Productivity Studies Utilizing Recombinant CHO Cells In Stirred-Tank Bioreactors: A Comparative Study Between The Pitch-Blade And The Packed-Bed Bioreactor Systems

Taylor Stephen Hatton
Utah State University

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PRODUCTIVITY STUDIES UTILIZING RECOMBINANT CHO CELLS IN
STIRRED-TANK BIOREACTORS: A COMPARATIVE STUDY BETWEEN THE
PITCH-BLADE AND PACKED-BED BIOREACTOR SYSTEMS

by

Taylor S. Hatton

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy and Veterinary Sciences

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2012
ABSTRACT

Productivity Studies Utilizing Recombinant CHO Cells In Stirred-Tank Bioreactors: A Comparative Study Between The Pitch-Blade And The Packed-Bed Bioreactor Systems

by

Taylor S. Hatton, Master of Science

Utah State University, 2012

Major Professor: Dr. Kamal A. Rashid, Ph.D.
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A recombinat Chinese Hamster Ovary (rCHO) cell line designated as CHO SEAP was utilized in this investigation to optimize protein production. Two bench top stirred-tank bioreactors, namely a pitched-blade and a packed-bed basket bioreactor, were utilized for a comparative study to determine which bioreactor would produce the best results in terms of protein production. The objective of this research project was to provide basic data that shows cells cultured in a packed-bed basket bioreactor in perfusion mode will generate more protein product than cells in batch mode suspension culture with a pitched-blade bioreactor. The packed-bed bioreactor creates a homeostatic environment similar to the environment found in vivo, where waste products are constantly removed and fresh nutrients are replenished. Closed batch cultures do not provide a homeostatic environment. In batch culture systems, nutrients are depleted and waste products accumulate. The results from this experiment could help investigators involved in protein and/or vaccine production facilities select the appropriate bioreactor
and mode of operation to optimize cell productivity for generation of a specific protein product. CHO cells have been used for the production of vaccines, recombinant therapeutic proteins, and monoclonal antibodies, and these cells are now the cell line of choice in the biopharmaceutical industry. Traditional vaccine production methods in egg embryos are slow and outdated, whereas roller bottle-based cell culture techniques are time consuming and have limited scalability. These limitations justify the need for development of stirred tank bioreactors. Cells cultured in a packed-bed bioreactor are not exposed to hydrodynamic forces, as is the case with pitched-blade bioreactors, allowing for maximum growth and protein expression. This mode of operation involves the constant removal of media depleted of nutrients and the addition of fresh media with more nutrients to keep the cells growing. Long run times decrease the constant need for re-seeding cells and re-establishing seed cultures, thus, reducing setup time and labor dramatically. Secreted products are automatically separated from cells in perfusion, eliminating filtration and membrane fouling. A detailed description of both modes of operation are discussed in this thesis.

(74 Pages)
PUBLIC ABSTRACT

Productivity Studies Utilizing Recombinant CHO Cells In Stirred-Tank Bioreactors: A Comparative Study Between The Pitch-Blade And The Packed-Bed Bioreactor Systems

by

Taylor S. Hatton, Master of Science

The Center for Integrated Biosystems (CIB) at Utah State University proposed to demonstrate the added benefits of bench top stirred-tank bioreactors utilizing the packed-bed impeller system in perfusion mode for the production of recombinant proteins. In this study recombinant Chinese Hamster Ovary (CHO) cells were utilized for the production of a secreted protein in two bioreactor types: pitched-blade bioreactor operated in batch mode versus packed-bed bioreactor operated in perfusion mode. Our primary objective was to determine whether the packed-bed bioreactor is superior to a traditional pitched-blade bioreactor for the mass production of cell culture protein products. We hypothesize that the reduced culture stress and optimal growth conditions of the packed-bed basket system will result in a higher yield of protein product compared to the traditional pitched-blade system.

The pitched-blade impeller is commonly used for the growth of shear sensitive animal cells. The impeller provides gentle mixing of the media without damaging the cells. The packed-bed basket impeller is composed of two metal perforated screens. In-between the screens are Fibra-Cel disks which create a bed to which suspension cells are entrapped. Cells cultured in a packed-bed bioreactor are growing in a shear free
environment as they are not exposed to hydrodynamic forces allowing for maximum growth and protein expression.

Three independent experiments were performed in each bioreactor system described. The results from these experiments could help investigators involved in protein and/or vaccine production facilities select the appropriate bioreactor and mode of operation to optimize cell productivity for a specific protein product.
To my Parents
Paul and Debbie Hatton
and my beautiful wife
Elise K. Hatton
ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. Kamal Rashid for his willingness to take me on as a graduate student with very little knowledge of cell culture practices and turn me into a modern cell culturist. The skills I have acquired through Dr. Rashid’s patience and guidance in modern cell culture techniques will be invaluable to me in my future career. I am also deeply grateful for the other outstanding professors of my graduate advisory committee, including Dr. Abby Benninghoff, Dr. Kerry Rood, and Dr. Charles Miller, for their willingness to support and guide me in my graduate work. I would like to give a special thanks to Dr. Abby Benninghoff for going above and beyond in her support for me. I would also like to give special thanks to Dr. Kerry Rood for having a watchful eye and referring me to work under Dr. Rashid.

I would like to give special thanks to Shaun Barnett for sharing his knowledge of the bioreactors and other instruments and helping me learn how to use and set them up. I would also like to thank Ken Olsen and Darlene Orduno, two outstanding staff assistants, for their support and help in ordering supplies for the project. Without their valuable help, the project would not have gone forward. I would also like to thank Vikram Gossain, Ma Sha, and New Brunswick Scientific, an Eppendorf Company, for their support of the project in loaning us the two bioreactors and for financial support as well. I would also like to thank Dean Cockett and the Utah Agricultural Experiment Station, Utah State University, for the grant money donated for the project. The donation helped to make the financial burdens of the project much easier to handle. I would also like to thank Vitaliy Gavrilyuk and CDI Bioscience for donating the rCHO Cells for use in the project. Special thanks to Parveen Parasar for his willingness to take time away from his
schedule and show me how to best run the Secreted Alkaline Phosphatase Reporter Gene Assay Kit. I would like to thank him for showing me how to run the plate reader computer program. I would also like to thank the Center for Integrated Biosystems and Utah State University for providing great facilities and professors and six incredible years of education.

Last but not least, I would like to thank my loving parents, Paul and Debbie, my siblings, Elisa, Philip, and Jessica, and loving wife, Elise, for their invaluable encouragement and support of me in my daily pursuits of my education. Without their love and examples I would not be the person I am today.

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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
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<td>mM</td>
<td>Micromolar</td>
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<td>µL</td>
<td>Micro-Liter</td>
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<td>µmol</td>
<td>Micro-mol</td>
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<tr>
<td>U</td>
<td>Unit</td>
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## Abbreviation Key

<table>
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<th>Definition</th>
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<tr>
<td>ALKP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PLAP</td>
<td>Placental alkaline phosphatase</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenylphosphate</td>
</tr>
<tr>
<td>rCHO</td>
<td>Recombinant Chinese Hamster Ovary</td>
</tr>
<tr>
<td>RTD</td>
<td>Resistance temperature detector</td>
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<tr>
<td>SEAP</td>
<td>Secreting alkaline phosphatase</td>
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## Definitions

**Unit (U):** The amount of enzyme that hydrolyzes 1 µmol if p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 mL in 1 minute at 37°C.

**Batch Culture:** A closed system in which cells are grown in a fixed volume of culture media under specific environmental conditions, such as temperature, agitation, and aeration, until a certain density is reached or for a set time frame. The cells are then harvested and processed as a batch, usually before all nutrients are depleted.

**Perfusion Culture:** An open system in which cells are maintained in the bioreactor either by a cell retention device inside the vessel or by filtration, sedimentation, or centrifugation of the cells. Such devices or methods allow for nutrient solutions to be fed into the system and for spent media with toxic byproducts to be removed.
CHAPTER 1
INTRODUCTION

Animal cell culture began in the late 19th century. In 1885, German zoologist Wilhelm Roux maintained the neural plate from a chicken embryo in a warm saline solution for several days, an accomplishment not yet obtained by the scientific elite of the day [1]. In the early 1900s, American Scientist Ross Harrison succeeded in maintaining amphibian nerve fibers in conditions suitable for proliferation of the fibers. Harrison placed disaggregated fragments of frog tissue in coagulated frog lymph and later observed outgrowths of new nerve cells from the tissue. Harrison’s work laid the foundation for modern cell culture and aroused the interest of leading scientists of the period [2]. Rous and Jones were the first scientists to use trypsin, a proteolytic enzyme, to make unicellular suspensions of cells in culture [3]. Alexis Carrel introduced the next major advancement in cell culture, the Carrel flask, which was the precursor to the modern T-flask. Carrel was also the first cell culturist to demonstrate that animal cells could be grown without the use of antibiotics. Carrel’s complex sterile techniques and Carrel flask helped reduce contamination and increased tissue handling [4]. Early work in cell culture relied on undefined media as the source of nutrients. Albert Fischer was the first to develop a defined media for cell culture, CMRL 1066, which he developed in 1948 [5]. In 1956, Harry Eagle developed Minimal Essential Medium (MEM), a medium still commonly used today [6]. The increased emphasis in chemically defined media and aseptic technique helped solidify the future of animal cell culture.

Innovations in animal cell culture techniques continued throughout the next 50 years. During the 1950’s the first continuously cultured human cell line, HeLa [7] was
established from cervical tissue. Scientists also shifted their focus to development of
defined media for cell culture, such as Eagle’s Basel Media (BME), Eagle's Minimum
Essential Medium (MEM) and CMRL 1066 (Connaught Medical Research Laboratories)
[8, 9]. These defined media formulations provided researchers the opportunity to utilize
serum-free cell culture techniques in the 1960s [10], followed by the first sterile plastic
cell culture vessels and development of many suspension cell lines in the 1970s. In 1975,
Kohler and Milstein were the first group of scientists to produce a monoclonal antibody
(mAb)-secreting hybridoma cell line. Their discovery set off a revolution in mAb
production in all areas of basic research [11, 12]. Many specialized cell lines were
developed in the 1980s, including cell lines for scale-up and production of mAbs. The
packed-bed bioreactor and Fibra-Cel disks used in the present study were also developed
in the 1980s. The human genome project of the 1990s lead to profiling the transcriptome
and proteome of many cell lines commonly used in bioprocessing. The early 21st
century was the era of omics and stem cell research [8]. As technology continues to
progress the future is open to many more advances in animal cell culture.

In recent years, the Chinese hamster ovary (CHO) cell has become an important
expression system of recombinant proteins for many biopharmaceutical companies. CHO
cells and two murine myeloma cell lines, NS0 and SP2/0, are currently the three main
mammalian cell lines which produce FDA approved products. Of the 21 FDA approved
products produced by mammalian cells, five were produced using CHO cells [13]. The
use of recombinant CHO (rCHO) cell lines in stirred-tank bioreactors is becoming a
method of choice for the scale-up of protein products.
An rCHO cell line designated as CHO SEAP was utilized in this investigation to optimize growth conditions, growth parameters and protein production. The pitched-blade and packed-bed bioreactors (Figure 1), both bench top stirred-tank bioreactors, were utilized for this comparative study to determine which bioreactor would produce the best results in terms of protein production. The overall hypothesis for this research project was that cells cultured in a packed-bed basket bioreactor in perfusion mode would generate more protein product than the cells cultured in the pitched-blade bioreactor in batch mode. The packed-bed bioreactor creates a homeostatic environment similar to the environment found in vivo where waste products are constantly removed and fresh nutrients are replenished. Batch cultures do not provide a homeostatic environment due to the depletion of nutrients and the accumulation of waste products [14]. The results from this experiment could help investigators involved in protein and/or vaccine production facilities select the appropriate bioreactor and mode of operation to optimize cell productivity for a specific protein product.

**Figure 1.** (Left) Packed-bed bioreactor and (right) pitch-blade bioreactor. (With permission from NBSC)
CHO cells have been used for the production of vaccines, recombinant therapeutic proteins and monoclonal antibodies. These cells are now a cell line of choice in the biopharmaceutical industry [15-18]. Traditional vaccine production methods in egg embryos are slow and outdated, whereas roller bottle based cell culture techniques are time consuming and have limited scalability. These technological limitations demonstrate the need to develop more rapid and scalable methods for vaccine production to serve human and livestock populations worldwide. Suspension cells coupled with stirred-tank bioreactors is a technology that will provide large-scale protein and vaccine production with less labor and expense. Two modes of operation in stirred-tank bioreactors were investigated in this study: batch and perfusion. A batch culture is a closed system in which cells are grown in a fixed volume of culture media under specific environmental conditions, such as temperature, agitation, and aeration, until a certain density is reached or for a set time frame. The cells are then harvested and processed as a batch, usually before all nutrients are depleted. A perfusion culture is an open system in which cells are maintained in the bioreactor either by a cell retention device inside the vessel or by filtration, sedimentation, or centrifugation of the cells. Such devices or methods allow for nutrient solutions to be fed into the system and for spent media with toxic byproducts to be removed.

Cells cultured in a packed-bed bioreactor are not exposed to hydrodynamic forces, like pitched-blade bioreactors, thus allowing for maximum growth and protein expression [19]. Also, cells in these packed-bed bioreactors may be maintained for weeks or even months on a perfusion culture system. This operation method involves the constant removal of media depleted of nutrients and the addition of fresh media with more
nutrients to keep the cells growing. Long run times allowed with the packed-bed bioreactor decrease the constant need for re-seeding cells and re-establishing seed cultures, thus reducing setup time and labor dramatically. Perfusion techniques also allow for higher cell densities, reaching as high as a 5- to 10-fold increase compared with batch cultures [20]. The packed-bed bioreactor produces high yields of secreted products from all types of animal cells [21]. The entrapped cells avoid shear from impellers, gas bubbles and pumps and allow higher mixing speeds, thereby increasing mass transfer of nutrients and oxygen. Secreted products are automatically separated from cells in perfusion which eliminates filtration and membrane fouling. Perfusion techniques in pitched-blade bioreactors are cumbersome and complex, usually requiring one of three techniques: centrifugation, filtration or sedimentation. The packed-bed bioreactor eliminates the need for all of these techniques as the cells are trapped in-between the Fibra-Cel disks allowing for media changes without the loss or damage of cells [22].

Animal cells lack a cell wall and are therefore vulnerable to shear effects inside bioreactors. Most of the cell damage arises from gas sparging aeration and mechanical agitation [23, 24]. Tramper, et al. suggested three areas of a bubble column where cells may experience shear stress: bubble formation at the sparger tip, the bubble ascending region and bubble disengagement at the gas-liquid interface. Murhammer, et al., contend that the influx of media into the space vacated by detaching bubbles from the sparger is a source of hydrodynamic stress deleterious to surrounding cells [25]. Although rising bubbles create little shear stress [26], they can lead to cell entrainment [27]. Entrained cells are subjected to bubble disengagement at the gas-liquid interface shown in Figure 2 (next page). As bubbles burst, high speed liquid jets are created causing hydrodynamic
stress. Although the pitched-blade impeller is designed for shear sensitive cells, the rotating blade can damage cells at high agitation rates [28]. The rotation creates a vortex which may introduce bubbles into the system leading to bubble entrainment. Additives like fetal bovine serum (FBS) and chemicals like Pluronic F68 are effective means of protecting cells from hydrodynamic stress [25]. The use of these additives requires their removal in any downstream processing. The packed-bed bioreactor is not subjected to the same hydrodynamic forces since the cells are sequestered by the Fibra-Cel disks thus eliminating the need for protective agents described above.

Figure 2. Bubble disengagement at the gas-liquid interface. (A) Bubble rises to the surface. (B) Bubble bursts causing the bubble film to rapidly reced. (C) The high pressure casued by the bubble bursting causes two liquid jets to form, one downward into the medium and one upword into the gas headspace.
Packed-bed bioreactors have been compared with microcarrier systems [19], and perfusion systems have been compared to fed-batch systems [29, 30]. Perfusion and batch cultures are two common modes of operation for scale-up of mammalian cells in culture. However, to date, no studies have compared the productivity of protein-secreting cells in packed-bed bioreactors in perfusion mode to pitched-blade bioreactors in batch mode. Therefore, the primary objective of this study was to determine whether cells cultured in a packed-bed bioreactor in perfusion mode would generate more protein product than cells cultured in a traditional pitched-blade bioreactor in batch mode. Cell densities of about $5 \times 10^5$ cells/mL were used for seeding each bioreactor. The experimental design utilized three separate runs for each bioreactor type for validity and reproducibility. Three strategies were implemented to monitor cell yield, viability and protein production. Growth of cells in the pitched-blade bioreactor will be measured by offline samples and cell count via Trypan Blue staining and the Countess instrument (Invitrogen, Life Technologies). A minor limitation to the packed-bed bioreactor is the inability to obtain direct cell counts and viability. Therefore, cell growth cannot be measured directly in the packed-bed bioreactor. Glucose up-take and lactose production were measured in the pitched-blade and packed-bed bioreactors utilizing the YSI 2700 Select Biochemistry Analyzer (YSI, Inc., Yellow Springs, OH). Both instruments are available in the bioprocessing facility at the Center for Integrated BioSystems. Protein concentrations were monitored every 24 hours in each bioreactor for the duration of each experiment using an enzyme assay (AnaSpec, Freemont, CA). All offline samples were plotted on a graph every 24 hours to monitor the progress of each bioreactor. We expect that the results of this research project will aid in the introduction of packed-bed basket
bioreactors in perfusion mode as a desirable and productive approach to mass production of CHO and other cell lines in large scale biopharmaceutical production including animal vaccines to keep the sources of the food supplies safe for consumption.
CHAPTER 2
MATERIALS AND METHODS

2.1 Cell Line

The CHO cell line was first established in 1957 by Dr. Theodor Puck. While at the University of Colorado he isolated the cells from the ovary of a female Chinese hamster [18]. CHO cells have become a well proven system for the production of proteins suitable for human use and therefore all experiments utilized a proprietary recombinant CHO cell line provided by CDI Bioscience, Inc. (Madison, WI). The rCHO cells were originally developed from the CHO-S (Invitrogen) cell line. The rCHO cell line is defined by the developer as SEAP (Alkaline Phosphatase secreting) Suspension Cells. The rCHO cells were engineered with the IPTG-regulated RP Shift vector so that the rCHO cells stop replicating and shift to protein production when induced with IPTG. CHO cells carry out many human congruent, post-translational modifications and have an inherent resistance to viral infection, making them a choice expression system [31].

Figure 3. Suspension rCHO cells.
2.2 Growth Medium

All experiments utilized CD-CHO medium (Gibco, Life Technologies, Grand Island, NY) which is a chemically defined, protein-free medium used for high density growth of rCHO cells. CD-CHO medium is free from any animal origin products, which will eliminate any batch to batch variability simplifying any downstream processing and reduce cost. The media contains 6.3g/L glucose and was supplemented with final concentrations of 8 mM L-glutamine and 100µg/mL of an antibiotic/antimycotic solution (G418).

![Metabolic pathways for glucose and glutamine in mammalian cells](image)

**Figure 4.** Metabolic pathways for glucose and glutamine in mammalian cells. (Ref. 32)
2.3 Seed Culture Development

Frozen stocks of rCHO cells were thawed at 37°C then transferred to T-75 flasks with CD-CHO serum-free medium and allowed to expand. Once a sufficient number of cells were achieved, sterile disposable spinner flasks were utilized to further expand the cells. The spinner flasks contain a magnetic stirring bar to mix the cell suspension when placed on a magnetic stirring plate. The seeded spinner flasks were then placed on a magnetic stirring plate inside a CO₂ incubator at 5% CO₂ and 37°C and allowed to grow for two days. The spinner flasks were then sub-cultured and seeded into new spinner flasks and allowed to reach confluency. Subculture of the cells continued until a sufficient number of viable cells was achieved for use as a seed culture at the density of approximately 5 x 10⁵ cells/mL.

2.4 Bioreactors

Advanced bench-top stirred-tank bioreactors were utilized to scale up the rCHO cells. The New Brunswick Scientific Company (NBSC) Bioflo 310 bioreactor allows the operator to control and monitor many parameters such as dissolved oxygen (DO), pH, temperature and impeller speed via a microprocessor control system. All this is made possible with a 15” touch screen which clearly displays all set points, present values, loops, cascades and more on the summery or synoptic screens. The Bioflo 310 can be used for batch, fed batch or continuous (perfusion) culturing of cells. Each vessel is surrounded by a water jacket and capped with a stainless steel headplate. The headplate has many customizable ports for pH and DO probes, addition and sample ports, sparger, a thermowell for a resistance temperature detector (RTD), an exhaust condenser, etc. The vessels are autoclavable for sterilization purposes. The motor attaches to a magnetic drive
coupling which is located in the center of the headplate. The motor ranges in speeds from 25 to 500 RPM (+5 rpm). The RTD can detect the temperature inside the vessel between 5°C above coolant temperature to 80°C (+0.1°C). A Gel-filled pH probe can sense the pH ranging on the scale from 2-12 (+0.01). The DO electrode has a range of control from 0-200% (+1%).

![Figure 5](image.png)

**Figure 5.** (A) New Brunswick Scientific’s CelliGen 310 bioreactor system. (B) Summary screen shows convenient view set points, current values, cascade loops and more. (With permission from NBSC)

2.4.1 Pitched-Blade Impeller

For these experiments two different bioreactor systems were utilized. The first bioreactor system utilized the pitched-blade impeller. The blades on the pitched-blade impeller are flat and set at a 45° angle. This blade orientation gives good axial and radial mixture of the media while also increasing the oxygen mass transfer rate and disruption of bubbles released from the sparger. The pitched-blade impeller is designed to minimize stress of mixing on shear-sensitive cells [33].
2.4.2 Batch Process (Pitched-Blade Bioreactor)

Three experimental trials were performed using the pitched-blade bioreactor in batch mode in a 2.2 L vessel (1.7 L working volume). The vessel was equipped with a pitched-blade as described above. For each trial, the bioreactor was allowed to operate until the cell concentration reached approximately $2 \times 10^6$ cells/mL, at which the cells were induced with IPTG. The growth and productivity was documented for the duration of the batch experiment. All of the experimental trials had the following parameters: agitation speed set to 120 rpm ($\pm 5$ rpm); dissolved oxygen at 35% ($\pm 1\%$); temperature at 37°C ($\pm 0.1^\circ C$); and pH at 7.1 ($\pm 0.01$).

2.4.3 Packed-Bed Basket Impeller

The second bioreactor system was equipped with the packed-bed basket impeller. This bioreactor system is suitable for both anchorage dependent and suspension cells. With this system there is no need to adapt anchorage dependent cells to suspension. The packed-bed basket impeller is commonly used in the production and collection of extracellular proteins [21]. The packed-bed basket impeller incorporates a basket with two horizontally positioned perforated metal screens. Fibra-Cel disks are placed in between the metal screens creating a bed to entrap suspension cells or provide a surface
for attachment of anchorage dependent cells. The Fibra-Cel disk bed provides a culture environment that allows freshly oxygenated media to slowly pass over the cells while also providing protection from external shear forces [33]. The rotation of the impeller creates a negative pressure that draws media up through the hollow center shaft where the sparger introduces oxygen to the media. The media flows in a spiral direction from the bottom of the vessel up the central shaft and out the three discharge ports at the top. The media then trickles down through the packed cell bed saturating it with nutrients and oxygen. The packed-bed basket bioreactor is the ideal system to use when a product is secreted out of the cell. Because cells immobilized in the Fibra-Cel bed, samples of media can easily be removed without the loss of cells or culture disruption.

Figure 7. (A) Packed-bed bioreactor. (B) Basket apparatus for packed-bed bioreactor. (With permission from NBSC)

2.4.4 Fibra-Cel Disks

The Fibra-Cel disks consist of sheets of polyester and polypropylene ultrasonically bonded together and then punched to a diameter of 6mm round disks.
They are then washed in methanol, electrostatically treated and tested for endotoxins, cytotoxicity, bioburden and viral degradation. Fibra-Cel disks create approximately 1,200cm$^2$/g of surface area and are optimized at 100 grams of disks per liter of media. A 1.7L working volume bioreactor containing 85g of Fibra-Cel disks creates a total surface area of 102m$^2$ (335sq. ft.). The surface created by the Fibra-Cel disks is equivalent to the same area available with 120 roller bottles (850cm$^2$), 453 t-flasks (225cm$^2$) and 4,080 t-flasks (25cm$^2$).

**Figure 8.** (A) Fibra-Cel disks. (B) High resolution micrograph of a Fibra-Cel disk indicating the polyester mesh with polypropylene support. (With permission from NBSC)

### 2.4.5 Perfusion Process (Packed-Bed Basket Bioreactor)

Three experimental trials were performed using the packed-basket bioreactor operated in perfusion mode in a 2.2 L total volume vessel (1.7 L working volume). The vessel was equipped with a basket, as described above, containing 85g of Fibra-Cel disks. The perfusion process was initiated once the cells reached the exponential phase. Each bioreactor was allowed to run for 12 days once perfusion started. The perfusion process utilized in these experiments required the removal of the media from the bioreactors over
several minutes. On day 1 of perfusion, 0.5 L of the media was removed from the vessel and replaced with fresh medium using a peristaltic pump. On day 2 1 L of media similarly replaced; subsequently, 2 L of culture media was replaced every other day (up to day 15). The growth and productivity was documented for the duration of the perfusion experiment. Otherwise, all experimental trials had the same growth conditions (temperature, oxygen, pH) as the batch process.

2.5 Project Timeline

Figure 9 details the timeline followed for each bioreactor system. The pitched-blade bioreactor, shown in blue, was seeded with $5 \times 10^5$ cells/mL on day zero followed with an initial glucose and lactate measurement and initial cell count and viability. Glucose and lactate levels were measured and cell count and viability were checked on days 1 and 2. The cells were induced with IPTG on day 3 or 4; depending on when the cell density reached $2 \times 10^6$ cells/mL. Glucose and lactate levels, along with cell count and viability, were measured pre and post induction and every day thereafter for the duration of the experiment. Each experiment in the pitched-blade bioreactor lasted approximately 9 days. The packed-bed bioreactor, shown in brown, was seeded with $5 \times 10^5$ cells/mL on day zero followed with an initial glucose and lactate measurement. Glucose and lactate levels were measured on days 1 and 2. Day 3 began the perfusion process where 0.5L of media was removed from the bioreactor and replaced with fresh media. On day 4 1L of media was replaced. On day 5 and every-other day thereafter, 2L (complete volume) of media was replaced with fresh media containing IPTG. Glucose and lactate measurements were taken pre and post perfusion on all three days. On day 6 and every-other-day thereafter, glucose and lactate measurements were taken.
2.6 Secreted Alkaline Phosphatase (SEAP)

SEAP is a reporter gene that is used to control the level of protein expression at the transcriptional and post-transcriptional levels. SEAP was created by truncating Placental Alkaline Phosphatase (PLAP) at the 489th amino acid by the addition of a termination codon. There are some significant advantages to the truncated form of PLAP. The truncated form liberates PLAP from the cell surface membrane allowing it to be secreted. This truncation also creates a thermally stable protein at 65°C. At this temperature other isozymes of alkaline phosphatase will be inactivated. Both of these advantages allow for easy sampling and testing of SEAP [34, 35]. CHO cells have been engineered to secrete alkaline phosphatase (ALKP). ALKP’s are naturally occurring enzymes found in many body tissues, especially the liver, bone and kidney. Although ALKP’s function is not fully understood, ALKP’s hydrolyze monophosphate esters in proteins, nucleotides and other molecules in tissues where they reside [36]. The levels of ALKP in the media were measured using a SEAP enzymatic assay and then plotted on a graph.

Figure 9. Established timeline for each bioreactor system. The pitched-blade bioreactor is shown in blue and the packed-bed bioreactor is shown in brown.
2.7 SEAP Enzymatic Assay

The SensoLyte pNPP Secreted Alkaline Phosphatase Reporter Gene Assay (AnaSpec, Freemont, CA) is a powerful tool used in the detection of alkaline phosphatase. The gene expression levels of Alkaline Phosphatase can be determined with the use of the Alkaline Phosphatase Reporter Gene Assay. All experimental runs were followed by the SEAP Enzymatic Assay and the results from each bioreactor type were compared to see which bioreactor allowed for more protein production. All cell free supernatant samples were incubated at 65°C for 30 minutes and 125µl of cell free supernatant were diluted in 125µl of PBS. Fifty microliters of the diluted supernatant was then inserted into four individual wells per sample of a 96 well plate. Various known dilutions of calf intestinal alkaline phosphatase (CIAP), used as the standard, were also added to each plate. Fifty microliters of a 1:100 solution of pNPP and 2X assay buffer were then added to each sample. The plate was incubated at 37°C for 60 minutes and then immediately measured on a SpectraMax Plus plate reader at 405nm to determine

Figure 10. PLAP amino acid sequence with truncated SEAP amino acid sequence in black. Source: http://www.ncbi.nlm.nih.gov/protein/178476?
the endpoint reading. The change in ALKP production from each experiment was plotted on a graph and compared between each bioreactor system.

2.8 Glucose and Lactate Analysis

The YSI 2700 Select Biochemistry Analyzer (YSI, Inc., Yellow Spring, OH) was utilized to check the glucose and lactate levels in the medium every 24 hours for the duration of each experiment. This instrument provides fast and precise analysis of nutrients or byproducts in the media.

2.9 Cell Count and Viability

The Countess Instrument (Invitrogen, Life Technologies), an automated cell counter, was utilized to count viable cells and calculate cell % viability in the pitched-blade bioreactor. This instrument uses plastic disposable slides with two enclosed chambers. The counting occurs at the center of these chambers where the total volume counted is 0.4μL. The volume equates to counting four 1 mm x 1 mm squares on a standard Hemocytometer. The Countess comes with a special trypan blue stain to only be used with this machine. Conventional cell counting with Hemocytometer and microscope will also be used.

2.10 Growth Curve

The progress and behavior of the rCHO cells were monitored by developing a growth curve. A growth curve is ideal for determining the correct seeding density that will give a short lag period which is the first phase, or the stage directly following a subculture. Cells during this phase are adjusting to the new environment. There is very little growth of cells and sometimes even a loss of cells during this phase is possible. The
second phase is the log or exponential growth phase. As the name implies there is an exponential increase in cell numbers with a growth rate of 90-100%. This is the optimal time for product formation and sampling. The population doubling time can be measured in the middle of the exponential phase. The third phase is the plateau phase where cell proliferation is reduced to 0-10%. In this phase the culture will reach confluency which will give the maximum cell density that can be reached in the vessel. Cells for experimental purposes should always be taken from the middle of the log phase to the plateau phase. Several parameters can be measured from the established growth curve. The first parameter is the lag time which is the adaptation time the cells need to again reach the initial seeding density in the lag phase. The second parameter is the doubling time which is the time needed for the cells to increase twofold in the middle of the exponential phase and subsequent doublings that take place before the cells enter senescence or cell death phase. The third parameter is the saturation density which is defined as the maximum number of cells per mL [8]. Seven 60mm tissue culture dishes were seeded with $10^4$ cells in 5 mL media. One dish was counted every other day over a 14-day period and the points were plotted on a graph. 5 mL of fresh media were added to the remaining culture dishes on days 4 and 12. Cells were spun down on day 8 and resuspended in 5 mL of fresh media. The growth curve helped in selecting the optimal time in the exponential phase for induction. During this stage cells are viable and actively dividing.
CHAPTER 3
RESULTS

3.1 Pitched-Blade Bioreactor Operated in Batch Mode

3.1.1 Cell Density and Viability

Figure 11 shows cell growth and viability in three independent experiments in the pitched-blade bioreactor. Seeding densities were $0.29 - 0.57 \times 10^6$ cells/mL as calculated by trypan blue staining utilizing the Countess cell counter. A slight decrease in cell density occurred in experiments 1 and 2 between days 3 and 4, suggesting that the optimal time for induction occurred on day 3. At the time of induction, cell densities were $1.5 \times 10^6$ cells/mL in both experiments 1 and 2 (Fig. 11A-B). The maximum cell density observed in experiment 1 ($2.7 \times 10^6$ cells/mL) was relatively lower than in experiment 2 ($3.5 \times 10^6$ cells/mL). Due to the rapid exhaustion of glucose in experiment 3, it was necessary to induce the cells on day 3. Cell density in experiment 3 was $2.0 \times 10^6$ cells/mL at the time of induction, and the maximum cell density observed was $2.2 \times 10^6$ cells/mL (Fig. 14C). Cell viability was greater than 90% in all experiment trials with the pitched-blade bioreactor.

3.1.2 Glucose Utilization and Lactate Production

Glucose is the main energy source for cell proliferation and ALKP production. Thus, glucose levels and cell densities were expected to directly correlate with ALKP production in each experiment. Because lactate is a secondary energy source, lactate levels were expected to decline following this initial increase and the utilization of glucose in the media. Lactate metabolism is beneficial to the system by reducing a major metabolic by-product from the system [37, 38]. Glucose levels measured at the time of
induction were 2.6 g/L in experiments 1 and 2, while glucose levels measured at the time of induction in experiment 3 were nearly depleted at 0.8 g/L (Fig. 12). Glucose levels in the first two experiments were sufficient to sustain ALKP production. However, glucose consumption by the cells in experiment 3 depleted amounts necessary to support the production of ALKP. Lactate levels in experiment 3 were nearly 1.5 g/L higher than the previous experiments, suggesting that cell replication may have been inhibited (Fig. 12C). Similar results were reported by others using CHO and NSO cells [37].

3.1.3 ALKP Production

Figure 13 shows the concentrations of ALKP measured daily or at the time of cell harvest in each of the three independent replicated experiments utilizing the pitched-blade bioreactor. ALKP concentrations steadily increased over the six culture days post induction. However, a decrease in ALKP activity was observed at the time of harvest in trials 2 and 3, possibly due to degradation of the ALKP protein at the end of the experiment [39, 40]. Serum free media was utilized for growth of rCHO cells in this study. Thus, ALKP was susceptible to the action of proteases made by the rCHO cells [41]. As compared to the first trial, the increased cell density observed in experiment 2 indicates that cell proliferation was more predominant than the production of ALKP (Fig. 13). As reported above, glucose levels and cell density in trial 3 were the lowest of all the replicate experiments, and these observations likely account for the low amount of ALKP detected.
Figure 1. Growth of rCHO cells in pitched-blade bioreactor system. Values shown are the cell density and viability on each day of culture, and each panel represents an independent experiment trial (A, trial 1; B, trial 2; C, trial 3). The dashed line indicates the time of induction of ALKP production by IPTG.
Figure 12. Glucose consumption and lactate production by rCHO cells cultured in the pitched-blade bioreactor system. Values shown are the concentrations of glucose and lactate in the culture media measured daily, and each panel represents an independent experiment trial (A, trial 1; B, trial 2; C, trial 3). The dashed line indicates the time of induction of ALKP production by IPTG.
Figure 13. ALKP production by rCHO cells cultured in the pitched-blade bioreactor system. (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP occurred on culture day four for experimental trials 1 and 2 and on day three for experiment 3. (B) Values shown are the media ALKP concentrations at time of cell harvest for all three experimental trials; also shown is the mean total production of ALKP ± SEM for all three experimental trials.
3.2 Packed-Bed Bioreactor Operated in Perfusion Mode

3.2.1 Cell Density and Viability

The packed-bed bioreactors in all three replicate experiments were seeded with 5.0 x 10^5 cells/mL. However, because of the presence of the Fibra-Cel discs, it was not possible to sample the cells directly during culture to determine yield and viability. Therefore, cell count and viability were not monitored on a daily basis; rather, the rate of glucose consumption was used as a surrogate to approximate changes in cell density [15]. The growth of cells in the packed-bed reactor was estimated using the average glucose consumption rate and rate growth of cells in the pitched-blade bioreactor. Glucose consumption rates were markedly similar in both the pitched-blade and packed-bed bioreactor systems up to day 4 (Fig. 14). However, after day four of culture, glucose utilization in the packed-bed bioreactor continued to increase exponentially, while the trend for glucose consumption in the pitched-blade bioreactor increased linearly. Increased glucose consumption observed on culture day five suggests that cell density in the packed-bed bioreactor had likely increased.

3.2.2 Glucose Utilization and Lactate Production

Most of the glucose consumption by the cells in experiment 1 was observed prior to media exchanges during the perfusion process (Fig. 15A). Cells in experiment 2 utilized the least amount of glucose in the media prior to repeat media exchanges (Fig. 15B), whereas the majority of glucose used by the cells in experiment 3 occurred within the first 24 hours after each media exchange (Fig. 15C). As previously observed with the pitch-blade bioreactor system, media lactate concentrations increased in response to decreasing glucose availability in all three trials (Fig. 15.)
3.2.3 ALKP Production

Concentrations of ALKP in the three independent experiments utilizing the packed-bed bioreactor are shown in Figure 16. Following initial expansion culture of rCHO cells for five days, ALKP production was induced every two days with a media exchange containing IPTG. We determined previously that continuous culture with IPTG in the media yielded greater production of ALKP compared to a transient exposure to the inducing agent (data not shown). A modest increase in ALKP production was observed at each media exchange, although the level of ALKP varied by induction day and by experiment trial (Fig. 16). The correlation between glucose utilization and ALKP production observed previously supports the inference that the rate of glucose consumption in experiment 1 and 2 was conducive to the production of moderate amounts of ALKP. However, the pattern of glucose utilization and ALKP was notably different in trial 3 (Fig. 16C). The rapid exhaustion of glucose and a presumed high cell density were likely contributing factors to the noticeably greater amounts of ALKP observed in experiment 3 compared to the other trials.
Figure 14. Comparison of glucose up-take by rCHO cells in pitched-blade and packed-bed bioreactor systems. Values shown are the mean daily glucose media concentrations consumed $\pm$ SEM of three replicate experiments.
Figure 15. Glucose consumption and lactate production by rCHO cells cultured in the packed-bed bioreactor system. Values shown are the amounts of glucose and lactate measured in the culture media at each media exchange. The time and volume of the media exchange is indicated at each dashed line. Induction of ALKP activity by IPTG began on culture day 5 and continued every two days throughout the remainder of the experiment. Results of three experimental trials are shown (A, trial 1; B, trial 2; C, trial 3).
Figure 16. ALKP production by rCHO cells cultured in the packed-bed bioreactor system. (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP began on culture day five and continued every two days for the remainder of the experiment. (B) Stacked bar charts show the cumulative production of ALKP throughout the experiment, with each bar representing a perfusion. Also shown is the mean total production of ALKP ± SEM of all three experimental trials.
3.3 Comparison of Bioreactor Systems for ALKP Production

A major objective of this study was to compare the pitched-blade and the packed-bed bioreactors run in their routine operating modes (batch and perfusion, respectively). The average total ALKP production per experiment trial is shown in Figure 17; overall, the packed-bed bioreactor system produced ALKP to a much greater extent (nearly 3-fold greater) compared to the packed-bed system. This pattern is retained even when accounting for different volumes of culture media used in the two bioreactor systems (1.7 L for the pitched-blade system compared to 1.3 L for the packed-bed system). The total amount of ALKP measured after five media exchanges in the packed-bed bioreactor was 10,500 U compared to 4,640 U in the pitched-blade bioreactor, or approximately a 2.25-fold greater production of ALKP in the packed-bed bioreactor.

![Bar graph comparing ALKP production by rCHO cells cultured in the pitched-blade and packed-bed bioreactor systems.](image)

**Figure 17.** Comparison of ALKP production by rCHO cells cultured in the pitch-blade and packed-bed bioreactor systems. Values shown are the mean ALKP concentration ± SEM for three replicate, independent experimental trials using either the pitch-blade or packed-bed bioreactor systems.
3.4 Growth Curve Results

Figure 18 shows results from a 14-day growth curve experiment. Three groups of seven 60mm tissue culture dishes were seeded with $1 \times 10^4$ cells in 5 mL media. One dish was counted every other day for 14 days and the total cell count per plate was used to plot the growth curve. Cells were in the Lag phase for a very short period of time as the cells quickly started to divide. Cells quickly entered the Log phase which lasted approximately 9 days. Cells entered the Plateau stage on day 10 reaching a maximum density of $1.7 \times 10^7$ total cells on day 12. These growth results suggest that days 5 to 7 were the optimal times for induction. During this period cells are viable and rapidly dividing.

**Figure 18.** Growth curve of CHO-SEAP cells grown in suspension in plastic 60 mm tissue culture dishes. Seven tissue culture dishes were seeded with 10,000 cells. (A) First four days of growth. (B) Complete growth curve.
CHAPTER 4
DISCUSSION AND CONCLUSIONS

Although the bioprocessing industry is currently dominated by the well-proven batch culture process, results of this study suggest that a perfusion mode of operation in a packed-bed culture environment may prove superior in some applications. The perfusion mode of cell culture offers some significant advantages to large-scale cell producers, such as lower set-up and maintenance costs and greater performance [42, 43]. By virtue of its design, the packed-bed bioreactor may be used continuously for months at a time when operated in perfusion mode. The limitation of 15 days was for establishing basic data on the performance of rCHO cells in the bioreactor for commercial production of biopharmaceuticals. The three-dimensional environment of the Fibra-Cel disks provides optimal culture conditions for cell growth and allows for new infusions of media into the system without cell loss. The shear-free culture environment of the packed-bed system offers more flexibility for researchers utilizing anchorage-dependent cell lines for the production of proteins or vaccines. Cell line maintenance is reduced since preparation of new seed cultures is not required. Moreover, initial setup effort associated with the packed-bed bioreactor is substantially less than would be required using the pitched-blade system, which requires multiple, shorter cultures to equal the production of a single culture with the packed-bed system. The perfusion process also offers several advantages, such as the removal of toxic metabolites or excreted proteases and the infusion of fresh nutrients to maintain optimal culture conditions. Protein product quality is likely to be higher with the perfusion system, as freshly secreted cellular proteins may be removed periodically by media exchanges. In case of products that are highly labile,
rapid removal from the packed-bed bioreactor is possible with the perfusion system [39]. However, a disadvantage of the packed-bed bioreactor is limited scalability. The packed-bed system is currently limited to a maximal operating volume of about 100 L, although larger scale packed-bed reactors may be available in the future. Additionally, when considering the cost-benefit of the packed-bed system, the cost of Fibra-Cel disks should be taken into account. Although this cost is not significant considering the value of the protein product.

Although the pitched-blade bioreactor requires less monitoring over the duration of an experiment, as cells are cultured until nutrient depletion has occurred, this approach does require significant initial input in terms of labor and resources. Multiple culture runs, and thus multiple seed cultures and system preparations, are required to match the output of the packed-bed system. In addition, application of a perfusion operation mode to pitched-blade systems requires pumping the cells through a membrane filtration device (e.g., hollow fiber columns) to prevent cell loss [44]. This process can impair bioreactor sterility and cause potential damage to the cells. Finally, cell product concentration may be comparatively low, as exemplified in this study, compared to the initial resources (volume of culture media) invested in the culture system. Given these drawbacks of the pitch-blade system, the added cost of the Fibra-Cel disks ultimately may be irrelevant in a cost-benefit analysis.

In summary, the results of this study, detailed in table 1, show that the packed-bed bioreactor operated in perfusion mode is superior to the pitched-blade bioreactor operated in batch mode for growth of rCHO cells secreting ALKP. Given the greater productivity of cells cultured in the packed-bed bioreactor and the multitude of advantages of this
system operated in perfusion mode, researchers desiring to scale up mammalian cell culture for protein production should strongly consider this strategy.

**Table 1: Comparison of the Pitched-Blade and Packed-Bed Bioreactors**

<table>
<thead>
<tr>
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<th>Pitched-Blade Bioreactor</th>
<th>Packed-Bed Bioreactor</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>Starting cell number (10^5 cells)</td>
<td>2.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Highest cell number (10^9 cells)</td>
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<tr>
<td>Duration (h)</td>
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<td>215</td>
</tr>
<tr>
<td>Maximum protein produced (U/ml)</td>
<td>3.57</td>
<td>2.47</td>
</tr>
</tbody>
</table>

*Note. *Counts calculated prior to bioreactor seeding.

**Counts calculated from a sample of Fibra-Cel disks removed from the bioreactor at the end of the run, Inaccurate.

The approach used in these experiments can now be modified in a number of ways. While the present experiments utilized a recombinant CHO cell line, many other cell lines engineered to secrete proteins can be utilized in similar investigations to generalize the utility of the system. With the development of single use bioreactor systems, it is desirable to conduct a comparative study between glass and disposable vessels to determine the feasibility of single use bioreactors. Conducting these experiments with a continuous perfusion mode of operation may also be desirable. In this model media is constantly removed and replaced with fresh media for the duration of the project. This modified perfusion process could potentially reduce the levels of toxic byproducts inside the culture vessel, thereby increasing cell productivity. Repeating these experiments in larger scale bioreactors is also important for process optimization and design.
REFERENCES


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APPENDICES
APPENDIX A

Stirred-Tank Bioreactor preparation as modified from the manufacturer manual of operation

INITIAL PREPERATION OF VESSEL ASSEMBLY

Clean glass vessel jacket and stainless steel headplate and base. Use a soft brush or sponge, warm tap water and mild cell culture detergent to clean all parts. Rinse several times with tap water until the detergent is removed. Rinse one final time with distilled water and allow vessel to dry.

Check the integrity of the O-ring at the base of the vessel. Replace O-ring if necessary.

Siliconize the O-ring at the base of the vessel assembly with food grade Silicon Grease. When the O-ring is seated in the O-ring groove, the O ring should extend above the level of the metal plate.

Place the glass vessel on the metal base and secure the bolts by finger tightening until the base and vessel are firmly attached to each other. Do not over tighten as this will result in a poor seal and excessive wear on the O-ring.

BASKET IMPELLER ASSEMBLY

Before assembling the basket make sure to understand the correct positioning of the basket.

Insert the bottom of the basket into the vessel. The rubber gasket around the basket insert will need to be moistened to assist in pushing the basket to the bottom of the glass vessel. Make sure to keep basket horizontal as pressure is applied on the basket insert.

Once properly installed make sure bottom basket insert is level to avoid any shearing of the impeller against the inner draft tube.

Fill the basket with Fibra-Cel disks. 100g/L of basket volume is the recommended filling density. The basket volume is about 50% of the working volume. Vessel to be used has a total working volume of 1.7 liters. 85g of Fibra-Cel disks was used.

Insert the top of the basket into the vessel. The rubber gasket around the basket insert will need to be moistened to assist in pushing the basket into the glass vessel. Align the small and large holes in the top insert with the appropriate stainless steel well tubes in the bottom insert.
ASSEMBLING THE HEADPLATE

Check the integrity of the O-ring on the headplate and each port. Replace any O-rings if necessary.

Siliconize the large O-ring on the headplate with food grade Silicon Grease.

Place the headplate on the glass vessel allowing the basket impeller to be positioned in the central draft tube. The headplate for the pitch blade system is not restricted in its placement.

Align the headplate on the vessel so that the sampler port is directly above the small well in the basket.

Insert the Harvest tube until it reaches the bottom of the glass vessel and fasten it to the headplate. Attach silicone tubing at the appropriate length to the exposed tube and secure with a tie wrap. The opposite end of the tubing will have an Aseptiquik Connector (Colder Products Company) attached to it.

![Figure 19. Aseptiquik connector](image)

Insert the Thermowell into the headplate. The thermowell only extends partially down the glass vessel, just above the top of the basket. In the pitch blade system the thermowell goes down close to the bottom of the glass vessel.

Insert the Addition ports into the headplate. The addition port has three addition tubes. In this setup only one of the ports will be used to add base into the system. The other two ports will be sealed off by securing a small piece of silicon tubing attached to each of the two unused ports.
**Insert the Inoculation port** into the headplate. Attach silicone tubing at the appropriate length to the exposed tube and secure with a tie wrap. The opposite end of the tubing will have an Aseptiquik Connector (Colder Products Company) attached to it.

**Insert the Sampler port** with the attached valve into the headplate. Connect the sample bottle and rubber bulb.

**Insert the Exhaust Condenser** onto the headplate. The exhaust condenser is secured by a ring nut that needs to be finger tightened. Attach a small piece of silicone tubing to the outlet with a 0.2µ filter at the end of the tubing.

**Insert the Sparger port** into the headplate. *(Only necessary on the pitch blade system.)*

**Insert the Dissolved Oxygen (DO) probe** into the headplate. First check the integrity of the DO probe membrane. Second replace the electrolyte solution inside the DO probe that surrounds the sensor. Lightly wet the DO probe to assist in inserting it into the appropriate port in the headplate.

Plug any remaining ports in the headplate with stainless steel blind stoppers.

Secure the headplate to the glass vessel by finger tightening the thumb screws a little by little. Tighten the screws in a star pattern and not one at a time in a circular pattern. Do not over tighten as this will result in a poor seal and excessive wear on the O-ring.

**CALIBRATE THE pH PROBE**

Connect the pH probe to the pH connector cable that is connected to the control cabinet. Do not twist the cable to prevent damaging it. After removing the protective red cap on the pH probe proceed to turn the probe to make the connection.

Prepare pH calibration buffers. Place approximately 15mL of pH 7.0 and 4.0 solutions into 50mL tubes.

**Calibrate the ZERO with buffer pH 7.0.** Select the calibration screen on the Bioflo 310 consol screen and press pH under loops. Rinse pH probe with distilled water and then place it in tube containing pH 7.0 and allow the probe to equilibrate for a few minutes. Touch the SET ZERO edit box and enter 7.0 and press OK. Press the Set Zero button.

**Calibrate the SPAN with buffer pH 4.0.** Rinse pH probe with distilled water and then place it in tube containing pH 4.0 and allow the probe to equilibrate for a few minutes. Touch the SET SPAN edit box and enter 4.0 and press OK. Press the Set Span button.

Disconnected pH probe from cable and place red protective cap on the pH probe.
Insert the pH probe into the headplate. Use CAUTION and wear protective gloves when installing probes in case of accidental breakage. Lightly wet the pH probe to assist in inserting it into the appropriate port in the headplate.

- NOTE: the pH calibration should be checked after autoclaving. Before inoculating, take a sample of media and using an offline pH meter, compare it to the pH from the control cabinet. Adjust any differences with the SET ZERO procedure.

CONNECTING AND DISCONNECTING VESSEL WATER SUPPLY

- Before proceeding be sure to read and follow these procedures.

Connecting vessel: 1) Connect vessel Water Out line,
2) Connect vessel Water In line,
3) Connect Main Water In line,
4) Turn ON main power switch on cabinet.

Disconnecting vessel: 1) Turn OFF main power switch on cabinet,
2) Disconnect Main Water In line from cabinet,
3) Disconnect vessel Water In line,
4) Disconnect vessel Water Out line.

INSPECTION FOR FUNCTIONALITY BEFORE AUToclAVING

Fill the water jacket with water. After the water supply has been properly connected to the vessel following the Connecting Vessel Water Supply procedure press the temperature control loop on the consol screen. Enter the temperature setpoint at least 12°C below the current value and turn the temperature control to ON. The water jacket will fill with water.

Temperature Functionality Test. Insert the RTD sensor into the thermowell and connect the sensor to the appropriate cable on the consol cabinet. Set the temperature to 37°C and observe the change in temperature on the control screen.

Sparging Functionality Test. Connect the gas line on the consol cabinet to the vessel assembly. On the control screen turn the gas on. Observe bubbles coming from the top of the central shaft in the basket bioreactor and the sparger at the bottom of the pitch blade bioreactor.

Agitation Functionality Test. Attach the motor to the magnetic drive coupling and connect the motor to the appropriate connector on the consol cabinet. Set the agitation to
100 RPM and observe the turning of the discharge tubes in the basket bioreactor and the turning of the pitch blade in the pitch blade bioreactor.

**Dissolved Oxygen Probe Functionality Test.** Connect the DO probe to the appropriate cable on the consol cabinet. Observe any changes in the Present Value (PV) on the control screen.

**PREPARING VESSEL FOR AUTOCLAVE**

**Fill the glass vessel with Phosphate Buffer Saline (PBS).** Place a funnel in one of the free ports in the headplate and fill glass vessel with PBS. The amount of PBS depends on volume of vessel assembly. Vessel to be used has a total working volume of 2.0 liters. Approximately 1.5 liters of PBS will be used.

**Fill the water jacket half full with water.** After the water supply has been properly connected to the vessel following the Connecting Vessel Water Supply procedure press the temperature control loop on the consol screen. Enter the temperature setpoint at least 12°C below the current value and turn the temperature control to ON. The jacket will then begin to fill with water. Once the jacket is half full, turn the temperature control to OFF.

**Disconnect** all water lines following the Disconnecting Vessel Water Supply. Also disconnect all other probes, sensors and gas lines from the headplate.

**Cover any filters with tin foil** before autoclaving to allow the filters to vent during the autoclaving process. The foil also prevents moisture from clogging them up.

**Secure all tubing** with a plastic clamp. To ensure that the clamps do not fail during autoclaving, place a plastic cable tie around the clamp.

**Prior to placing the vessel in the autoclave** be sure to loosen the sampling bottle and the four thumb screws connecting the headplate to the glass vessel by ½ turn. This allows pressure to equalize and to prevent the glass vessel from breaking during the autoclave process.

**Sterilize vessel assembly** for 30 minutes after autoclave reached 121°C. The liquid cycle with slow exhaust should be selected on the autoclave.

**Remove vessel assembly** from the autoclave using appropriate gloves. Immediately finger tighten the thumb screws and the sample glass bottle. Allow the vessel assembly to cool at room temperature before connecting it to the consol.

**CONDITIONING VESSEL ASSEMBLY FOR INOCULATION**
Place vessel assembly next to the console cabinet.

Connect vessel and condenser Water In and Water Out lines following the Connecting Vessel Water Supply procedure.

Connect condenser Water In and Water Out lines following the Connecting Vessel Water Supply procedure. The water line out connects to the quick connect fitting at the top of the condenser. The water in line connects the quick connect fitting at the bottom of the condenser.

Insert the RTD sensor into the thermowell and add ~2mL of distilled water or glycerol to the thermowell.

Fill the vessel jacket with water. After the water supply has been properly connected to the vessel following the Connecting Vessel Water Supply procedure press the temperature control loop on the consol screen. Enter the temperature setpoint to 37°C and turn the temperature control to ON. The jacket will then begin to fill with water to slowly cool the vessel assembly.

Connect the pH probe to the pH probe cable.

Connect the DO probe to the DO probe cable.

Attach the motor to the magnetic drive coupling and set the agitation to 100 RPM.

Connect the gas line to the sparger inlet line on the vessel.

CALIBRATE THE DO PROBE

Connect the DO probe to the DO connector cable that is connected to the control cabinet. Do not twist the cable to prevent damaging it. After removing the protective cap on the DO probe proceed to connect the cable to the DO probe.

Calibrate the ZERO. Select the calibration screen on the Bioflo 310 consol screen and press DO under loops. Disconnect the DO probe from the cable and allow the probe to equilibrate for a few seconds. Touch the SET ZERO edit box and enter 0.0 and press OK. Press the Set Zero button.

Calibrate the SPAN. Connect the DO probe the DO probe cable. Select agitation under loops and turn the agitation to 100. Under the gas flow loop, turn on the gas flow and exit. Under the O2 loop, sparge in 100% oxygen and allow medium to mix for a few minutes. Touch the SET SPAN edit box and enter 100 and press OK. Press the Set Span button.
REMOVE PBS FROM VESSEL

**Turn off the agitation** control loop from the consol screen.

**Turn off the gas** control loop from the consol screen.

**Turn off the temperature** control loop from the consol screen and remove RTD sensor from thermowell.

Pitched-Blade Bioreactor

**Disconnect all cables and tubing** from the vessel assembly making sure that all tubing is clamped off.

**Under a laminar flow hood**, connect harvest port tubing to a peristaltic pump. Start pumping PBS into a collection bag.

Packed-Bed Bioreactor “Click-Pull-Click”

**Turn off the agitation and gas flow** control loops from the control screen.

**Connect the appropriate blue (Waste collection bag) and white (Harvest port) AseptiQuik Connectors.** Once properly connected a click sound will be made. Pull the provided tabs from the center of the connected pieces. Turn the blue and white connectors in opposite directions to seal the connection. Another click sound will be made. Using a peristaltic pump start pumping the PBS from the vessel into the waste collection bag.

> **NOTE:** Due to the amount of times required to stop the reactor to remove media from the packed-bed bioreactor, AseptiQuik Connectors are used to steriley connect all components to the bioreactor. With this method there is no need for a laminar flow hood when using the packed-bed bioreactor.

ADDING MEDIUM TO THE VESSEL

Pitched-Blade Bioreactor

**Under a laminar flow hood**, connect the medium addition line to a peristaltic pump. Start pumping medium into the vessel. Allow medium to condition to 37° C before adding to bioreactor vessel.

Packed-Bed Bioreactor “Click-Pull-Click”

**Connect the appropriate blue (Media bag) and white (Media addition port) AseptiQuik Connectors.** Once properly connected a click sound will be made. Pull the
provided tabs from the center of the connected pieces. Turn the blue and white connectors in opposite directions to seal the connection. Another click sound will be made. Using a peristaltic pump start pumping media from 10L bag into the bioreactor. Allow medium to condition to 37°C before adding to bioreactor vessel.

INOCULATION

Pitched-Blade Bioreactor

Under a laminar flow hood, connect the medium addition line to a peristaltic pump. Start pumping medium containing cells into the vessel.

Reconnect the vessel to the consol and turn on all control loops.

Measure the pH of a sample of medium. Take a sample of medium from the vessel to an offline calibrated analytical pH meter. Adjust the pH calibration ZERO point if there is a change in the pH reading.

Packed-Bed Bioreactor “Click-Pull-Click”

Connect the appropriate blue (Media bottle containing cells) and white (Cell addition port) AseptiQuik Connectors. Once properly connected a click sound will be made. Pull the provided tabs from the center of the connected pieces. Turn the blue and white connectors in opposite directions to seal the connection. Another click sound will be made. Using a peristaltic pump start pumping the cell suspension from media bottle into the bioreactor.

Turn on the agitation and gas flow control loops. Set the agitation speed to 40rpm for one hour to allow cells sufficient time to accumulate in the Fibra-Cel disk bed. Increase speed to desired level.

Measure the pH of a sample of medium. Take a sample of medium from the vessel to an offline calibrated analytical pH meter. Adjust the pH calibration ZERO point if there is a change in the pH reading.

GROWTH CONDITIONS

Set the temperature setpoint to 37°C under the temperature control loop.

Set the agitation setpoint to 120 RPM under the agitation control loop.

Set the pH setpoint to 7.1 under the pH control loop.

Set the DO setpoint to 35% under the DO control loop.
Set the Gas Flow setpoint to 0.5% under the gas flow control loop.

CULTURE MONITORING

Take daily samples of media from the vessel assembly using the sampler port.

Analyze the samples for glucose consumption and lactate production offline using the YSI 2700 Select Biochemistry Analyzer.

Freeze a small sample for later use. This sample will be used to analyze the amount of protein in the media. The cells in the samples from the pitched-blade bioreactor will be used for RNA analysis.

CELL COUNT AND VIABILITY

Turn on the countess instrument to display the startup screen. The countess is preset to cell mode.

Mix 10µL of cells with 10µL of supplied trypan blue stain in a microfuge tube. Gently mix the sample by pipetting up and down.

Add 10µL of the sample mixture to each chamber labeled A and B on the provided slide. Insert the slide, sample chamber A first, into the slide inlet on the instrument then press the Count Cells button on the touch screen. The image may be adjusted using the Zoom button and quadrants can be viewed using the grid. The image can also be fine tuned using the focus knob on the instrument.

➤ NOTE: Accurate cell counts will not be taken in the packed-bed bioreactor due to the lack of cells in sample.

INDUCION OF PITCHED-BLADE BIOREACTOR

Connect the appropriate blue (Media bottle with IPTG) and white (Addition port) AseptiQuik Connectors. Once properly connected a click sound will be made. Pull the provided tabs from the center of the connected pieces. Turn the blue and white connectors in opposite directions to seal the connection. Another click sound will be made. Using a peristaltic pump start pumping IPTG into the bioreactor. Allow medium to condition to 37°C before adding to bioreactor vessel.
PERFUSION AND INDUCTION OF PACKED-BED BASKET BIOREACTOR

**Start the perfusion 2-3 days post inoculation** by removing 0.5L of media from the vessel. Immediately replace with 0.5L of fresh media.

**On day two of perfusion,** remove 1L of media from the vessel. Immediately replace with 1L of fresh media. Allow medium to condition to 37°C before adding to bioreactor vessel.

**Add IPTG to remaining media in 10L bag.** Once the second perfusion has been performed add the appropriate amount of IPTG to the remaining media. Use the second Aseptiquik Connector attached to the media bag, connect it to a media bottle containing the IPTG. Pump IPTG into the media bag.

**On day three of perfusion,** remove the complete contents of the vessel. Immediately replace with media that contains IPTG in fresh media.

**On days four through fifteen of perfusion,** media will be removed every other day to allow for a longer run time.

➢ **NOTE:** The packed-bed basket assembly allows for complete medium exchange without the loss of any cell mass.

SHUT DOWN

**Pitched-Blade Bioreactor.** Once the viability in the bioreactor falls below 50% turn off all control loops on the console.

**Immediately after shut down** start to clean all parts of the bioreactor. Add bleach or CiDecon to the cell suspension to kill the cell mass. Using soap, clean all parts of the headplate and vessel and flush all addition, harvest and sample ports with hot water.

**Packed-Bed Bioreactor.** On day 15 after analyzing the media sample remove the media in the vessel.

**Take the vessel under the laminar flow hood** and remove a small sample of disks and weight them for later use. Weight out the remaining disks in the bioreactor for a total weight of the disks.

**Immediately start to clean** all parts of the bioreactor. Add bleach or CiDecon to the vessel to kill any cells that remain. Using soap, clean all parts of the headplate and vessel and flush all addition, harvest and sample ports with hot water.
APPENDIX B

Freeze & Thaw Cells
Modified from the manufacturer Protocol

Equipment:

**Laminar Flow Hood** such as BioGARD Hood, model B60-112, The Baker Company, Sanford ME 04073.

**Centrifuge** such as Eppendorf Centrifuge, model 5804R, Eppendorf, Enfield CT, 06082.

**Automated Cell Counter** such as Countess Automated Cell Counter, Invitrogen, Grand Island, NY 14072.

**Incubator** such as CO₂ Incubator, model HERAcell model 150i, Thermo Scientific, Asheville, NC 28801.

**Water bath** such as Dual Chamber Water Bath, model 188, Precision Scientific, Chicago, IL 60647.

**Pipetman** such as Pipetman Classic, model p20, p200 and p1000, Gilson, Inc., Middleton, WI 53562.

**Pipet-Aid** such as Labnet FastPette, Labnet International, Inc., Woodbridge NJ, 07095.

**Nitrogen Tank** such as Thermolyne, model Locator 4, Barnstead International, Dubuque IA, 52004.

**-80°C Freezer** such as Isotemp basic, Model U86-25D2, Thermo Fisher, Asheville NC, 28801.

Supplies:

Permanent marker

50 mL conical tubes

Cryovials

T-75 Flasks

70% Ethanol
Gloves
Kimwipes
DMSO
Freezing container
Pipets (10 mL & 25 mL)
Pipet tips (10 – 1000 µl)

CD-CHO Medium (1000 mL)
- from Gibco, Grand Island, NY 14072
- cat. # 10743029

L-Glutamine
- Mediatech Inc. Glutamine 200MM
- cat. # MT25005LI

Antibiotic-Antimycotic
- Mediatech Inc. cellgro > 100mL
- cat. # MT-30-004-CI

Countess Cell Counting Chamber Slides
- Invitrogen, Grand Island, NY 14072
- cat # C10228

**Procedures:**

1. Thaw PACE CHO-KS Cell Line
   a. Cells are supplied at 1 X 10^7 cells per vial in 10% DMSO in CD CHO medium.
   b. Store cells in liquid nitrogen until ready to thaw.
   c. Thaw cryovial in a 37°C water bath for 2 min.
   d. Pipet out cells in 0.5 mL CD CHO medium into 10 mL total CD CHO (to remove trace amounts of DMSO).
   e. Centrifuge cells for 3 minutes at 1200 rpm at room temperature.
f. Aspirate supernatant above the pellet.

g. Resuspend pellet in 25 mL of CD CHO medium.

h. Transfer cells to a t-75 flask in a 37°C incubator, 5% CO₂.

i. Passage a minimum of twice before use.

2. Freeze PACE CHO-KS Cell Line

   a. Cells are grown to a density of approximately 1 x 10⁶ cells/mL. Viability should be at least 90%.

   b. Centrifuge cells at 1,200 rpm for 3 min at room temperature and carefully aspirate medium.

   c. Resuspend cells in cell freezing medium to obtain the final density of 1 X 10⁷ viable cells/mL.

   d. Aliquot 1 mL of the cell suspension into each cryovial.

   e. Transfer vials to -80°C freezer. Frozen vials are transferred to liquid nitrogen for long-term storage.
APPENDIX C

SensoLyte pNPP Secreted Alkaline Phosphatase Reporter Gene Assay
Modified from the manufacturer Protocol
(With permission from AnaSpec)

Equipment:

**Laminar Flow Hood** such as BioGARD Hood, model B60-112, The Baker Company, Sanford, ME 04073.

**Incubator** such as CO₂ Incubator, model HERAcell model 150i, Thermo Scientific. Asheville, NC 28801.

**Water bath** such as Dual Chamber Water Bath, model 188, Precision Scientific, Chicago, IL 60647.

**Pipetman** such as Pipetman Classic, models p20, p200 and p1000, Gilson, Inc., Middleton, WI 53562.

**Pipetman** such as Fisherbrand, model Finnpipette-12 50-300µl, Thermo Scientific. Asheville, NC 28801.

**Absorbance Microplate Reader** such as SpectraMax Plus, model 384, Molecular Devices, Sunnyvale, CA 94089.

Supplies:

Permanent marker
70% Ethanol
Gloves
Kimwipes
96-Well plate
PBS
Pipet tips (10 – 1000 µl)
Sterile Disposable Reagent Reservoirs
SensoLyte pNPP Secreted Alkaline Phosphatase Reporter Gene Assay Kit
- AnaSpec, Freemont, CA 94555
- Kit Size 500 Assays (96-well)
- cat # 71233

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Component A: pNPP, Placental alkaline phosphatase</td>
<td>1 vial</td>
<td></td>
</tr>
<tr>
<td>Component B: 2X Assay buffer</td>
<td>30 mL</td>
<td></td>
</tr>
<tr>
<td>Component C: Stop Solution (Optional)</td>
<td>30 mL</td>
<td></td>
</tr>
</tbody>
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INTRODUCTION

The placental alkaline phosphatase is the most stable isoenzyme among the four mammalian alkaline phosphatases and it only exists naturally in the placenta of higher primates. These characteristics make placental alkaline phosphatase the enzyme of choice to serve as a reporter gene for the analysis of promoter activity and gene expression in cell culture or animals. The natural form of placental alkaline phosphatase is membrane-anchored. The recombinant form of placental alkaline phosphatase is the secreted alkaline phosphatase (SEAP).\(^1,2\)

The SensoLyte® pNPP Secreted Alkaline Phosphatase Reporter Gene Assay Kit provides a convenient colorimetric assay of placental alkaline phosphatase for both secreted and membrane-bound forms by using pNPP as a phosphatase substrate. The absorbance signal can be read at 405 nm.

References


**Procedures:**

1. Prepare placental alkaline phosphatase containing sample.
   
   1.1 Collect the supernatant of cell culture medium.
   
   Note: the supernatant can be stored at -70°C for later use.

   1.2 Heat the culture supernatant at 65°C for 10-30 min to inactivate the endogenous non-specific alkaline phosphatase. Then cool down to room temperature.

2. Prepare stock solution (first time preparation only).
   
   2.1 **pNPP stock solution**: Reconstitute by adding 250 µL of deionized water into the pNPP vial (Component A). Mix the reagents well. The stock solution will be good for 3-4 weeks if stored at -20°C.

3. Prepare pNPP reaction mixture.
   
   3.1 Dilute pNPP stock solution 1:100 with 2X assay buffer (Component B). Prepare fresh reaction mixture for each experiment.

4. Start the placental alkaline phosphatase detection.

   4.1 Add 50 µL/well of supernatant or cell extract. Include a mock-supernatant (such as PBS) to serve as a negative control.

   4.2 add 50 µL/well of pNPP reaction mixture. Mix the reagents by gently shaking the plate for 30 sec.

4.3 Measure absorbance:

   **For kinetic reading**: Immediately start measuring absorbance at 405 nm continuously and record data every 5 min for 30-60 min.

   **For end-point reading**: Incubate the reaction for 30-60 min. Optional: add 50 µL/well of stop solution (Component C). Shake
the plate on a plate shaker for 1 min before the reading. Measure absorbance at 405 nm.

Note: If the amount of SEAP is low in the sample, the incubate time can be prolonged to overnight.
APPENDIX D

Miscellaneous Equipment Used

Figure 20. Disposable spinner flasks. (Left) 500 mL, (Right) 125 mL.

Figure 21. The YSI 2700 Select biochemistry analyzer.

Figure 22. The Countess instrument, an automated cell counter.
Figure 23. AseptiQuik connector (Colder Products, Part # AQC17006HT & AQC22006HT). Allowed for sterile connections of tubing without the need for laminar flow hood.

Figure 24. (A) Packed-bed basket bioreactor. (B) Pitched-blade bioreactor connected to the BioFlo 310 console.