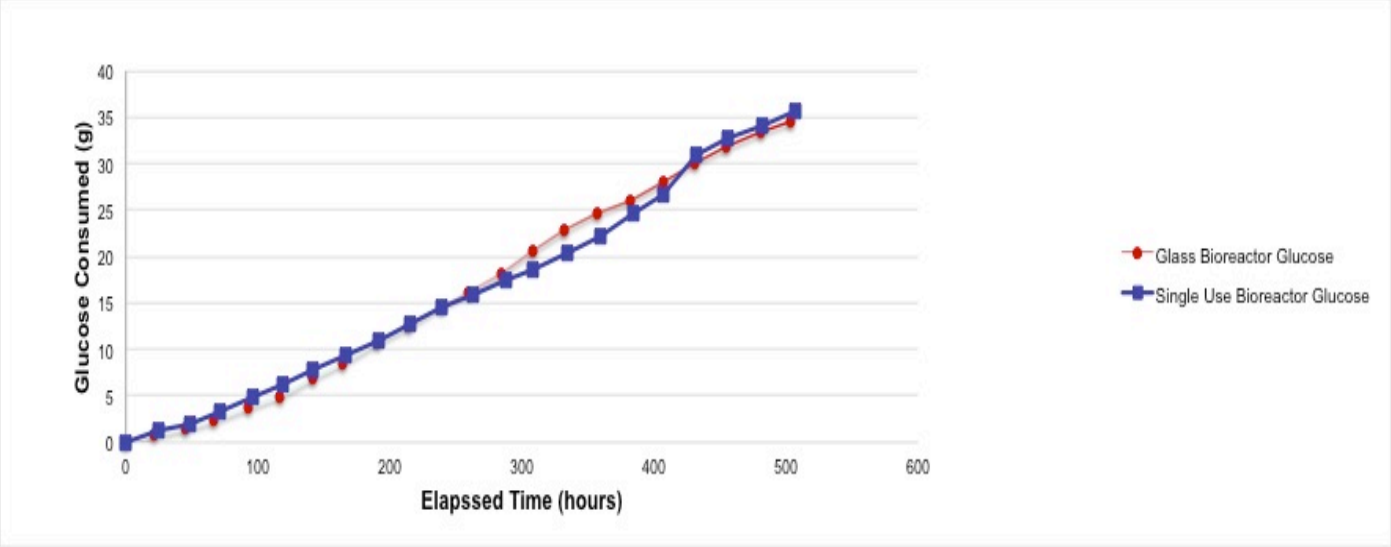


Figure 4 is a chart of total glucose consumption within each vessel over the course of 21 days. Note that the red line represents glucose consumption within the glass vessel, and the blue line represents glucose consumption (in grams) within the single-use vessel. Within both the glass and single-use vessels, glucose consumption rose at a consistent rate over the course of 21 days within the packed-bed bioreactor system. This data indicates that there was almost no variation between conditions provided by the single-use and glass vessels, and the Vero cells remained within the exponential growth phase for 21 days.

Figure 5: Glucose Consumption Rate

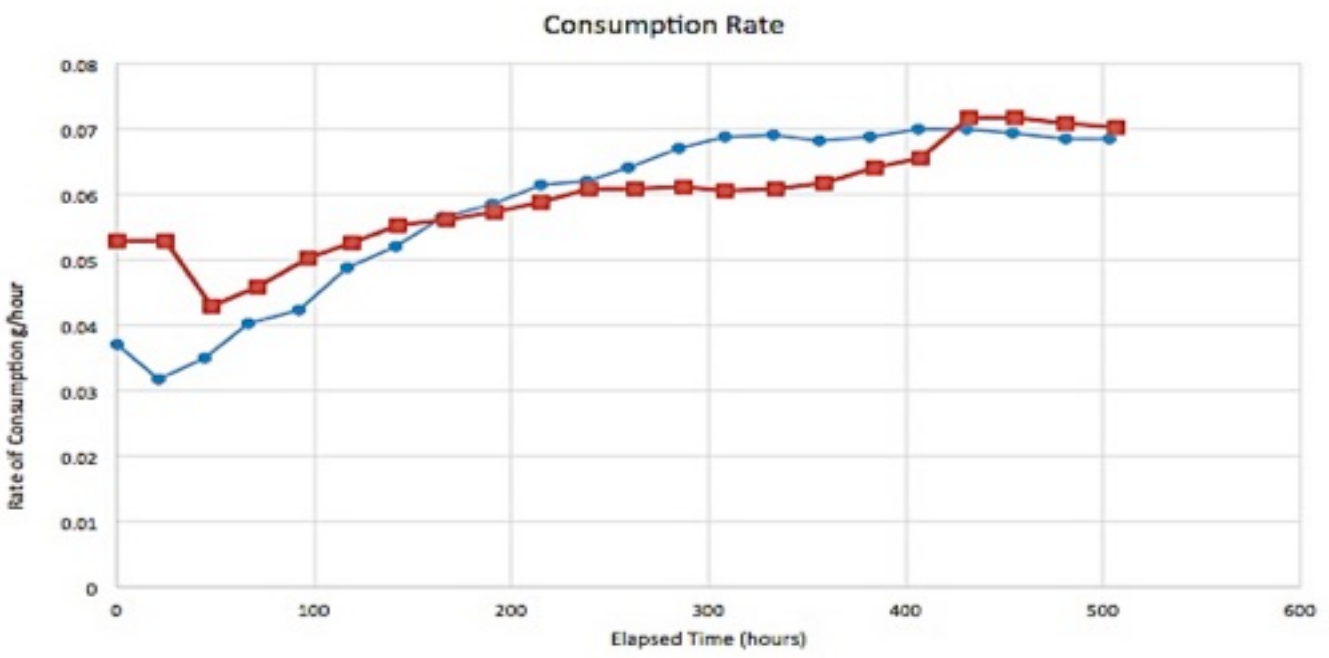


Figure 5 is a chart of the glucose consumption in grams per hour within each vessel over the course of 21 days. Note that the blue line represents glucose consumption within the glass vessel, and the red line represents glucose consumption within the single-use vessel. The rate of consumption was initially slow, as the cells adapted to their environment. Then around 100 hours post-inoculation, the cells began to consume glucose at an increasing hourly rate, indicating that conditions within the bioreactor remained stable. The increasing rate is consistent with the cells continuing to grow exponentially.

Figure 6: Grams of glucose Consumed Hourly

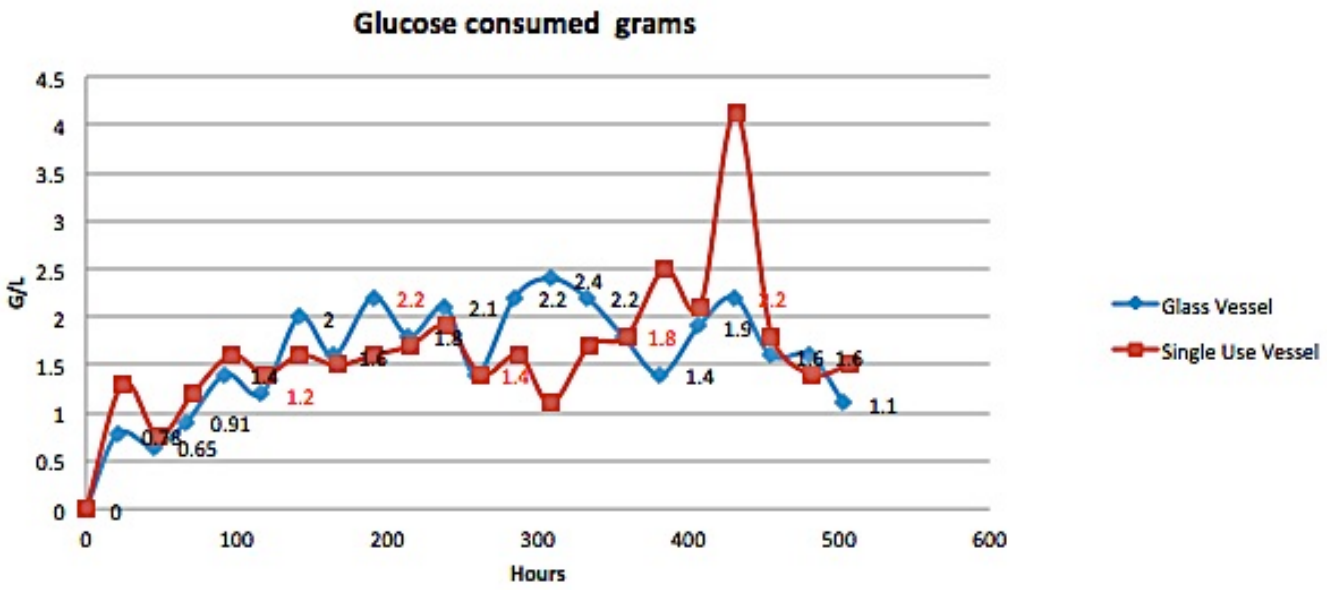


Figure 6 is a chart of the grams of glucose/liter consumed hourly within each vessel. Note that the blue line represents glucose consumption in grams per liter in the glass vessel, and the red line is within the single-use vessel. This data indicates that the grams of glucose/liter consumed hourly were indicative of the cellular need for more glucose or media exchanges. Sharp peaks within glucose consumption are synonymous with increased cell growth and are synonymous with a glucose addition.

Lowering the seeding density for inoculation of a bioreactor significantly decreases time, cost, and risk of contamination during the scale-up process. A typical bioreactor using pitched-blade agitation and microcarrier beads for adherent cell growth must be inoculated with $1.7x10^6$ cells/mL, however a packed bed bioreactor can be seeded at a much lower density. Here I determined if Vero cells could be seeded in a packed bed bioreactor with Fibra Cel disks at lower densities and achieve high levels of growth. Seeding densities are shown below in Figure 7.

Figure 7: Seeding Densities (*literature based)

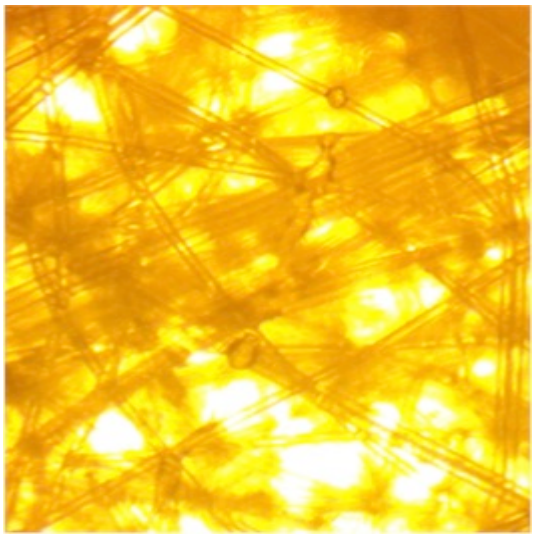
	Pitched Blade*	**Packed-Bed Single-Use**	**Packed-Bed Glass Vessel**
Seeding Density (cells/mL)	$1.7x10^6$	$1.0x10^5$	$1.0x10^5$

As indicated by Figure 7, this seeding density of $1.0x10^5$ cells/mL in a 5-liter vessel for the packed-bed bioreactors (glass and single-use) is much lower than the industry standard of seeding with $1.7x10^6$ Vero cells/mL. By charting glucose uptake and lactate production, this indicated that cells continued to grow in the exponential phase, and expected growth was achieved over 21 days. There was no significant variation in cell growth from the glass vessel to the single-use vessel. This experiment was performed in order to determine if a packed-bed bioreactor with FibraCel disks could be seeded with Vero cells at a lower than typical density, as well as whether or not improved cell yield would be possible after using such a low seeding density.

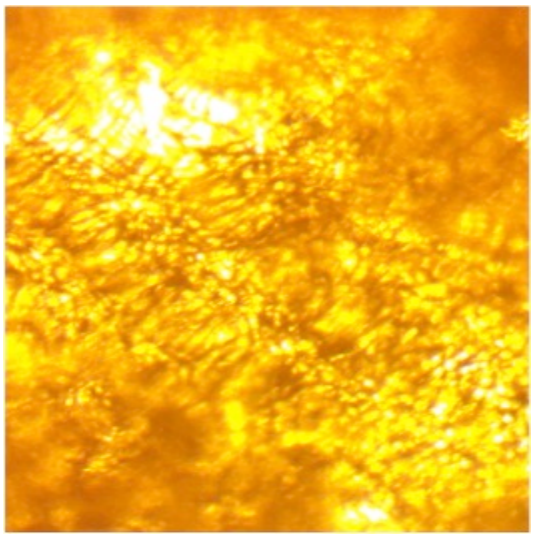
In Figure 8 on the left panel is an image of a FibraCel disk before cell growth within the bioreactor, and the right panel is a photo from the single-use bioreactor after 21 days in culture with Vero 76 cells anchored to the surface. By photographing the disks before cell growth, it was possible to obtain a control to ascertain how much cell growth was obtained on each disk. The surface of the FibraCel disk full of Vero cells indicates that cell growth and yield were very successful within the packed-bed bioreactors at a low seeding density.

Figure 8: FibraCel Disks- With and Without Cells

FibraCel Surface- No Cells



FibraCel Surface- With Cells



DISCUSSION

This project proved that Vero Cells can successfully be grown in a packed-bed bioreactor system, in both a single-use or a glass vessel at the 5L benchtop scale. A packed-bed bioreactor can be seeded at a lower than typical density, which significantly decreases time, cost, and human risk during the seed train and scale-up process. The packed-bed bioreactors were seeded at $1.0x10^5$ cells/mL, when a traditional vessel with Vero cells on microcarriers would have to be seeded around $1.7x10^6$ cells/mL. Based on glucose uptake and lactate production, cells within the packed-bed bioreactor face less shear-effects within both glass and single use systems, and grow to a yield as high, if not higher, than as in a traditional bioreactor. Anchorage dependent cells exhibit sufficient cell-to-cell communication within the packed bed of FibraCel Disks, allowing for greater proliferation and yield. It is also important to note that the glass and single-use vessels produced comparable results. Taken together, the results presented here suggest that the use of Vero cells in the packed-bed bioreactor with FibraCel disks is a worthwhile method for pursuing a streamlined scale-up process for vaccine production, which will ultimately lead to production efficiency and lowered costs.

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