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EFFECT OF DIFFERENT FEEDS AND GAS SPARGERS ON MONOCLONAL ANTIBODY YIELDS AND INTEGRITY IN STIRRED TANK BIOREACTORS

A Major Qualifying Project Report

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ABSTRACT

The production of monoclonal antibodies (mAbs) is fast becoming a staple of pharmaceutical development. Understanding how differing feeds and bioreactor setups affects the production of mAbs is important for improving yields. In the process of manufacturing mAb IPTG1, an anti-phosphotyrosine antibody produced at Millipore, this project monitored cell viability and secreted antibody yields under a variety of culture conditions, including different media feeds and different gas spargers.

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BACKGROUND

Since the development of the first cultured mammalian cell line in 1952, cell culture and the bioprocess industry have grown enormously. Increased knowledge of biology has facilitated pharmaceutical advancements and scientific research, especially in the production of monoclonal antibodies (mAbs) for targeting specific proteins (Bakalar, 2012). The cells, methods, and biological products are all important aspects of the process of bioproduction.

Bioprocessing

According to the Commission on Life Sciences (1992), Bioprocess engineering can be defined as "…the sub-discipline within biotechnology that is responsible for translating the discoveries of life science into practical products, processes or systems that can serve the needs of society." A wide variety of products have been manufactured using bioprocess engineering, but the best characterized is the biopharmaceutical. The FDA first approved a biopharmaceutical created from mammalian cells in1986 for Muromonab CD3, an immunosuppressant drug given to reduce acute rejection in patients with organ transplants (Emmons and Hunsicker, 1987).

Bioprocessing begins with the adaptation of a specific biological function to produce a protein or other biological molecule of choice. Today, a common method of bioproduction is to attach a gene of interest to a selectable marker, such as an antibiotic resistance gene or dihydrofolate reductase, to allow the selection of cells containing the recombinant DNA (rDNA). Cells that have not successfully integrated the gene of interest usually do not survive the selection agent (Chusainow et al., 2009). Once a stable

cell clone has been developed containing the rDNA, the cells are expanded to produce the molecule of interest. The expansion usually begins in a simple flask, and then proceeds to larger scale bioreactors – devices designed to grow larger cultures of cells to produce significant amounts of the product (Bioreactor, 2013).

Bioreactors

Bioreactors are used to grow large amounts of cells in a controlled environment produce a product. The modern bioreactor comes in sizes ranging from benchtop 3 liter reactors, to industrial reactors that can hold over 10,000 liters of culture (Plunkett, 2011). All bioreactors have a few basic requirements: a sterile environment, a way to provide necessary cellular nutrients, a system to monitor the conditions inside a culture, and a means to stimulate the cells to produce product.

First and foremost, a sterile environment is very important to the health of the culture. Mammalian cells are grown in a rich medium that is highly susceptible to contamination by outside organisms. These contaminants will deprive nutrients from the growing cells, either making it more difficult to produce the protein product needed or choking out the productive cells. Most reactors are sterilized before use by autoclaving, however some companies have moved on to single-use reactors to remove that necessity. Nutrients and gases are also added to the reactor using a sterile approach. Necessary gasses depend on the type of cell, but often include: air, oxygen, or nitrogen for cell health, and carbon dioxide to help control the pH of the reactor. Gases typically pass through sterile filters on their way into the reactor, and will also often pass through a type of sparger to release gases into the culture itself, rather than direct absorption through the

liquid surface. Two common sparger types are sintered and open pipe; each has their pros and cons. Sintered spargers release smaller bubbles which allow faster absorption, however the size of the bubbles produced can be fatal to cells. Open pipe spargers require more gas flow for oxygen absorption, but they tend to produce less cell shear (Sieblist et al., 2011).

Cells are sometimes provided with nutrients or glucose feeds during bioreactor runs. These depend on the process and are developed based on the needs of the cells. The addition of gasses and nutrients to a reactor, and the growth of the cells, changes the conditions inside the vessel. The changing environment must be monitored. Bioreactor systems are connected via computers and probes to monitor pH, dissolved oxygen, temperature, and other variables.

Finally, depending on the type of cultured cell, reactors may need to be agitated to prevent clumping, cell death, and lackluster growth. There are a variety of ways to accomplish this, however the most common uses a stirred tank reactor. Using an agitator, a stirred tank reactor allows for equal distribution of nutrients and cells, and prevents the clumping of suspension culture (Sieblist et al., 2011).

A variety of different strategies are used to ensure bioreactors produce the highest levels of protein possible. The most basic of these is the feed strategy.

Feed Strategies

There are three major feed strategies for bioprocess in bioreactors: batch culture, fed-batch culture, and perfusion (Hu et al., 2011). The batch culture strategy provides all nutrients in the initial media provided to the cells. While this makes the process much

simpler than fed-batch or perfusion and reduces the risk of contamination to the reactor, the limited amount of nutrients prevents the cells from achieving their maximum production ability. Fed-batch cultures allow a set amount of nutrients added to the cultures as they are growing which allows for cells to grow to higher densities and to increase product yield (Hu et al., 2011). Fed-batch is much simpler than perfusion culture. Perfusion culture completely replaces the media of the reactor with a continuous feed throughout the growth process. This allows the reactor to be run indefinitely, however it is not a simple or realistic approach for labs with limited resources. It is also more difficult to scale up (Whitford, 2006). Often the conditions in a bioreactor are initially simulated in a shake flask study to determine the optimal feed strategy and expected growth of the cells.

Once the reactors are grown to the optimum cell density, the protein product of interest is purified from the culture or medium. This is known as downstream bio-proccessing. But before this process can begin, there needs to be confirmation of the protein being produced.

Chinese Hamster Ovary Cells

Chinese Hamster Ovary (CHO) cells are a widespread cell type frequently used in mammalian cell culture and bio-production. These cells were first isolated for use in mammalian culture from an ovary of a Chinese hamster in 1957 by Dr. Theodore T. Puck. Since then, they have widely been utilized to produce antibodies and other proteins for therapies of a variety of different ailments (Jayapal et al., nd). In fact, 70% of therapeutic recombinant proteins on the market have been estimated to be made in CHO

cells (Kim et al., 2012). These cells are advantageous for many reasons, including their resiliency and ability to grow in both suspension and adherent cultures. However, what may be their most valuable characteristic is the DHFR gene deficiency in some mutant strains (Wurm and Hacker, 2011). The purpose of the DHFR gene (dihydrofolate reductase) is to convert dihydrofolate to tetrahydrofolate, allowing CHO cells to synthesize specific amino acids (NCBI, 2013). In some CHO cell lines, the DHFR gene is inactive, making it difficult for the cells to effectively utilize such nutrients as purine, pyrimidine, or glycine. When rDNA encoding a desired product is transformed into DHFR-mutant cells, the gene of interest is attached to a DHFR gene. Only cells receiving WT DHFR from the rDNA construct will be survive on the selection medium (Camire, 2000). This method of selection provides a powerful way for scientists to transform DNA into cells and select for the recombinants.

Monoclonal Antibodies

Antibodies are an essential part of the mammalian immune system. These proteins bind to foreign proteins and cells such as bacteria and viruses, and trigger an immune response throughout the body. Each antibody detects a specific antigen, thus allowing for a very precise response (Bakalar, 2012). The monoclonal antibody (mAb) is a very common product of mammalian cell culture and bioproduction. The term monoclonal antibody refers specifically to an antibody derived from a single B-cell clone, so it has one type of specificity. mAbs are made in a laboratory. The first mAbs designed specifically for treatment of human diseases were developed in the early 1980s (Emmons and Hunsicker, 1987). Since then, the mAb industry has grown exponentially. As of

2010, there were seven approved mAb drugs on the market and over 130 more in clinical trials. The current approved mAbs treat diseases ranging from rheumatoid arthritis and lupus to melanomas (Nelson et al., 2010). mAbs are not just used for treating diseases however, they can also be used for diagnostic tests, and biological study.

The structure of the mAb (**Figure-1**) is very important for understanding how to use and confirm the presence of an antibody in a bioprocess culture. Antibodies are made of two identical strands known as heavy chains (green in the figure) and two identical light chains (yellow). These chains are bound together with disulfide bonds.

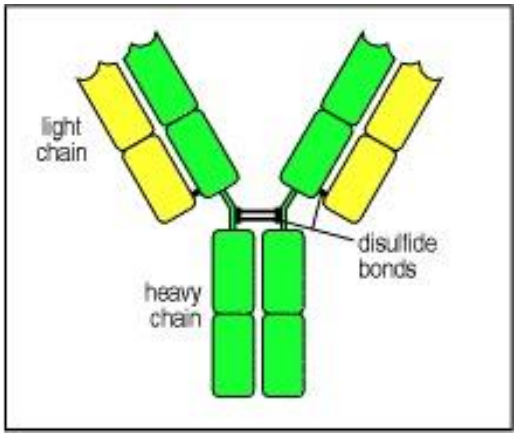

Figure-1: Diagram of a Typical Antibody. The structure is composed of two heavy chains (green) and two light chains (yellow), held together by disulfide bonds. (Janeway et al., 2001)

Y-shaped structure is integral to the functioning of each antibody, as it allows for binding to a specific antigen, while also permitting the antibody to bind to other antibodies and molecules to make higher order complexes. This characteristic is often exploited for scientific research and other applications (Janeway et al., 2001). Protein-A, a surface protein found in specific bacteria, readily binds to antibodies, so scientists frequently use Protein-A to both purify and quantify antibodies.

PROJECT PURPOSE

The purpose of this project is to prove that altering bioreactor feeds based on the analysis of cells and byproducts can increase the yield of a test mAb (IPTG1) being produced at Millipore Corp. This goal was accomplished by comparing cell viability and secreted antibody titers for various bioreactor feeds and gas spargers.

METHODS

Bioreactor Setup

CHO cells were thawed two weeks prior to use in Cellvento 200 chemically defined culture medium (Merck Millipore) supplemented with 4 mM glutamine and 20 μM puromycin. The puromycin is used as a selection agent to ensure all the cells are producing the desired antibody. Cells were passaged twice a week until they reached the necessary density, carefully keeping the cells in growth phase during this time, as overgrown cells tend to lag once added to a reactor.

The setup of a single-use stirred tank bioreactor varied from process to process. The reactors used were Mobius CellReady 3L single-use bioreactors. Reactors were completely set up the night before with 2 liters of Cellvento 200 medium and 4 mM glutamine. All the settings used for each reactor run are shown below in the table.

	Temperature	Agitator Speed	pH Set Point	DO Set Point	Sparger	Air Flow Rate	Oxygen Flow Rate
Run 1	36.8°C	180 RPM	7	50%	Open Pipe	0-100 mL/min	0-100 mL/min
Run 2	36.8°C	180 RPM	7	50%	Open Pipe	0-100 mL/min	0-100 mL/min
Run 3	36.8°C	180 RPM	7	50%	Sintered Sparger	0-100 mL/min	0-25 mL/min

The setup serves two purposes: 1) bring the medium up to running temperature and O_2 concentration, and 2) ensure the medium in the reactor was not contaminated by poor autoclaving of the DO or pH probes. The following day, the reactors were seeded at 0.5 x 10^5 cells per ml. Prior to being added to the reactor, cells were counted using a Beckman Coulter ViCell.

Feed Strategy

The bioreactors were fed according to the CellVento 200 guidelines. This requires three different feeds per reactor: a CellVento feed, a mixed supplements feed, and a cystine tyrosine feed. All reactors were fed on days 3, 5, 7 and 10 with all three feeds. Glucose was checked and supplemented when needed to maintain a concentration of 4 grams per liter. Over the course of the experiment, both the CellVento feed and the cystine tyrosine feed were modified to produce better growth and mAb titers in the reactors.

	CellVento 200 Feed	Mixed Supplements Feed	Cystine/Tyrosine Feed
Day 3	30 ml/L	3 ml/L	5.3 ml/L
Day 5	60 ml/L	6 ml/L	5.3 ml/L
Day 7	60 ml/L	6 ml/L	5.3 ml/L
Day 10	60/ml/L	6 ml/L	5.3 ml/L

Bioreactor Sampling

Reactors were sampled twice a day. A 3 milliliter purge was done using the sampling port and then 5 milliliter sample was withdrawn. This sample is immediately run on the Nova Biomedical BioProfile FLEX analyzer to accurately measure the carbon dioxide and oxygen concentrations. Following that, the sample was diluted 1 to 2 with accumax de-clumping agent, and the sample was run on the vicell in order to accurately count cell density and viability. The remaining portion of sample was spun down at a high speed for 5 minutes, and the supernatant which contained the secreted mAb product, was collected then placed in a -20°C freezer until titer measurements could be completed.

Measurement of mAb Titer

The antibody titer of the reactors was measured after each run was completed. This was measured using a ForteBio Octet instrument which uses Protein A sensors to quantify the amount of antibody present in the supernatant. First, medium was added to all the wells in column A of a 96 well plate to serve as a blanking solution for the assay. An antibody standard curve was created in the next two rows with known concentrations of IPTG antibody which was purified in-house. The medium samples were diluted and added to the wells. This assay was run using protein-A sensors and the direct quantification program on the instrument.

RESULTS

This project investigated whether altering bioreactor feeds based on the analysis of cells and by-products can increase the amount of a test mAb (IPTG1) produced. This was accomplished by comparing various bioreactor feeds while monitoring cell viability and antibody titers. Single-use 3-liter stirred tank bioreactors were used. Reactors 1 and 2 were set up identically (duplicates) at 36.8°C, 180 rpm stirring, 7.0 pH set point, dissolved O_2 set at 50%, Open pipe type gas sparger, 0-100 ml/min air flow rate, and 0-100 ml/min O_2 flow rate.

Using the commercially available feeds and growth medium discussed in Methods, a control run of reactors 1 and 2 was completed. **Figure-2** shows the cell viability and viable cell yield, while **Figure-3** shows the antibody yields. This initial run was performed to serve as a baseline in order to optimize cell viability and density for future runs with modified feeds. The data indicate that the cell viability, viable cell density, and antibody yields were very consistent between the two identical runs. Cell viability (solid curves) remained near 100% until around day-10 and then dropped slightly. Viable cell density peaked around day-6 at approximately 12 million cells per ml. IPTG antibody yields increased consistently throughout the runs, and peaked around days 10-11 at approximately 0.27 mg/ml.

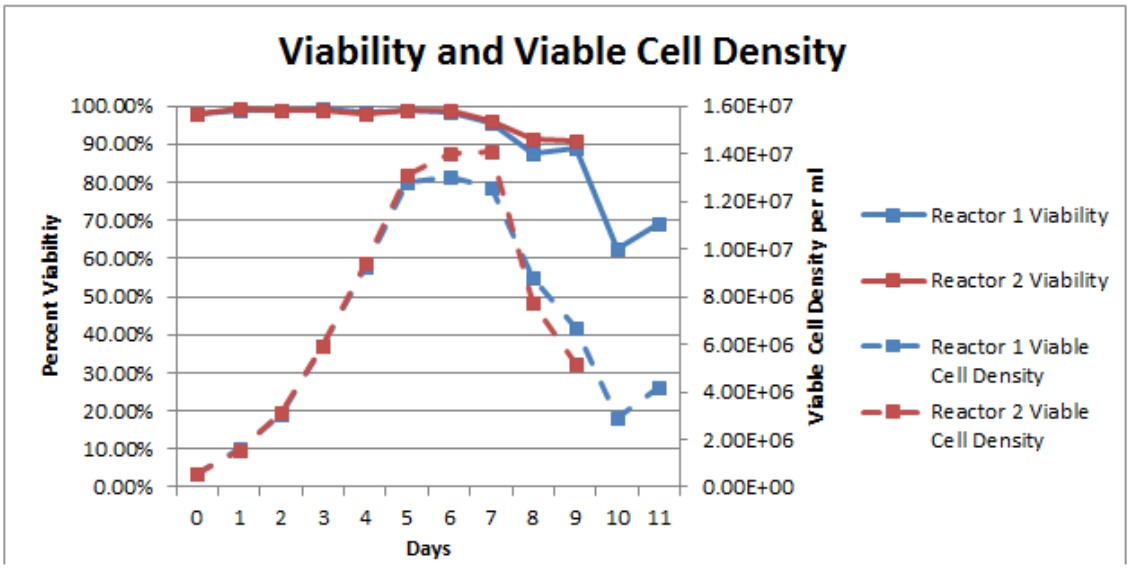

Figure-2: Plot of Cell Viability and Viable Cell Density for 11 Days. Although the viability (solid lines) for both reactors 1 and 2 drops considerably at day 7, the viable cell density (dotted lines) reached above 12 million cells per milliliter at peak.

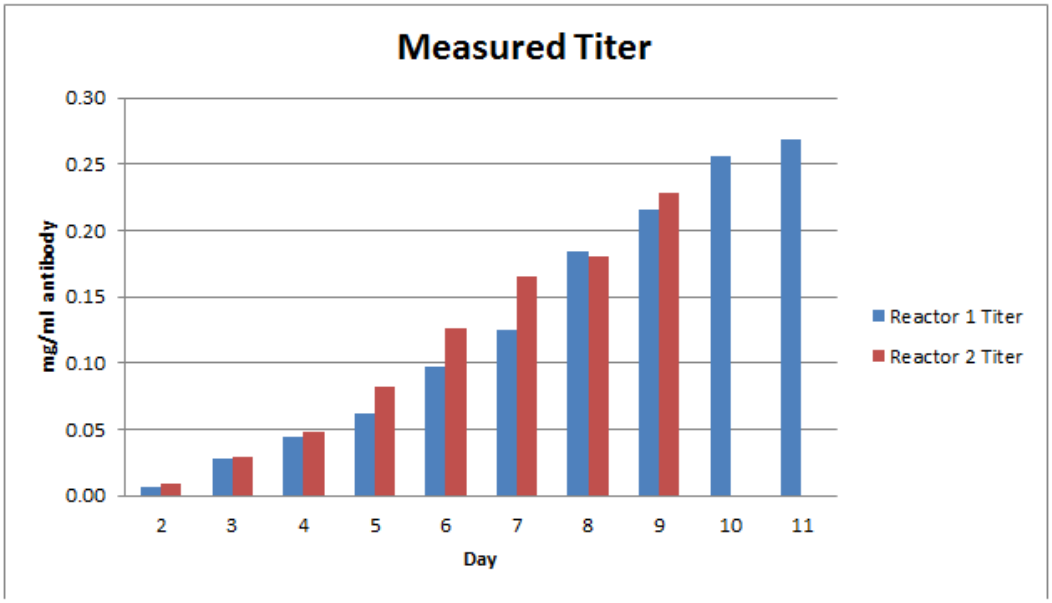

Figure-3: Plot of Antibody Yield (mg/ml). The yield was measured using a protein sensor in the octet assay. Both reactors reached a high of around 0.27 mg/ml of IPTG antibody.

Following the initial set of baseline reactors, a series of five different reactors were run with changes to their feeds: one control reactor, two reactors with a modified version of the CellVento 200 feed (referred to as modified feed A), and two reactors with a modified version of the CellVento 200 feed (referenced as modified feed B) (**Figures 4 and 5**). The reactors with the modified feeds were also run with double the normal amount of the cystine and tyrosine feed. The viable cell density was highest for the control reactor, peaking at approximately 17 million cells per ml. All other feed conditions produced approximately equal viable cell densities, slightly underneath the control reactor. The antibody yields were also highest for the control reactor, reaching 0.30 mg/ml at day-12. Feed-B produced the next highest yield at around 0.25 mg/ml at day-10.

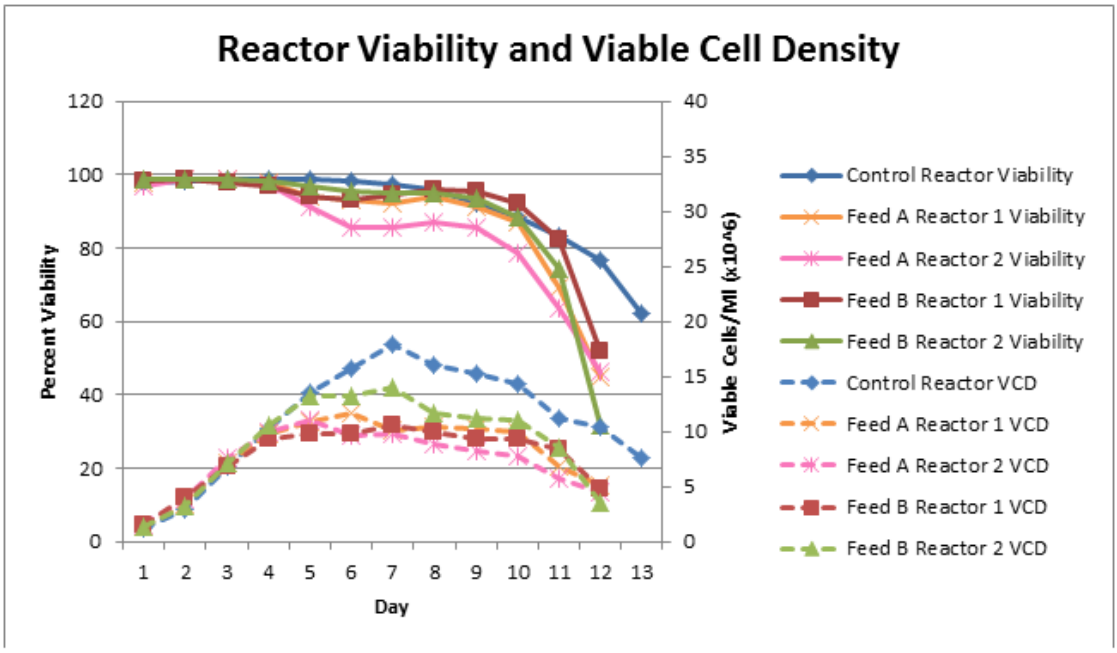

Figure-4: Plots of Cell Viability and Viable Cell Density For Reactors With Different Feeds. The viable cell density (dotted lines) reached much higher in the control reactors as it did in the reactors with variable feeds.

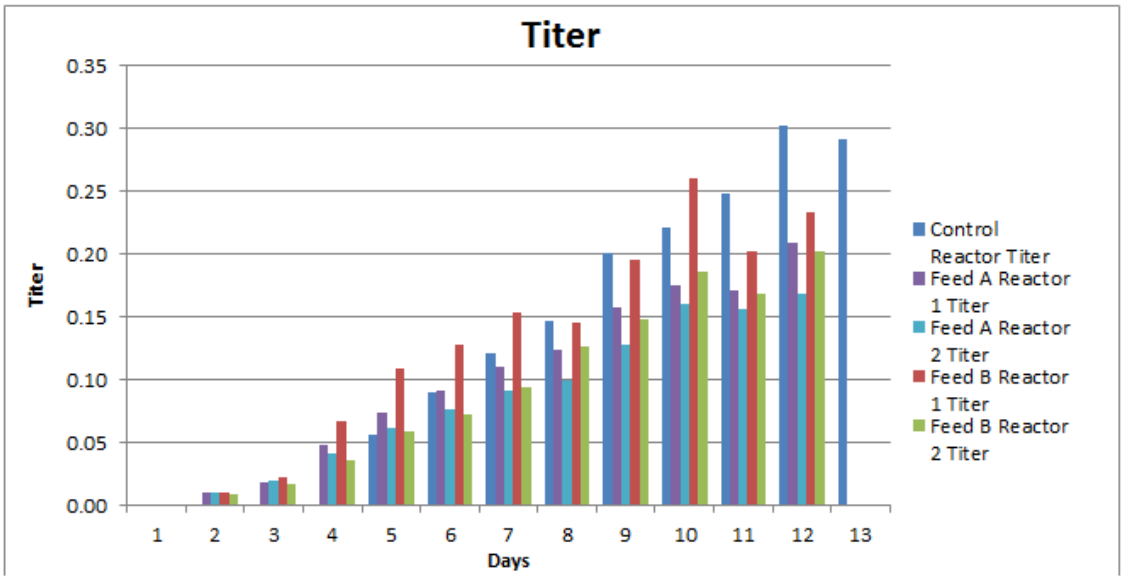

Figure-5: Plot of Antibody Yields (mg/ml) For Runs with Different Feeds. This graph of the reactor titer over tie also shows the control reactor made significantly more titer over time as well.

During the final days of this second set of reactors, crystals of unknown origin were seen while running cell counts. These crystals are shown in **Figure-6.** Because of the high levels of cystine added in the modified cystine tyrosine feeds, it was proposed that these crystals were cystine crystals forming after the nutrient had fallen out of solution. The presence of these crystals could explain the slower growth and lower viability of these reactors as the cells would be unable to take up the cystine in crystallized form. The crystals may also trap cells, killing them, which could have caused the lower viability.

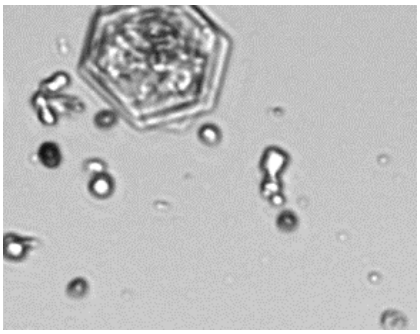

Figure-6: Photo of Presumed Cystine Crystals. Show is a photograph of crystals believed to be cystine precipitating out of the culture due to its high concentration. This picture is from Feed-A Reactor-2, however similar crystals were found in all reactors besides the control reactor. Photo taken using Beckman Coulter Vicell.

Another possible cause of the observed decreased viability and cell density of this reactor run was the high levels of carbon dioxide measured after the addition of the feeds (**Figure-7).** The cystine tyrosine feed reactor in particular had a very high pH, so CO_2 was added to the reactors to lower the pH, and was unable to be stripped away. This resulted in a high percent CO2 in the reactors, seen in the figure.

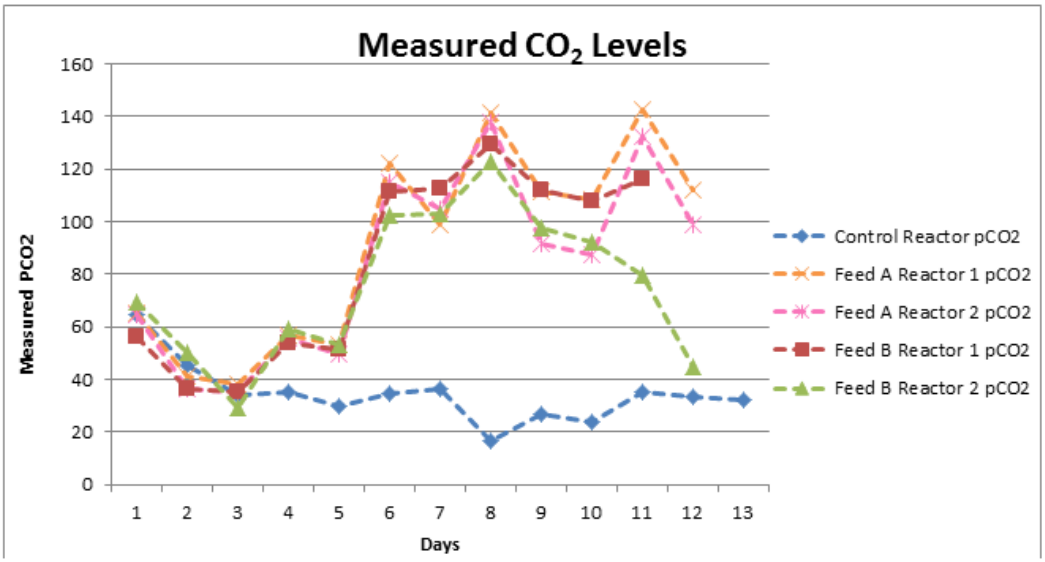

Figure-7: Measured Percent CO_2 in the Reactors With Different Feeds. CO_2 was added to lower the pH of the reactor. It was very high in the experimental reactors but remained at a reasonable level in the control reactor (blue dotted line).

One final set of reactors was run with a control reactor and two different modified feed reactors all with the normal concentration cystine/tyrosine feed, and one reactor with normal feeds but a low concentration of cystine/tyrosine. A different gas strategy was tested using a sintered-type gas sparger instead of an open pipe to remove the carbon dioxide faster from the culture (**Figures-8 and 9**). As shown in **Figure 8,** the cell viability and viable cell density of all these reactors with the sintered sparger were lower than the previous runs with the pipe sparger, including in the control reactor. The mAb titer of the reactor (**Figure 9**) is also low, likely reflecting the low growth of the cells.

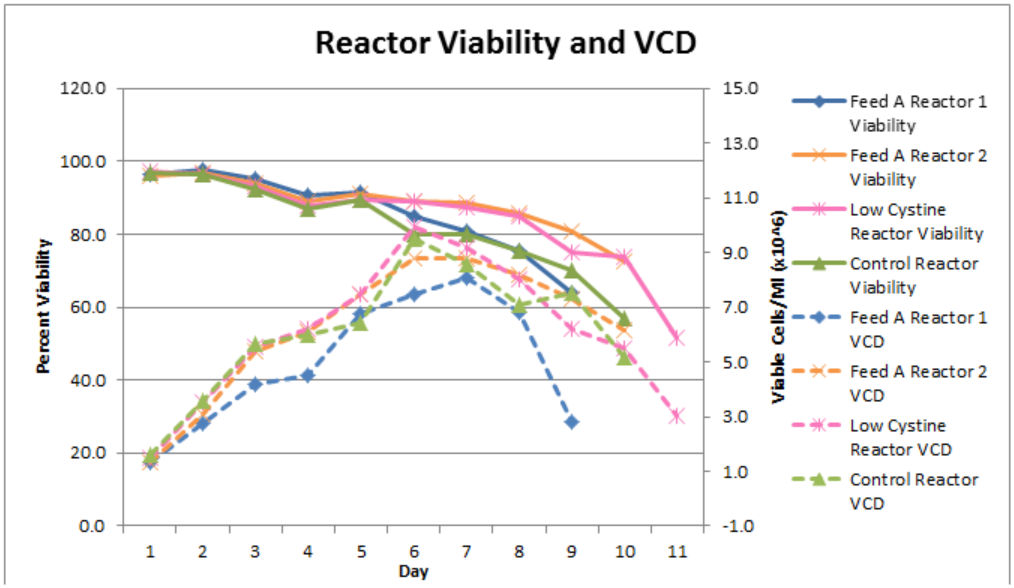

Figure-8: Plot of Cell Viability and Viable Cell Density Using a Sintered Type Gas Sparger. The solid lines measure the percentage of viable cells in the reactor, while the dashed lines measure the viable cell density.

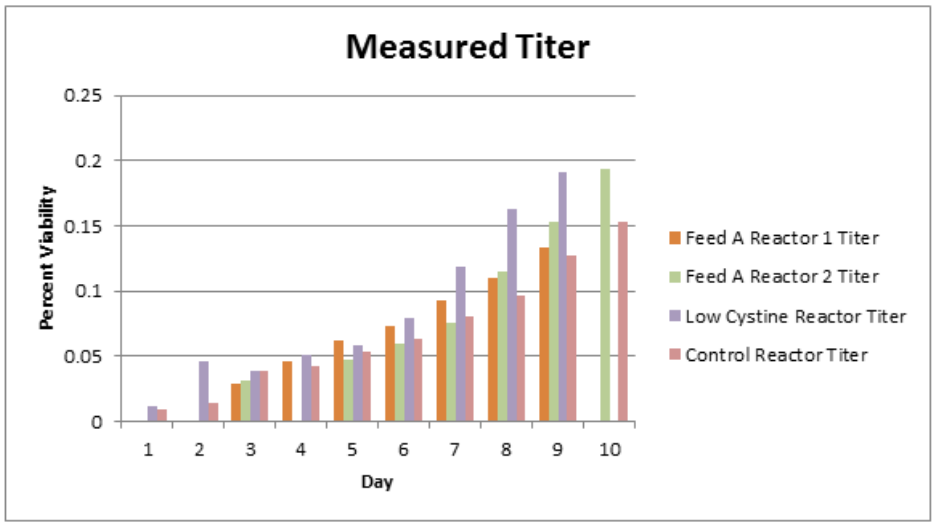

Figure-9: Plot of Antibody Yields Using a Sintered Type Gas Sparger. The mAb yields were lower than with the other runs, most likely due to the low cell density caused by the sintered sparger.

DISCUSSION

Over the course of three sets of reactor runs, it can be seen that small changes in media feed or in CO_2 content can make a great difference in the viability of the cells and the mAb yields. The dramatic effect of changing the gas strategy using different spargers was unexpected. While the effect of sparger type has been seen previously in reactors, it had not been evaluated with this cell line in this media (Chalmers, 1994). Adding the high concentration of cystine tyrosine to the feed also had a drastic effect on the growth of the culture. This feed had a high pH, so it was added much slower than the other feeds to prevent cell death. However, the amount of CO_2 added to counter the high pH was most likely more detrimental to the cells than the pH change would have been.

In future runs, less CO_2 will be added during feeding times, and the feeds will be added over the course of two hours rather than just one hour. This will hopefully allow less CO_2 build-up in the reactor, as the pH will be less dramatic during feeds. The data gathered from the high cystine tyrosine run may not be as accurate as the other runs since nutrients were precipitating out of the media as crystals. A change in titer growth due to changes in feeds cannot be proven if the nutrients are not in solution.

After optimizing the yield of antibody, in the future, western blots will be used to verify the produced antibody interacts with its cognate antigen and retains sufficient structure to be functional. This will also help ensure that the reactor processes and the feed changes have not negatively affected the functional domains of the antibody.

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