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GROWTH OF *PERKINSUS MARINUS* BY

BIOREACTOR FERMENTATION

By

Caitlin Murphy

B.S University of Maine, 2017

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Biomedical Engineering)

The Graduate School

The University of Maine

May, 2021

Advisory Committee:

Dr. Gerard Van Walsum, Associate Professor of Chemical and Biomedical Engineering, Advisor

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GROWTH OF *PERKINSUS MARINUS* BY

BIOREACTOR FERMENTATION

By Caitlin Murphy

Thesis Advisor: Dr. Gerard Van Walsum

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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Perkinsus marinus is a marine protozoan responsible for "Dermo" disease in the eastern oyster species, *Crassostrea virginica*. *P. marinus* has been detected along the Atlantic Coast of the United States and Mexico. In laboratory studies, many parasites need to be maintained *in vivo*, which complicates the study of the organism. However, *In vitro* cell culture for *P. marinus* at a scale of 1 mL was established in 1993 by several groups with the 1995 optimization (Gauthier & Vasta, 1993, 1995) considered the gold standard for small scale growth of this species. In addition to its importance as a parasite of an important food source, *P. marinus* is notable among easily cultured microbes in its ability to produce, fold and excrete very large, complex proteins. Such proteins could potentially be used for therapeutic applications, such as development of vaccines.

In order to be useful as an organism to produce vaccines, we need to learn how to grow the organism at larger scale. Transitioning the growth procedures from small culture vials to tenfold larger bioreactors is a first step towards achieving this scale up. Growing *P. marinus* in a bioreactor introduces new production variables and challenges, such as: aeration, pH control, temperature control, and cost of media. In this study we developed methods and procedures to grow *P. marinus* in bioreactors and have also developed a lower cost growth medium that reduced the cost of growth medium ingredients by about 60%. Higher

order cell culture comes with expensive media components, most notably fetal bovine serum (FBS), which provides essential growth factors and cytokines for growing cells. A promising replacement for FBS was found to be chicken serum (CS). It has the potential to provide growth factors and cytokines at a much lower cost than FBS.

SuperPro Designer v10 (Intelligen, Inc., Scotch Plains, New Jersey) is a process simulator software designed to analyze the techno-economics of commercial-scale bioprocesses. SuperPro Designer v10 was used to translate the experimental fermentation data from this study into a process model that provides insight into the financial feasibility of growing *P. marinus* at an industry scale.

ACKNOWLEDGEMENTS

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Thirdly, I would like to thank my committee for taking the time to read, what to me, feels like an insanely long thesis and for helping me along this journey.

From the bottom of my heart, thank you.

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    - Culture of Marine Protozoans Using Industrial Bioreactors Award #1701480

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

A	Absorbance Units
COA	Certificate of Analysis
CS	Chicken Serum
DC	Direct Cost
DFC	Direct Fixed Capital
DMEM	Dulbecco's modified Eagle medium
ECP	Extra Cellular Proteins
FBS	Fetal Bovine Serum
G	Grams
GFP	Green Florescent Protein
IC	Indirect Cost
KG	Kilograms
L	Liters
LOD	Loss on Drying
M	Molarity
mL	Milliliters
OC	Other Costs
ROI	Return on Investment
U	Units
WT	Wild Type
WC	Working Capital

CHAPTER 1

INTRODUCTION

Historical Information

Perkinsus marinus is an intracellular parasite that causes "Dermo" disease in the eastern oyster species, *Crassostrea virginica*, and has been detected along the Atlantic and Gulf coasts of the USA (Fernández Robledo et al., 2018) and Mexico (Ford, 1996). *P. marinus* was first described by Mackin et al. in 1950. Originally, *P. marinus* was named as *Dermocystidium marinum,* and placed in the phylum Apicomplexa. (Mackin, Owen, & Collier, 1950). In 1978, *D. marinum* was reclassified because the zoospores differed from all other apicomplexans and was renamed *Perkinsus marinus*, but remained in the Apicomplexa phylum (Levine, 1978). In 1982, Vivier argued that *P. marinus* was more closely related to dinoflagellates (Vivier, 1982). In 1997, it was argued that *P. marinus* was no longer to be included in the Apicomplexa phylum based on data from a non-coding structural gene. Instead, *P. marinus* was thought to be more closely related to the dinoflagellates, (Siddall, Reece, Graves, & Burreson, 1997) confirming Vivier's thoughts. In 1999, *P. marinus* was placed a in a new phylum, Perkinsozoa, which bridges the evolutionary gap between Apicomplexa and Dinoflagellates (Norén, Moestrup, & Rehnstam-Holm, 1999).

The Perkinsozoa phylum is an exclusively parasitic group with 3 families: Perkinsidae (infects shellfish), Xcellidae (infects fish), and Parviluciferaceae (infects dinoflagellates) and seven genera. The Perkinsozoa phylum also includes parasites which have been attributed to mass mortalities in amphibian populations in the United States (Gleason, Chambouvet, Sullivan, Lilje, & Rowley, 2014). Perkinsea hypnospore-like and trophozoite-like organisms were found were found in the liver, kidney, spleen and pancreas of frogs (Isidoro-Ayza, Grear, & Chambouvet, 2019). The Parviluciferaceae includes 4 genera: *Dinovorax, Snorkelia, Tuberlatum,* and *Parvilucifera*. The *Dinovorax, Snorkelia* (Reñé, Alacid, Ferrera, &

Garcés, 2017)*, Tuberlatum* (Jeon & Park, 2019) genera only includes one species per genus. *Parvilucifera* has the most species with 4 (*P. infectans, P. rostrata, P. corolla,* and *P.multicavata)* (Jeon & Park, 2020).

Life Cycle of *Perkinsus marinus*

P. marinus has three main life stages: trophozoite, hypnospore, and zoospores. The trophozoite stage occurs in the tissues of the live host. *P. marinus*, as a mature trophozoite, has been described as having a very large vacuole containing an inclusion body, which pushes the nucleus to one side of the cell (Mackin et al., 1950). Using an electron microscope, it can be seen that immature trophozoites contain: mitochondria with tubular cristae, smooth endoplasmic reticulum, a nucleus with a small nucleolus, virus-like particles and two centrioles (Perkins, 1996) (Figure 1). Proliferation within the host involves the trophozoite undergoing cycles of karyokinesis followed by cytokinesis, which will which will yield up to 32/64 daughter cells. The cell wall will then rupture allowing the daughter cells, or immature trophozoites, to be released. The immature trophozoites will then gradually begin to grow and become mature trophozoites (Perkins, 1996).

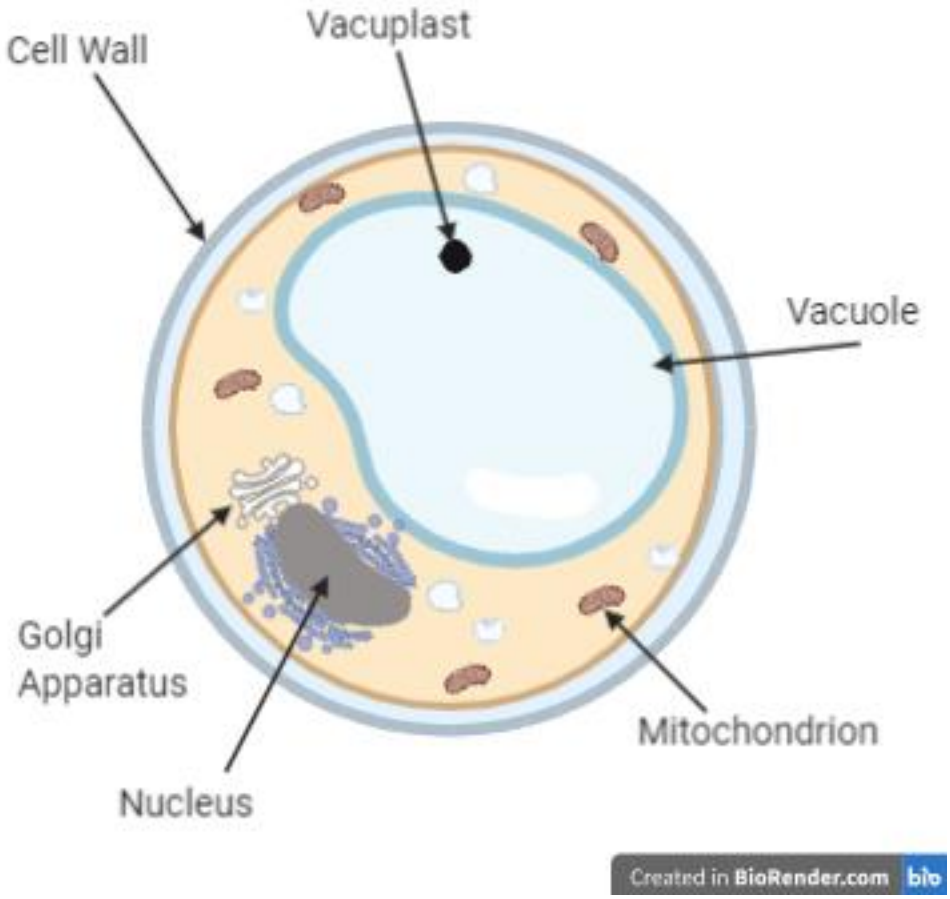

Figure 1 Diagram of *Perkinsus marinus* with selected features highlighted. Created with BioRender.com

Hypnospore has been observed when *P. marinus* were grown in fluid thioglycollate medium. It is characterized by a thick cell wall growing around the trophozoite. When hypnospores are isolated and returned to sea water, they will begin zoosporulation. It is believed that the hypnospore stage may be the dormant stage, which will allow *P. marinus* to live in unfavorable conditions (Perkins, 1996), such as extreme salinity and extreme temperatures. (Queiroga, Marques-Santos, De Medeiros, & Da Silva, 2016)

Zoospores are formed within the cell wall and are ellipsoidal in shape with normally two flagella, sometimes there is only one flagellum present. Zoospores contain a U-shaped large vacuole, a single Golgi body located next to the nucleus, one mitochondrion, lipid droplets, and a nucleus with well-defined

heterochromatin (Perkins, 1996). Zoospores will exit the zoosporangia through a single discharge tube, while occasionally, there will be two discharge tubes (Montes, Durfort, & García-Valero, 2005; Sunila, Hamilton, & Duncan, 2001) (Figure 2). However, zoosporulation has not been observed *in vitro* for *P.marinus* (Casas & La Peyre, 2013).

In the lab, we work with the trophozoite stage of growth (Figure 3).

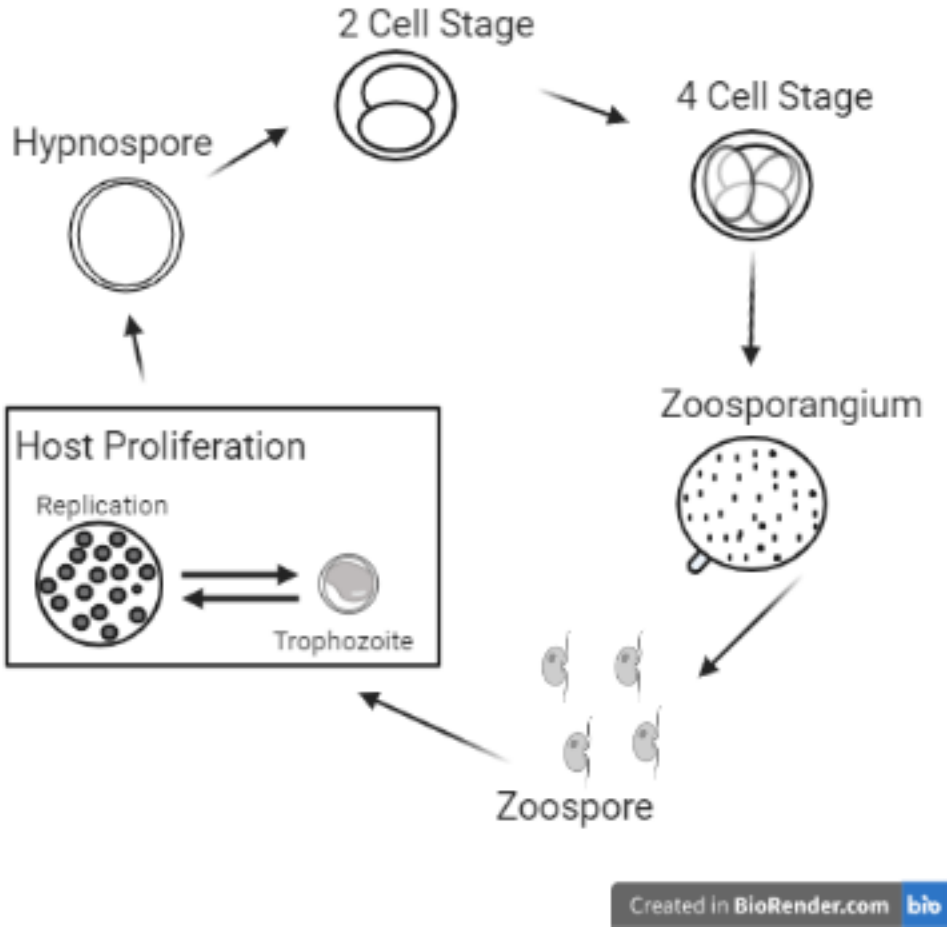

Figure 2 Diagram of the Life Cycle of Perkinsus marinus. Created with Biorender.com

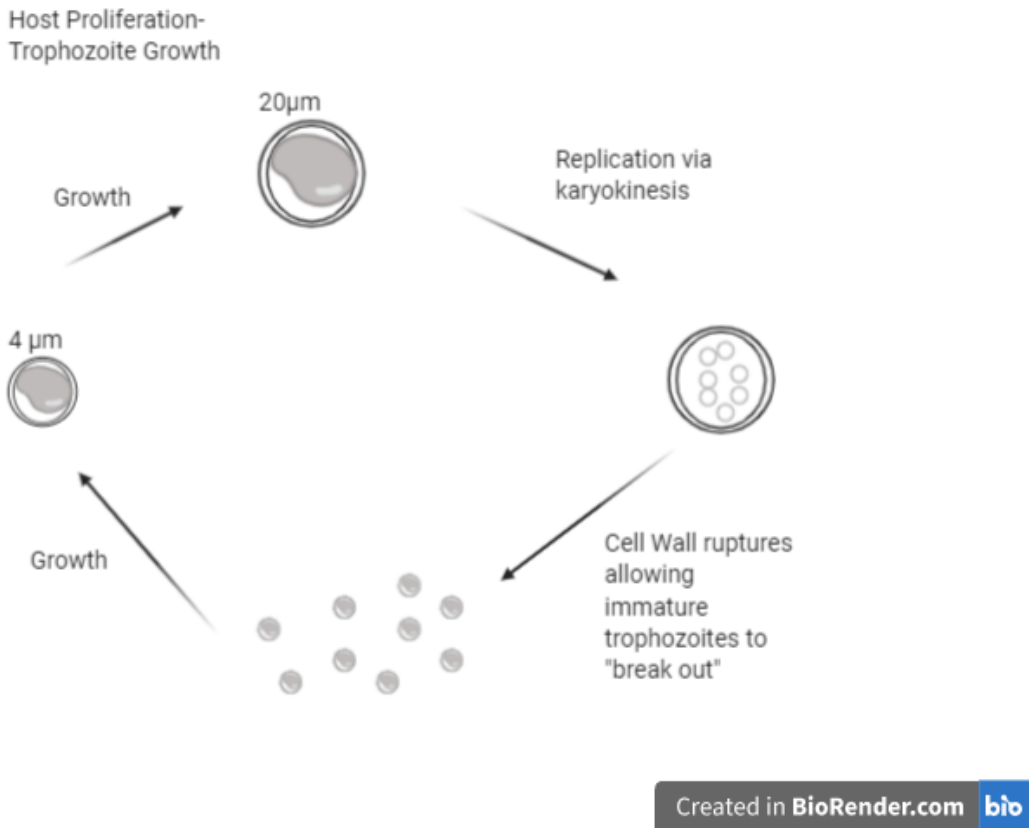

Figure 3 Diagram of Trophozoite Life Cycle of *Perkinsus marinus*. Created with BioRender.com

Genetic Modification

It has been shown that *p. marinus*, in the trophozoite stage of growth, is able to undergo genetic modification. Using a highly expressed gene, MOE, *P. marinus* is able to be transfected via a plasmid (Fernández-Robledo, Lin, & Vasta, 2008). Using an upgraded version of the plasmid, *P. marinus* was able to be transfected with two genes, MSP8 and HAP2, from *Plasmodium berghei*. Both genes are currently being considered as candidates for malaria vaccine development (Cold, Vasta, & Robledo, 2016).

Sakamoto et al. improved the selection process for genetic modification. They discovered that puromycin and blasticidin S are potent growth inhibitors against *P. marinus* (Sakamoto et al., 2019; Sakamoto, Kita, & Matsuzaki, 2016). Using both drugs allow for a more efficient selection process.

The selection process was improved further by the use of fluorescence activated cell sorting and an alternative protocol for transformation using glass bead abrasion was developed although with very low transfection efficiency (Faktorová et al., 2020).

More recently Yadavalli et al. developed a CRISPR/Cas9 system to genetically modify *P. marinus*. This system allows for greater selection in genetic modification (Yadavalli et al., 2021).

The genomes of *P. olseni* and *P. chesapeaki* were sequenced in 2021. This allows for a greater understanding of the *Perkinsus* species which will allow for better genetic modification (Bogema et al., 2021).

Vaccine Development

In 2003, extracellular proteins (ECPs) from *P. marinus*, grown in culture, were harvested from the media and then given to mice. The goal was to create antibodies against *P. marinus*; however, it was unsuccessful because the mice did not produce an adequate immune response (Earnhart & Kaattari, 2003). The two possible mechanisms for a suppressed immune response are the presence of PF68, an immunomodulatory surface-active agent, or by constituents of *P. marinus* secreted products (Earnhart & Kaattari, 2003). While the experiment may have been a failure in some respects, it opened the door for new possibilities in the study of parasitic diseases.

In 2014, wild-type *P. marinus* was given to humanized-mice expressing HLA-DR4 molecules and lacking expression of mouse MH-class II molecules. This was done so that the CD4 T cell responses in the mice were solely based on the human HLA-DR4 molecule. It was found that when the mice were fed *P. marinus*, an immune response against *P. marinus* was activated. However, there was no noticeable pathology found in the mice (Wijayalath et al., 2014). This exploratory research supports the potential use of *P. marinus* as an oral vaccine, although further research is needed before it can be approved as a vaccine for human consumption.

While malaria has been the subject of research for decades, developing a vaccine continues to be a goal for researchers today. An effective vaccine against *Plasmodium* eludes researchers due to its complex life cycle and massive antigenic variability, insufficient knowledge of the immune responses triggered by the parasite and the lack of adequate animal experimentation models (Garrido-Cardenas, González-Cerón, Manzano-Agugliaro, & Mesa-Valle, 2019). The life cycle of the malaria parasite *Plasmodium falciparum* involves two hosts. While the infected female Anopheles mosquito feeds on the blood of a human, the human host is inoculated with sporozoites. The sporozoites will then infect liver cells before maturing into schizonts, which will then rupture and release merozoites causing infection in the erythrocytes. After initial replication in the liver, the parasites will undergo asexual replication in erythrocytes. Once the parasite has entered the blood, it has entered into the clinical stage of malaria. Another Anopheles mosquito will come and feed on the infected human host. The parasite will then multiply inside the mosquito's stomach before making its way to the salivary glands and perpetuating the cycle. To add to the complexity, Plasmodium is an obligate intracellular parasite, meaning that it cannot replicate outside its host (Stanisic, Barry, & Good, 2013).

Targeting specific proteins of interest becomes challenging with Plasmodium parasites. Expressing proteins of interest in the in vitro model can be challenging even if successful. This can be overcome by over-expressing the specific protein in the in vitro model. However, the parasite would still need to be isolated from the host cell and then risk the contamination from host cell proteins. Yields of proteins can vary from 0.9 to 406.6 mg/L of media depending on the expression study (Mehlin et al., 2006). There's also the problem of amino acid substitutions and the presence or absence of post-transcriptional modification systems. This can lead to the synthesis of inaccurate protein structures and/or functions (Schneider, King, & Marletta, 2005). An important transcriptional process is glycosylation of proteins. However, there is still much to learn on this process in the Apicomplexa (Rodrigues et al., 2015) and its significance. In other phylum, glycosylation has a significant effect on protein half-life and function; hence

it must be efficiently controlled. A system that produces the correct proteins is vital to inducing an immune response.

One of the most popular systems to use to express apicomplexan proteins is the *Escherichia coli* expression system, partly because it is one the cheapest choices. The solubility problems of the recombinant product reflect that incorrect folding can lead to reduced antigenicity for vaccine applications. While there have been many advances to optimize protein production, including novel strains (Dutta, Ware, Barbosa, Ockenhouse, & Lanar, 2001), plasmids, and methodologies (Pandey et al., 2002), the soluble recombinant apicomplexan proteins remain low in prokaryotic systems (Aguiar et al., 2004; Mehlin et al., 2006; Vedadi et al., 2007).

Vaccine Delivery Systems

Vaccines have become a vital factor in reducing the prevalence of infectious diseases. The majority of vaccines are administered either by subcutaneous or intramuscular injection. The immune response is limited to systemic immunity (antibody production) against the pathogen or toxin with limited cellular immunity (T- and B-cell response) and weak protection generated at the mucosal surfaces (Vela Ramirez, Sharpe, & Peppas, 2017). When the activity of T and B-cells is induced, the adaptive immune system kicks into high gear. The lymphocytes will then differentiate into long-lived memory cells that will rapidly respond if the microbe is encountered in the future (Irvine, Swartz, & Szeto, 2013). Traditional vaccines used clinically contain either dead or live-attenuated microorganisms, inactivated toxins, protein subunits, and polysaccharide antigens or conjugates. Some issues with traditional forms of vaccines include pain and distress with injections and require highly trained personnel for administration. There is the possibility of needle-stick injuries or the reuse of contaminated needles. Also limited vaccine supply and/or production can prove problematic when a mass vaccination is necessary. Also, the conditions of storage of the vaccine can prove problematic. Most vaccines require a cold chain delivery system.

One way to combat some of the issues with vaccines is to deliver them orally. Turning the vaccine into a pill, reduces many of the costs of traditional vaccines: a cold chain delivery system is no longer required, the risks of using needles goes away, and the need for highly trained personnel goes away. Also, if a vaccine requires boosters, a person is more likely to take it in pill form than go back for a subsequent shot. They can take the pills on their own time as opposed to scheduled appointments. People are happy to do this to treat as simple a condition as a headache, taking pills throughout the day or once a day over a period of time. Taking multiple doses of an oral vaccine should not deter the success of the approach. Also, comparing the efficiency of oral vaccines vs injection vaccines is not a fair comparison. They induce an immune response in different parts of the body via different pathways (New, 2019).

Media Preparation and Replacements

One of the big challenges with parasitic growth is being able to grow the parasite without its host. Even though *Perkinsus* was discovered in 1950 (Mackin et al., 1950), a continuous *in vitro* method to grow the organism was not established until 1993. Gauthier & Vasta developed three possible media formulations all with the addition of 5% oyster serum. The three media formulations were Dulbecco's modified Eagles medium (DMEM) with 20% fetal bovine serum (FBS), DMEM:Ham's F-12 (1:1) with 10% FBS, or DMEM: Ham's F-12 (1:2) with 10mM Hepes/43 mM sodium bicarbonate or 100mM Hepes/7mM sodium bicarbonate. The minimal effective concentrations of antibiotics used to prevent contamination were 100 U/mL of penicillin G and streptomycin sulfate (Gauthier & Vasta, 1993).

The media formulation was then further refined in 1995. Gauthier & Vasta determined the optimum temperature, salinity and pH rangers were 28-30°C, 25-30 ppt, and 6.6-6.8 respectively. The optimal growth media formation was determined to be DMEM: Ham's F-12 (1:2) with the addition with 5% FBS. Higher concentrations of FBS (10-20%) were determined to be inhibitory. Oyster serum was determined to only enhance growth at low concentrations of FBS (0-.1%)(Gauthier & Vasta, 1995).

To investigate the inhibitory effect of high FBS concentrations, three major glycoproteins (transferrin, fetuin, and albumin) were tested in a serum free media to determine their effects on the proliferation of *Perkinsus.* At the concentrations tested in the study, fetuin enhanced the growth rate, albumin had a slight positive effect on the growth rate, and transferrin inhibited the growth rate (Gauthier, Feig, & Vasta, 1995)**.** This media formulation became the gold standard for *in vitro* culturing of *Perkinsus marinus.*

Twenty-One years after *in vitro* culture was established, a new way for *Perkinsus* to be cultured was developed. In 2016, Cold, et al. developed an agar based plating method for *Perkinsus marinus.* However, they still used the media formulation developed in 1995. They just modified and optimized it to contain agar (Cold, Freyria, Martínez Martínez, & Fernández Robledo, 2016).

In the past 25 years, the media formulation for *Perkinsus marinus* has not changed significantly. What has changed significantly is the price of the media. The cost of ingredients is only going up. One reason the cost is increasing is because FBS must come from cattle herds grown in USDA approved countries. Every batch/lot must be traceable back to its country, slaughter house, and herd of origin. All lots must be tested for viral contamination, sterility, endotoxin levels, mycoplasma content and other constituents (Minonzio & Linetsky, 2014). In 2019, FBS costed $185 for 100 mL and on Nov 8th, 2020 Fisher-Scientific listed FBS for $204 for 100 mL. Larger scale production reduces prices to an extent, however finding a suitable, cost effective replacement for FBS is challenging.

In 2018, a team of researchers were trying to determine the production of calcium-binding proteins in *Crassostrea virginica* in response to increased CO_2 concentrations. While *C. virginica* is the host of *Perkinsus*, this paper did not seem to directly apply to host-parasite interactions. What was interesting, was that in order to grow *C. virginica* they didn't use FBS to supplement the growth media. Instead, they used chicken serum (CS) (Richards, Xu, Mallozzi, Errera, & Supan, 2018). Because the host of *Perkinsus*

was able to be maintained by CS, this led to the possibility that it would be used as a replacement in the media formulation for *Perkinsus*. In terms of cost, on Nov 8th, 2020, Sigma-Aldrich listed chicken serum for $34.70 for 100 mL.

If FBS could be replaced with CS, this would be an 81% cost decrease for this one ingredient. With FBS in the media (using 2019 numbers), the whole media costs $123.98 per liter vs $48.67 per liter with CS in the media. Using CS, the overall cost would decrease by 60%.

Modeling Bioprocess

Modeling a simulation can be an invaluable tool to engineers in learning and researching a process. There are a number of different software programs used in academic and industry settings. Two of the most commonly used programs are AspenPlus (Aspen Technology, Inc., Bedford, Massachusetts) and SuperPro Designer v10 (Intelligen, Inc., Scotch Plains, New Jersey).

AspenPlus is a chemical process simulator that was initially developed primarily for the oil and petrochemical industries, and is best suited to continuous steady state processes. Since its initial development, it has broadened its applications to a greater diversity of industries and can now include aqueous processing environments, however it is still best suited to its original applications. AspenPlus comes in a variety of packages to suit the needs of research and industry. Given a process design and thermodynamic models, AspenPlus uses mathematical models to predict the performance of the process. The information is then used in an iterative fashion to optimize the design. The accurate modeling of thermodynamic properties allows AspenPlus to handle very complex and non-ideal chemical systems including multiple-column separation systems, chemical reactors, and distillation of chemically reactive compounds. One of the draw backs to AspenPlus is that it does not assist in designing the process. The user must have a solid understanding of the chemical engineering principles and processes required to

input parameters and evaluate the results obtained. This makes AspenTech more suitable for industrial chemicals and fuels, and for continuous processes.

SuperPro Designer v10 is another popular tool in research and industry. SuperPro Designer v10 is used to model, evaluate, and optimize batch or continuous processing as well as combinations of batch and continuous. Scheduling batch process operations is one of the key distinguishing features of SuperPro Designer v10, and this makes it particularly suitable for modeling fermentation processes, which due to their need for sterility are typically run-in batch mode. SuperPro Designer v10 has been used in variety of industries including: Biotech, Pharmaceutical, Specialty Chemical, Food Processing, Consumer Goods, Metallurgical, Materials, Water Purification, Wastewater Treatment, and Air Pollution Control. SuperPro Designer v10 allows manufacturing and environmental operation models in the same tool to allow users to optimize manufacturing while preventing pollution.

SuperPro Designer v10 has user friendly interface allowing for ease of use. Users create a flow diagram to model the process, declare the materials used in the process and initialize its operations. SuperPro Designer v10 has databanks that include physical and thermodynamic databanks for more than 1200 materials and the option to add materials to the databanks. SuperPro Designer v10 includes: models for over 140 unit procedures, rigorous reactor modules, material and energy balances, chemical component and mixture database, equipment and resource databases, equipment sizing and cost, thorough process economics, scheduling of batch operations, throughput analysis and debottlenecking, resource (utilities, raw materials, and labor) tracking as a function of time, waste stream characterization, environmental impact assessment, and compatibility with a variety of graphics, spreadsheet, and word processing packages. SuperPro Designer v10 can generate comprehensive reports that provide information on material and energy balances, equipment sizing, capital and operating cost estimation, throughput analysis, environmental impact assessment, and emissions of volatile organic compounds.

The modeling software chosen for this project was SuperPro Designer v10, for many of the reasons listed above.

CHAPTER 2

METHODS

Media Preparation

1) *P. marinus* was grown in DMEM: Ham's F12 (1:2) 5% FBS as reported elsewhere (Gauthier & Vasta, 1995).

All glassware needed for the experiment was autoclaved at 121°C for 1 hour. Glassware was then left under a UV light overnight to cool down before use the next day. All tubing used for sterilization was autoclaved at 121°C for 1 hour and then left under a UV light to cool down before use the next day.

Once vessels are cool, media was prepared in volumes of 2 liters using the following method: first dissolve 30 grams of artificial sea water in 1.6 liters of sterile water. Next add 10 grams of DMEM and 20 grams of Ham's F12, dissolving both completely. Then, add 24 grams of HEPES buffer and 0.6 grams $NaHCO_3$. Next add the antibiotics: Add 0.12 grams penicillin G and 0.26 grams streptomycin sulfate. If necessary, adjust the pH to 6.6 with NaOH or HCL. Next, add 100 milliliters of FBS. Finally, pass the media through a 0.2-micron sterilization filter. Media is stored in the refrigerator (≈4°C) until use.

2) Media was also prepared by replacing 100 milliliters of FBS with 100 milliliters of chicken serum (5% v/v)

3) For chicken serum optimization, DMEM: Ham's F12(1:2) was prepared with chicken serum concentrations of 0, 0.1, 1, 5, 10 and 20% v/v

Cells

Perkinsus marinus PRA-240 Wild Type and PRA-393 GFP mutant cells were obtained from Dr. José A. Fernández Robledo and Dr. Raghavendra Yadavalli (Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine) (Fernández-Robledo et al., 2008).

P. marinus was grown in T-75 flasks and kept in an incubator at 26-28°C.

First, warm media up to 28°C in a water bath. Next, in a biological safety hood, transfer 20 mL of media into T-75 flask using serological pipettor. Using serological pipettor, transfer 5 mL of media + cells into T-75 flask (cell density should be approximately 0.8 A). Place the T-Flask containing the cells in the incubator at 26-28°C and allow to grow until optical density is 0.8 A (approximately 8-10 days)

Bioreactors

The DAS-GIP system is a multicomponent system (Figure 4). The 4 vessels each have a volume of 400 mL, with a working volume of 300mL (Figure 5).

Figure 4 Das-GIP System A) Shows the whole Das-Gip System B) Shows the bioreactors in the system

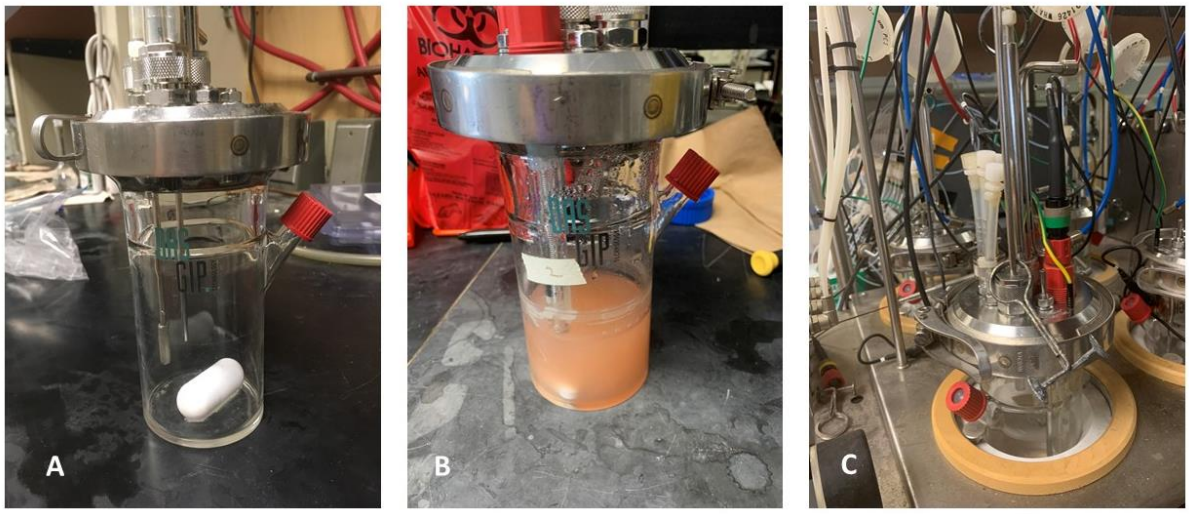

Figure 5 Bioreactors A) Shows an Empty Bioreactor B) Shows A bioreactor at the end of a fermentation C) Shows an empty bioreactor in the housing

Vessels were autoclaved for 60 minutes at 121°C. Vessels are then left under UV light for 24 hours to allow the glass to cool down. Media is then transferred into the vessels under a sterile hood. Vessels are then hooked up to the DAS-GIP system.

Variables to be controlled: pH, temperature

Variables to be measured: pH, temperature, redox – DAS-GIP logs measurements approximately every 30 seconds.

Aeration of the bioreactors was done by continuous purge of the head space instead of submerged sparging. When the sparger was immersed in the medium, the air caused the media to instantly start foaming. When bubbles start to form in the medium, it can lead to the wetting of exit vents and increasing the chance of bacterial contamination on a small scale. On a larger scale, we could have bacterial contamination and also lower cell growth. We were also concerned that an anti-foaming agent could have a negative impact on cell growth. The best option, at this small scale, was to aerate the system in the headspace. The surface area to volume ratio of the head space is 0.44 cm^{-1}. The surface area to volume ratio of the whole reactor is 0.8 cm^{-1}.

Vessels are inoculated with 15-20 mL of cells suspended in their medium with an optical density of 0.8. The system is allowed to run for 8-10 days. Cells are grown at 28°C, 200 rpm, aeriation of approximately 3.5-4 mL/s in the headspace, or 1.2-1.33 headspace vol/min. pH and Redox measurements are logged throughout the experiment by the DAS-GIP control system. 2 mL are taken out each day for analysis using a 3 mL syringe and a 22Gx4'' needle.

Flasks

5 mL of *P. marinus* were pipetted into 20mL of media in a T-75-Flask and allowed to grow 8-10 days before re-culturing. 1 mL was taken out each day for analysis.

Analysis

P. marinus was spun down at 3.6 x G for 1 minute using a Sorvall Legend Micro 21 centrifuge. The media was siphoned off for HPLC analysis using a 1000 µL pipettor. The cells were then resuspended in 1 mL of 0.5 M NaCl solution. The absorbance was taken using Genesys 10S UV-VIS Spectrophotometer at 600 nm.

Dry Weight

Method 1

To determine dry weight of cells, first spin down 1 mL of *P. marinus* cells at 3.6 x G for 1 minute using a Sorvall Legend Micro 21 centrifuge. Then, remove the media from the microcentrifuge tube using a 1000 µL pipettor. Wash the cells with 1 mL of 0.5M NaCl solution to remove excess media. Next, take the absorbance of the cells at 600nm using a Genesys 10S UV-VIS Spectrophotometer. Record the absorbance. Then, spin down 1mL of *p. marinus* cells at 3.6 x G for 1 minute using a Sorvall Legend Micro 21 centrifuge. Remove the sodium chloride solution from centrifuge tube leaving behind the cell pellet. Weigh the aluminum drying dish without cells and then transfer the cell pellet to an aluminum drying dish.

Wash centrifuge tube with 40 µL of 0.5M NaCl to get all the cells out of the tube and into the drying dish. (Mathematically, 40 µL of 0.5M NaCl weighs 0.0011688 grams. Subtract this number from the total at the end). Transfer the 40 µL to the aluminum drying dish and then place the aluminum drying dish in 100°C oven for 6-24 hours, until there is no decrease in weight. Place the drying dishes into a desiccator and allow them to cool completely. Weigh the dishes and record the weight and then subtract out the 40 µL of NaCl. Put the dishes back in the oven for an hour, then weigh them again once cool to determine if there is any decrease in the weight.

Method 2

First spin down 5 mL *P. marinus* cells in a 15 mL centrifuge tube at 360 x G for 5 minutes using an accuSpin™ 400 benchtop model. Decant the media from the centrifuge tube. Wash the cells with 1 mL DI water to remove excess media. Spin down the cells again. Remove the DI water from the centrifuge tube. Weigh the crucible and record the number. Transfer the cell pellet to a crucible. Wash the centrifuge tube with 40 µL of DI water and then transfer the 40 µL to the crucible. Place the crucible into a 100°C oven for 6-24 hours, until there is no decrease in weight. Place the crucibles into a desiccator and let cool for 1 hour. Weigh the crucibles and record the number. The crucibles are then ready for ashing.

Ashing Cells

First weigh the empty crucibles and record the number. Place the empty crucibles in the muffle furnace for 1 hour at 525°C. Using tongs, place the crucibles in a desiccator for 1 hour to cool down to room temperature. Weight the crucibles and record the weight. Add the dried cell sample to the dried crucibles and record the weight. Next, place the crucible holding the sample in the muffle furnace for 2 hours at 525°C. Using tongs, place the crucibles into a desiccator for 1 hour to cool to room temperature. Weigh the crucible and record the weight. Place the crucible back into the muffle furnace for 1 hour at

525°C. Again, place crucible into a desiccator for 1 hour to cool to room temperature. Weigh the crucible and record the weight to be sure that it is constant (±0.3mg).

High Performance Liquid Chromatography

Each sample was filtered through a 0.45 nylon micron filter after the addition of 4-5 drops of H_2SO_4 to acidify the sample. Each sample ran for 35 minutes at 45°C using an AminexHPx87H column. The following parameters were used for each run: 1 replicate for each run, 15 µL of sample were injected for the run, the mobile phase used was 5 mM H_2SO_4, the flow rate for the column was 0.6 ml/minute, 5g/L glucose standards were used to calibrate system, the detection system is the RID-10A (refractive index detector), and the column temperature was 45°C.

Super Pro

The different flowsheet configurations were simulated using Super Pro Designer (Intelligen, Inc., Scotch Plains, New Jersey) due to its capability to model bioprocess and unit procedures bank. See Chapter 4 for details.

CHAPTER 3

EXPERIMENTAL RESULTS

Table 1 summarizes the similarities and differences for each experiment detailing the number of vessels, cell types grown, which media was used, the air supply, HPLC data, absorbance data, and the purpose of each experiment.

Table 1 Experimental Differences

Experiment	Number of Vessels	Cell Type(s)	Media Type(s)	Air Supplied	HPLC data	Absorbance Data	Purpose
1	2	PRA-240 WT	FBS	No	No	Yes	Demonstrate Growth in bioreactor
2	2	PRA-240 WT	FBS	Yes	No	Yes	Demonstrate Growth in bioreactor
3	2	PRA-240 WT	FBS	Yes	Yes	Yes	Demonstrate Growth in bioreactor
4	4	PRA-240 WT	FBS	Yes	No	No	Maximize growth
5	4	PRA-240 WT	FBS & CS	Yes	No	Yes	Compare growth mediums
6	4	PRA-240 WT	FBS & CS	Yes	Yes	Yes	Compare growth mediums
Chicken Serum Optimization	Small Scale Flasks; N =3	PRA-240 WT	CS	Yes	Yes	Yes	Optimization of Growth medium
7	4	PRA-240 WT & PRA-393 GFP	FBS	Yes	No	Yes	Comparing wild type to modified cell types
8	4	PRA-393 GFP	FBS & CS	Yes	Yes	Yes	Compare growth mediums on modified cells

Experiment 1: First Attempt to grow *Perkinsus* in Bioreactors

The first experiment that was run with the bioreactors was a learning curve experiment. The absorbance, as displayed in Figure 6, shows that something was growing. However, it is my belief that the something growing was not *Perkinsus*, but bacteria because the absorbance doubled faster in Vessel 2 than in the small-scale experiments. Vessel 1 does not double as fast as vessel two, but I also believe that bacteria were growing in the vessel. Evidence for this is that once the cells were spun down, they should be a white-clear color. In this experiment, the cell pellet was tinted red. However, one can see in the redox, as displayed in Figure 7, is negative for the whole experiment indicating limited oxygen present. Lack of oxygen is likely, because the air was not turned on until the slight spike seen around time 25-4-19.

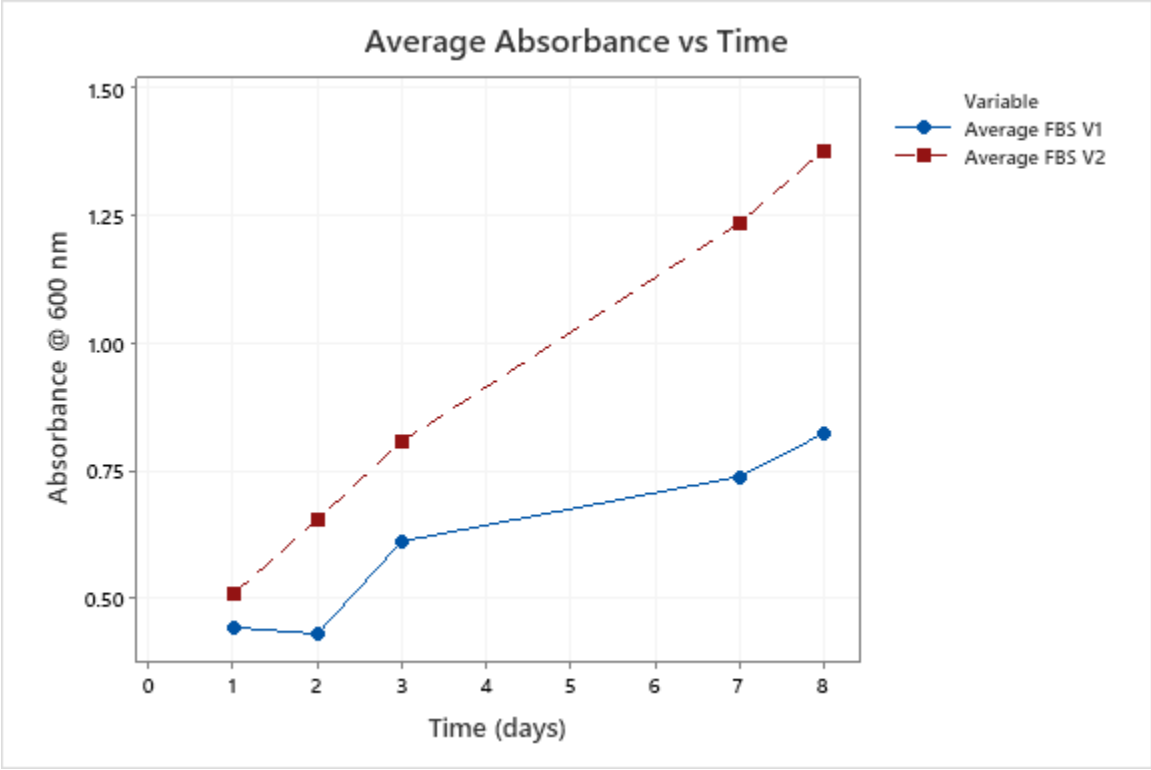

Figure 6 Growth Curve PRA-240 WT cells in FBS media

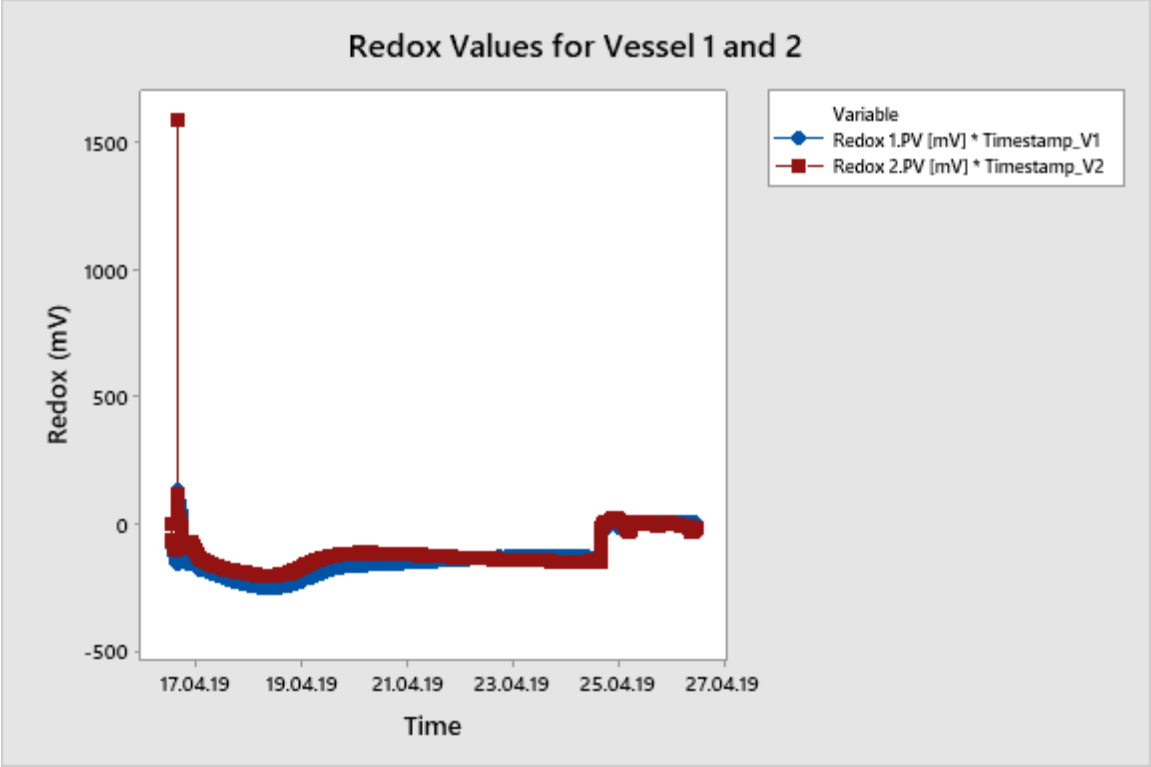

Figure 7 Redox Curve

Experiment 2: Second Attempt to Grow *Perkinsus* in Bioreactors

This experiment we fixed the air supply, so both vessels had oxygen throughout the whole experiment. As in Experiment 1, this experiment used 2 vessels, FBS Medium and wild-type cells. As can be seen in Figure 8, the growth curve aligns more closely with the known doubling time (16-24hrs) of *Perkinsus*. A One-Way ANOVA on the two growth curves, yields a P-value of 0.017 indicating that the two growth curves are significantly different. This could be attributed to slightly different inoculum sizes, or possibly the vessels got contaminated with bacteria. There was no visible evidence of contamination, but the redox graph, Figure 9, shows limited oxygen present at the beginning of the experiment with negative numbers for the first few days. Oxygen doesn't become saturated in the growth medium until after 7/5/19. We then see another spike downwards in the redox after 7/9/19.

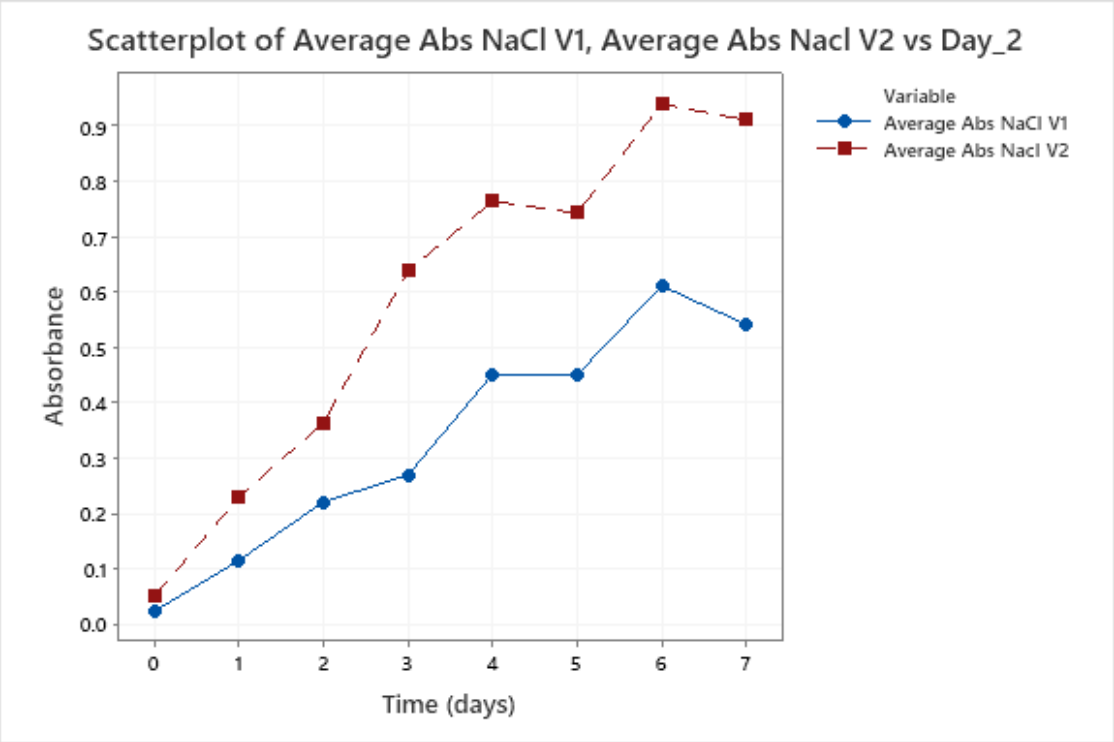

Figure 8 Growth Curve PRA-240 WT cells in FBS media

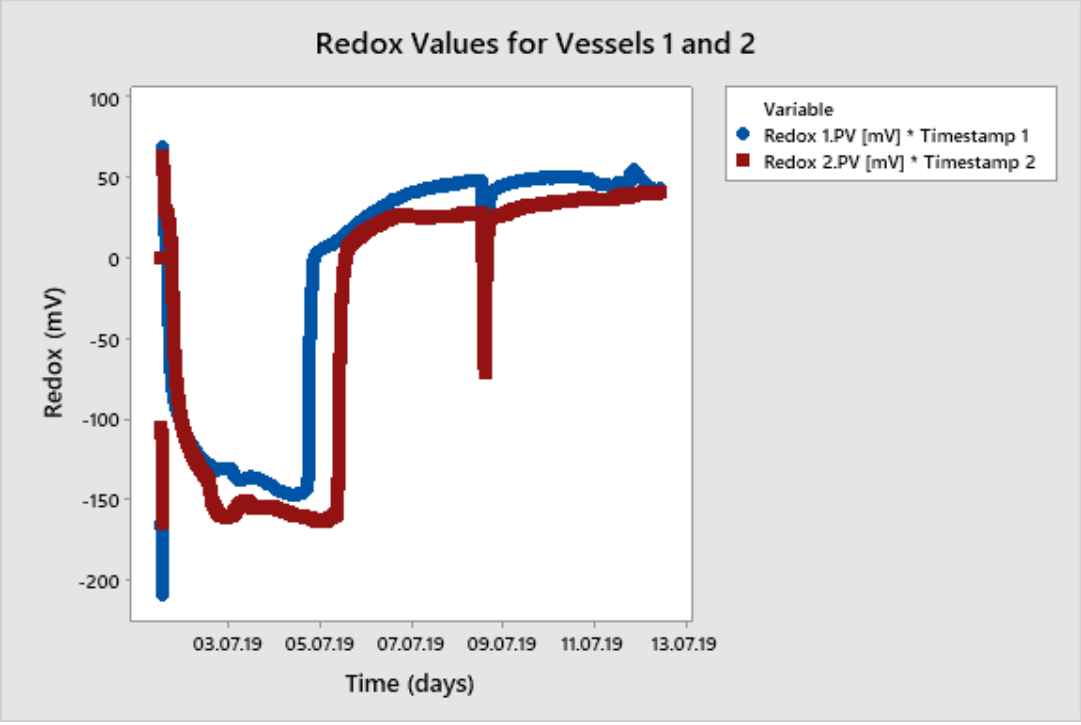

Figure 9 Redox Curve

Experiment 3: Third Attempt to Grow *Perkinsus* in Bioreactors

This experiment the two vessels were grown in FBS medium. A One-way ANOVA done on the growth curves, as displayed in Figure 10, gives a P-value of 0.089 indicating no significant difference between the two curves.

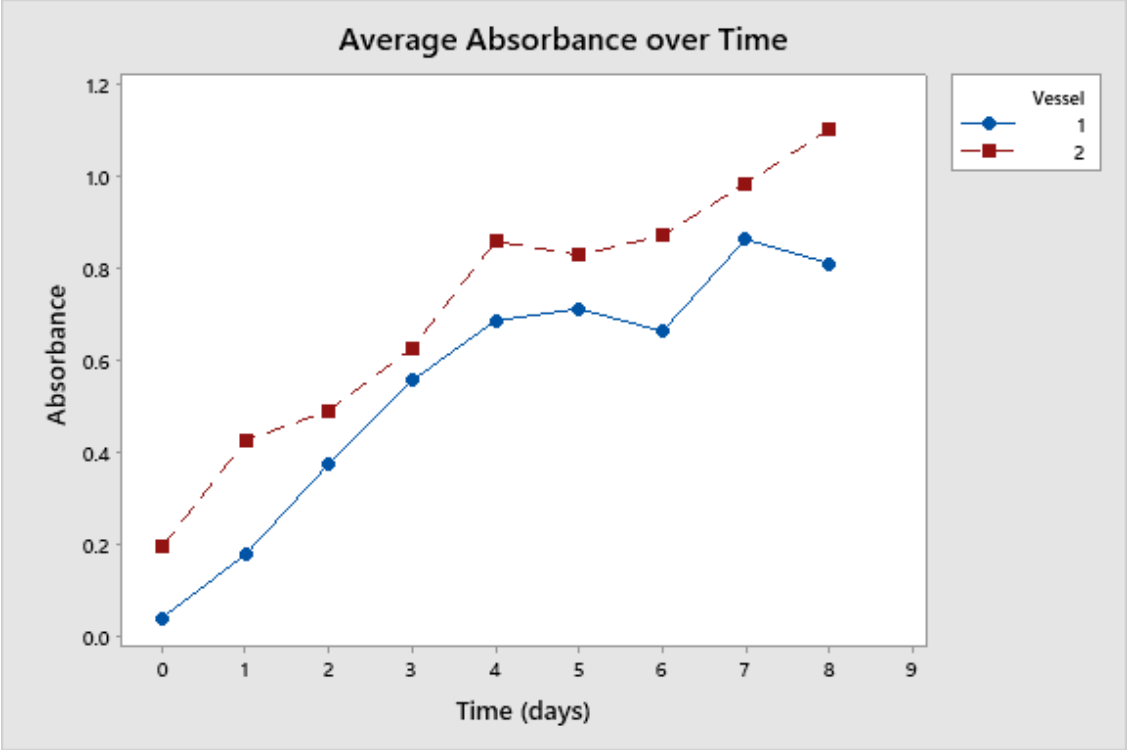

Figure 10 Growth Curve PRA-240 WT cells in FBS media

Under the microscope, the cell density increases by day 3 with the cells filling the field of view. The cells are uniform in size. Day 4 the cells look bigger under the microscope, and there is a higher number of larger cells while some cells remain smaller. By the end of the experiment, the cells have reduced in size. There are no detectable differences between the cells in the different vessels.

As can be seen in Figure 11, the Redox curves for the two vessels match shape very closely. The negative spike at the beginning indicates the time when the cells were injected into the vessel. The rest of the experiment, the redox values stayed positive indicating that oxygen was present the entire time. This is the first experiment where the Redox graph has stayed positive for the whole experiment, and

there were no visible signs of contamination. This leads to the idea that the redox curve could provide a real time indication of contamination.

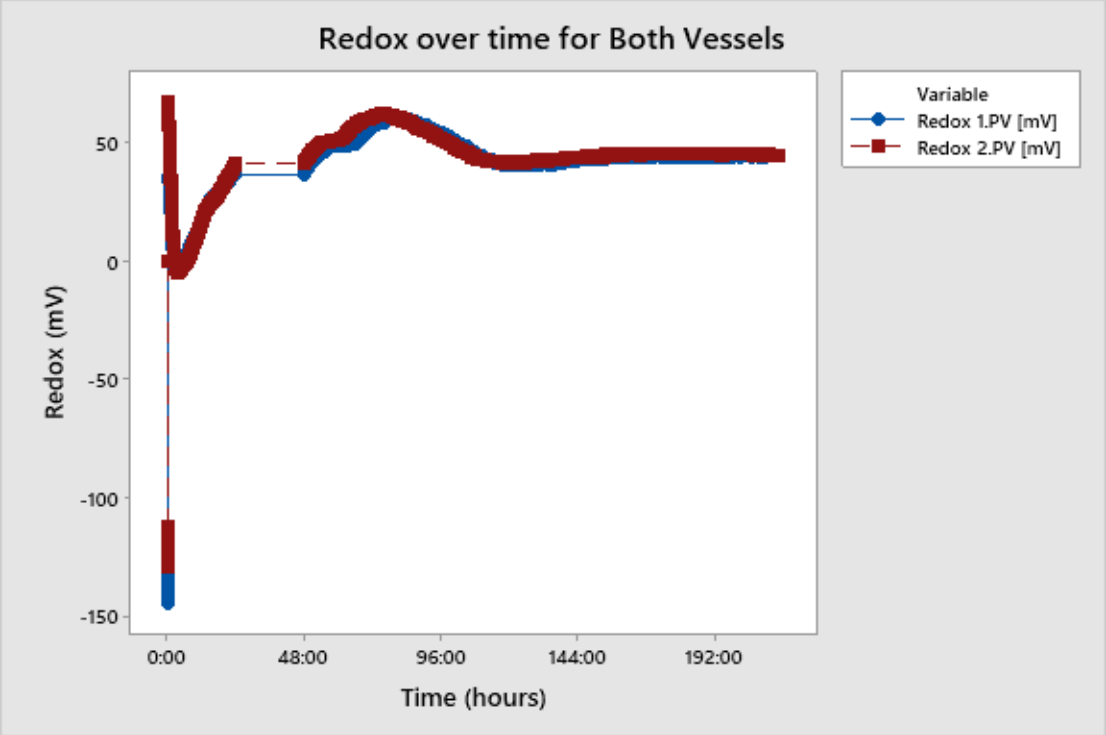

Figure 11 Redox Curve

This is the first experiment where HPLC was run to determine glucose concentrations in the media over the course of the fermentation. Figure 12 shows how the concentration of glucose decreases in the media over time. Both vessels follow a similar trend in that glucose is consumed during the fermentation. It is interesting to note that the levels detected do not go to zero. On day zero, the glucose levels are approximately 3.1 g/L and only drop to approximately 2.8 g/L. The difference between the start and end of the experiment is only 0.3 g/L. The glucose levels in media free from cells is slightly lower than media with cells because the inoculum has glucose present, slight raising the glucose levels. Figure 13 shows a zoomed in HPLC trace show detection of glucose.

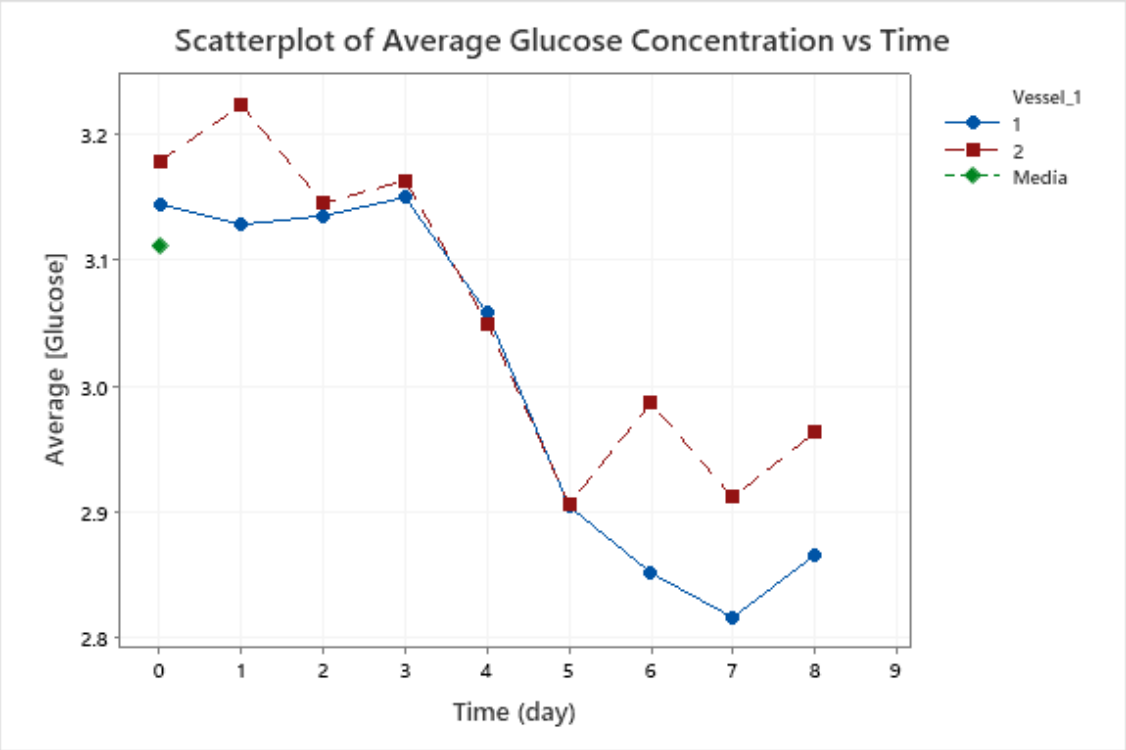

Figure 12 Glucose Concentration This graph shows how the glucose concentration was changing over the course of the fermentation

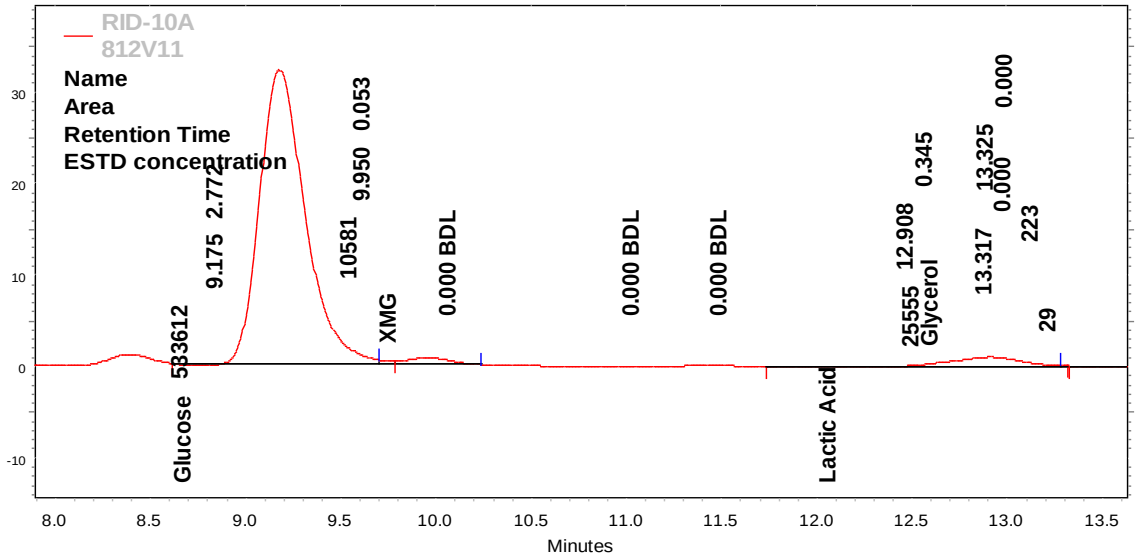

Figure 13 HPLC Trace

Experiment 4: Expanding from 2 Bioreactor Vessels to 4 Bioreactor Vessels

This experiment was expanded from 2 vessels into 4 vessels. Cells grown were wild-type in FBS Medium. The absorbance data were not recorded for this experiment partly because of the real time measurements of the redox, as displayed in Figure 14. Figure 14 indicates that negative values of vessels 2, 3 and 4 are all contaminated with bacteria. However, the shape of Vessel 1 matches the shape of the redox graph from experiment 3, which was a clean culture experiment. This indicates that out the 4 vessels, only vessel 1 was not contaminated and growing only *Perkinsus.* On Day 1, all the vessels appeared normal under the microscope. By Day 2, vessels 2, 3, and 4 had visible bacterial contamination while vessel 1 remained uncontaminated.

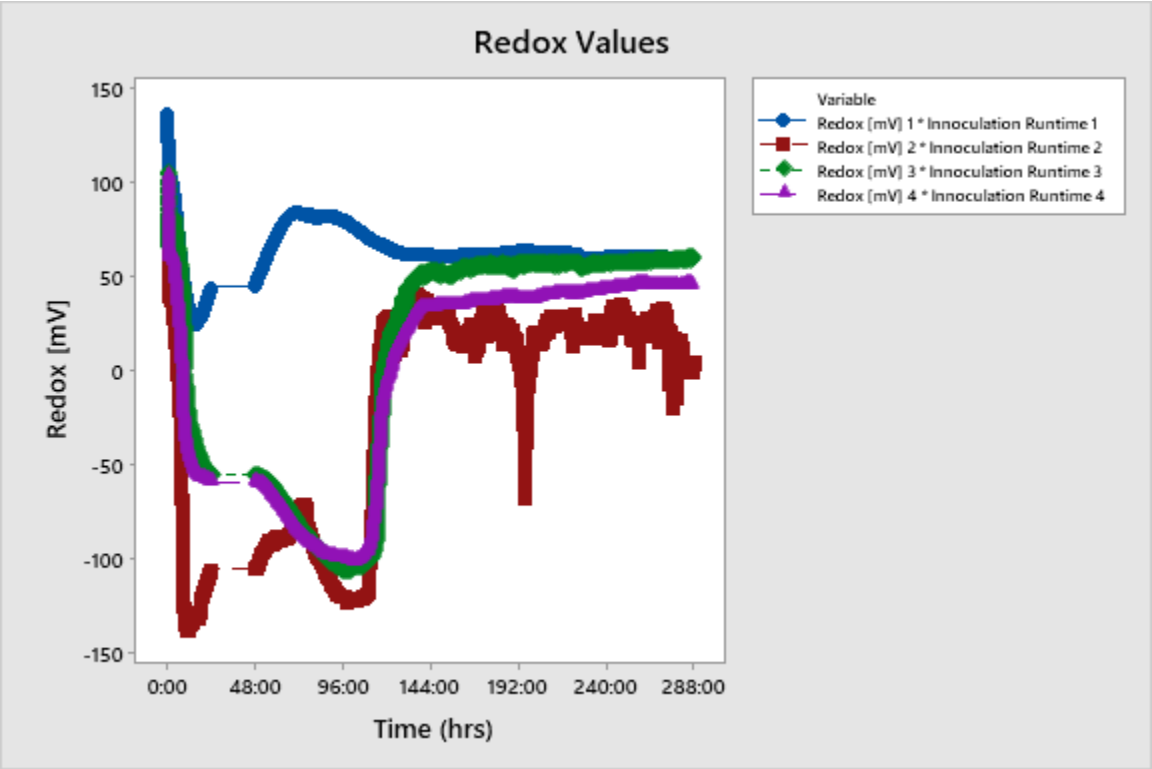

Figure 14 Redox Curve

Experiment 5: Comparing PRA-240 WT Growth in FBS Media to Growth in CS Media

This experiment compares growing PRA-240 WT cells in either FBS or CS media. Vessels 1 and 3 contain FBS media and vessels 2 and 4 contain CS media. The absorbance data at 600 nm is displayed in Figure 15.

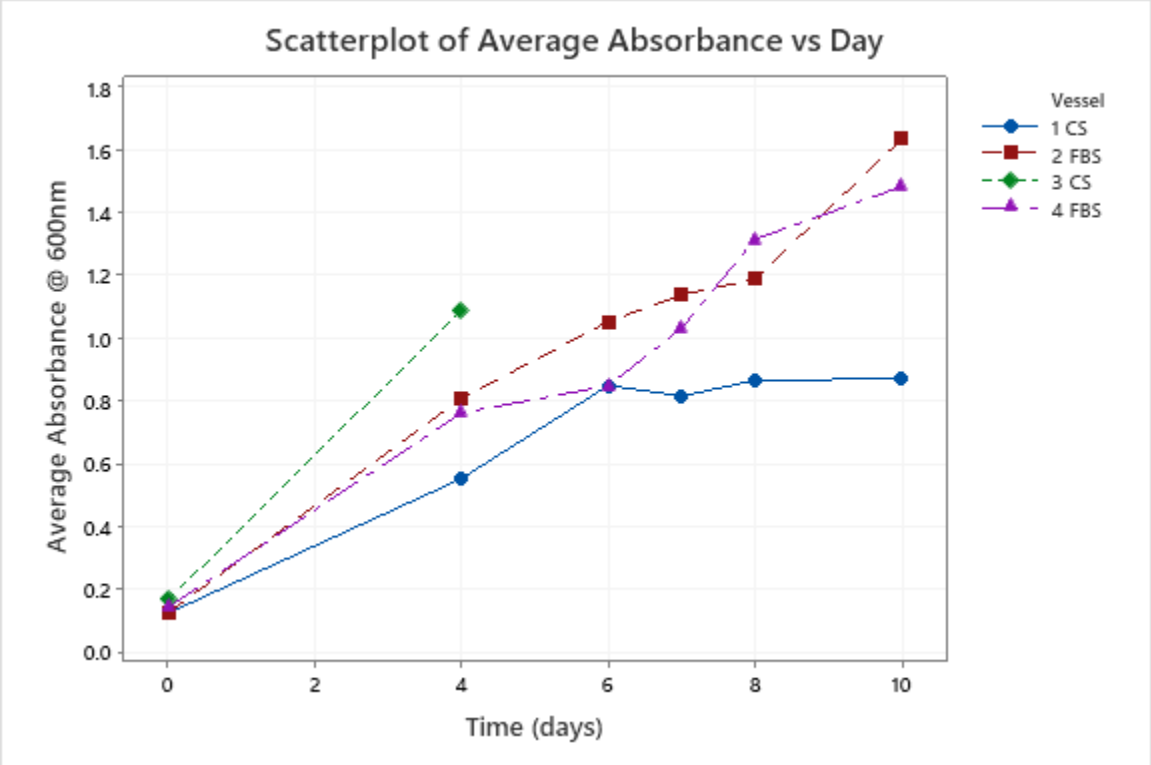

Figure 15 Growth Curve PRA-240 WT cells in FBS and CS media

Table 2 Grouping Information for Figure 15 Using the Tukey Method and 95% Confidence

Vessel	N	Mean	Grouping
2	12	0.991	A
4	12	0.933	A
1	12	0.6797	A
3	4	0.627	A

Means that do not share a letter are significantly different.

Absorbance data for vessel 3 was no longer recorded after day 4 because the jump in absorbance was too high to be growing only *Perkinsus*. The Redox graph, displayed in Figure 16, stays negative for the length of the experiment. The negative values, and lack of characteristic shape, indicated that vessel 3

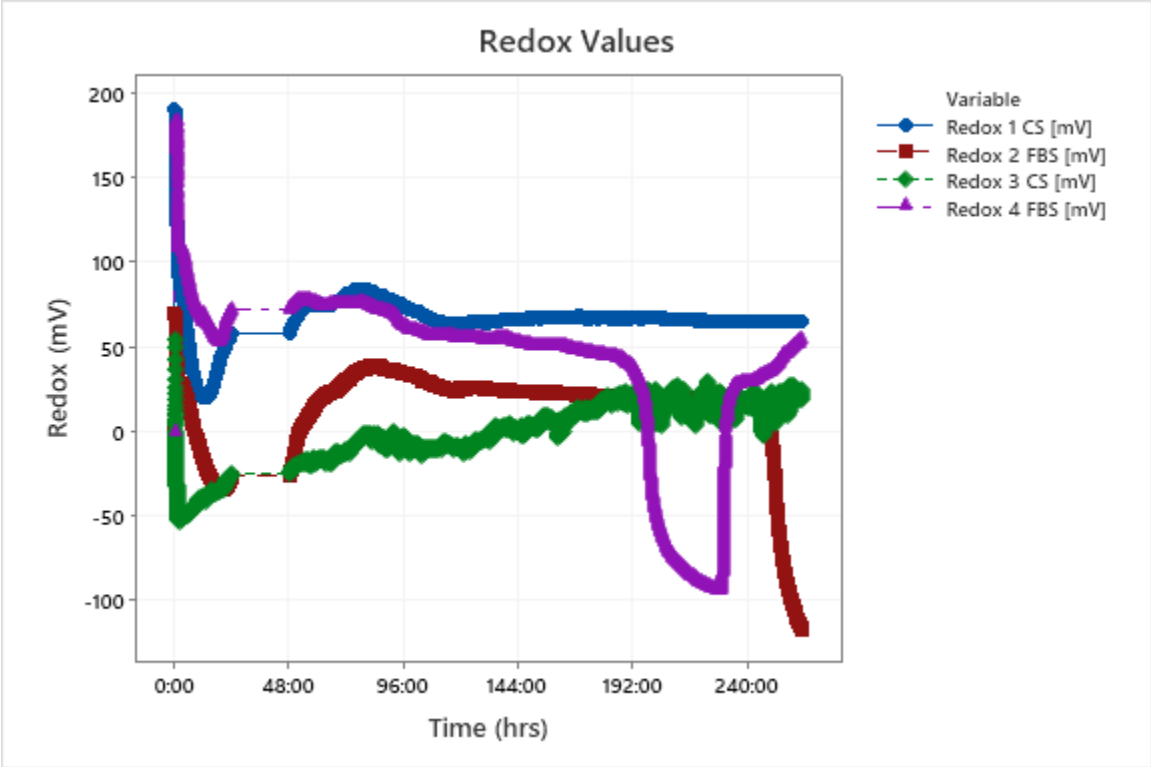

Figure 16 Redox Curve

was contaminated with bacteria. The other three vessels showed no signs of contamination until the end of the experiment when vessels 4 and 2 dipped into negative Redox values. Vessel 1's Redox stayed positive for the whole experiment and has the characteristic shape indicating that there was no contamination present. A One-Way ANOVA on the absorbance curve gives a P-value of 0.215 indicating no statistical difference between the growth curves.

Experiment 6: Comparing PRA-240 WT Growth in FBS Media to Growth in CS Media

This experiment compares PRA-240 WT cells grown in FBS or CS media and is a repeat of Experiment 5. Vessels 1 and 2 contain FBS media and vessels 3 and 4 contain CS media. Figure 17 displays the absorbance data over time for this experiment. A One-Way ANOVA done over the graph gives a P-value of 0.835 indicating no statistical difference in the growth of *Perkinsus* with either FBS or CS media.

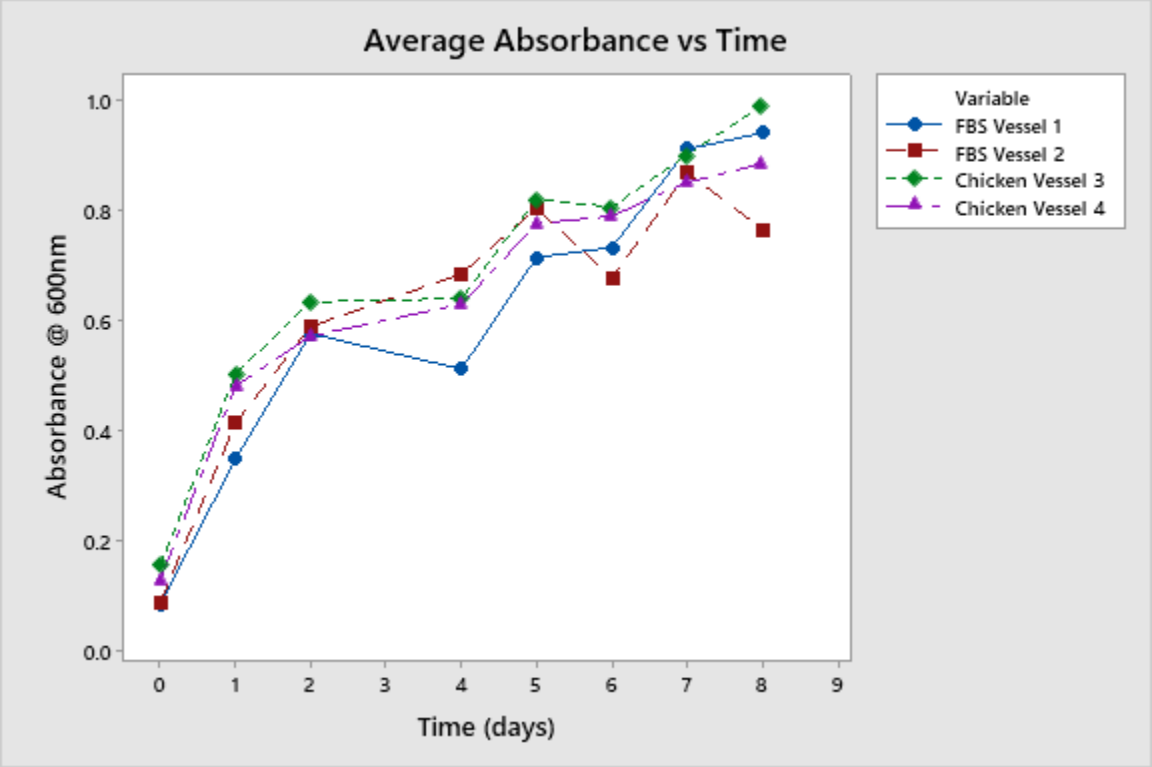

Figure 17 Growth Curve PRA-240 WT cells in FBS and CS media

Table 3 Grouping Information for Figure 17 Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Vessel 3	16	0.6889	A
Vessel 4	16	0.6390	A
Vessel 2	16	0.6156	A
Vessel 1	16	0.6026	A

Means that do not share a letter are significantly different.

In addition, there were no noticeable differences when looking under the microscope at the cells between the vessels. As displayed in Figure 18, The Redox values stay positive and are only negative when the cells were injected into the vessels. All 4 curves show the characteristic shape to indicate that only *Perkinsus* is growing in the bioreactors.

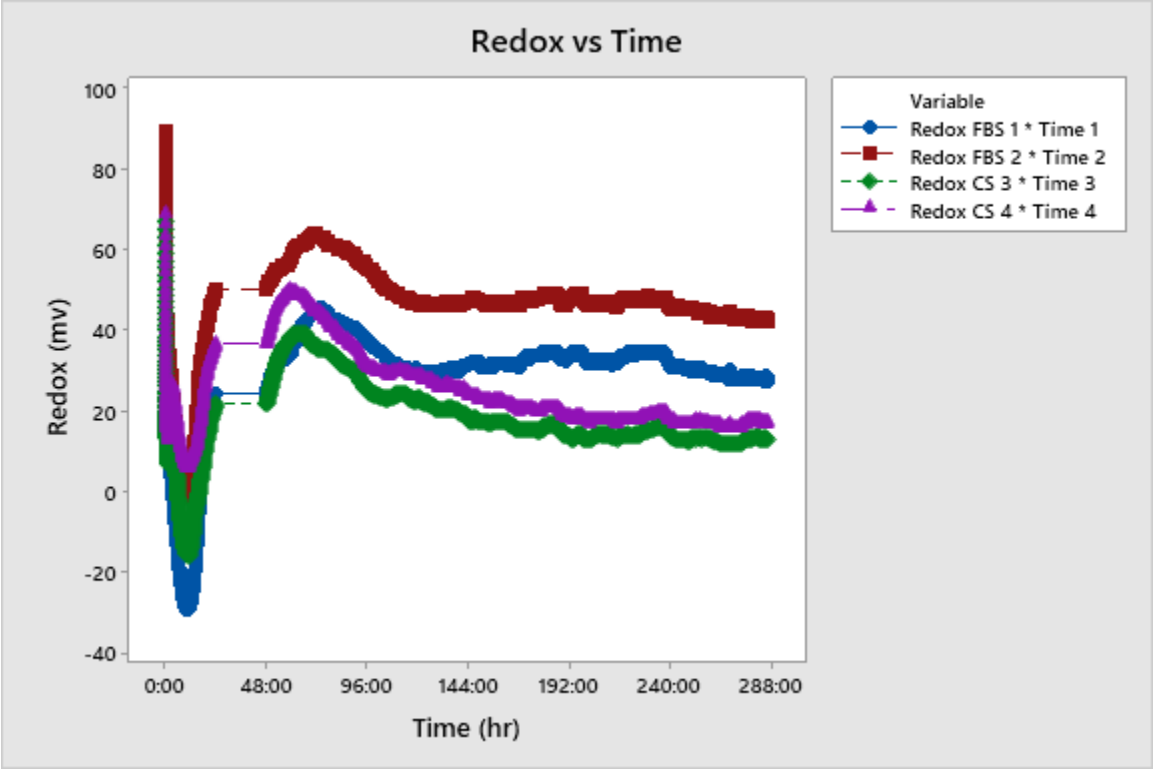

Figure 18 Redox Curve

Figure 19 shows how the average glucose concentration decreases over time. For the FBS vessels, vessel 1 starts at 2.1 g/L and ends at 1.6 g/L. Vessel 3 starts at 1.9 g/L and ends at 1.6 g/L. For the CS vessels, vessel 2 seems to not be very steady over time. There could have been issues with samples as they went through the HPLC. Vessel 4 starts at 1.95 g/L and ends with 1.5 g/L.

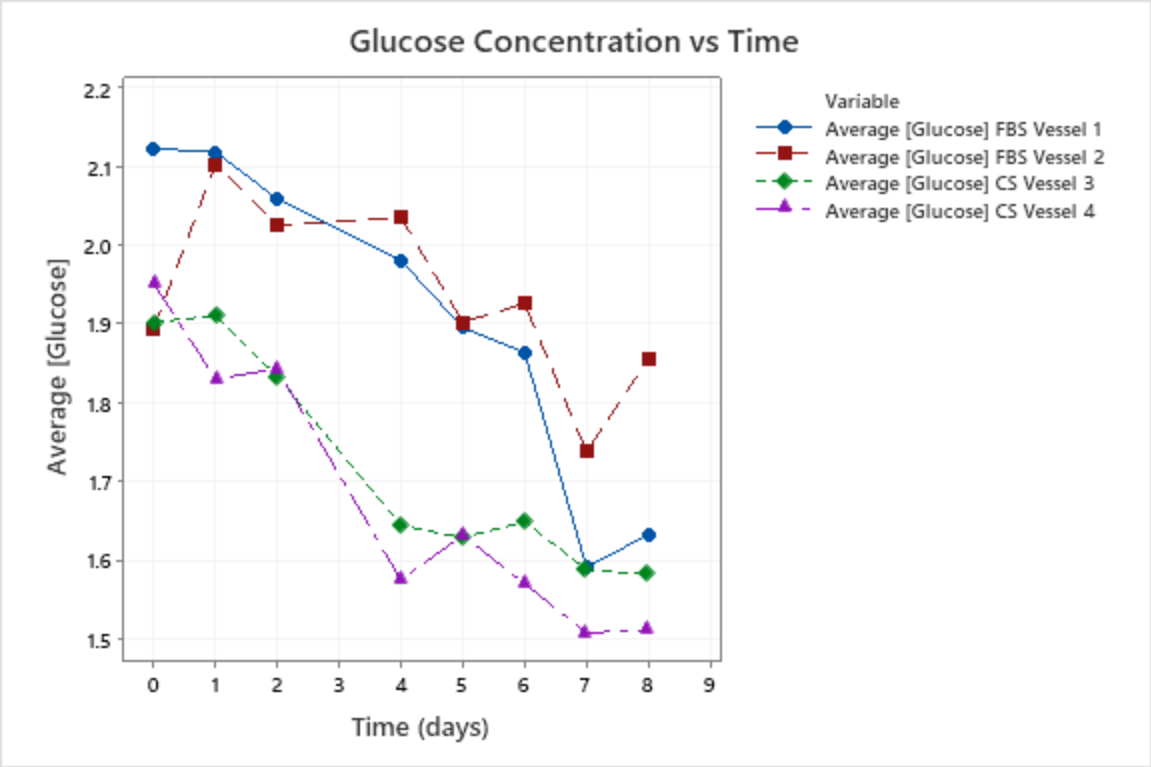

Figure 19 Glucose Concentration

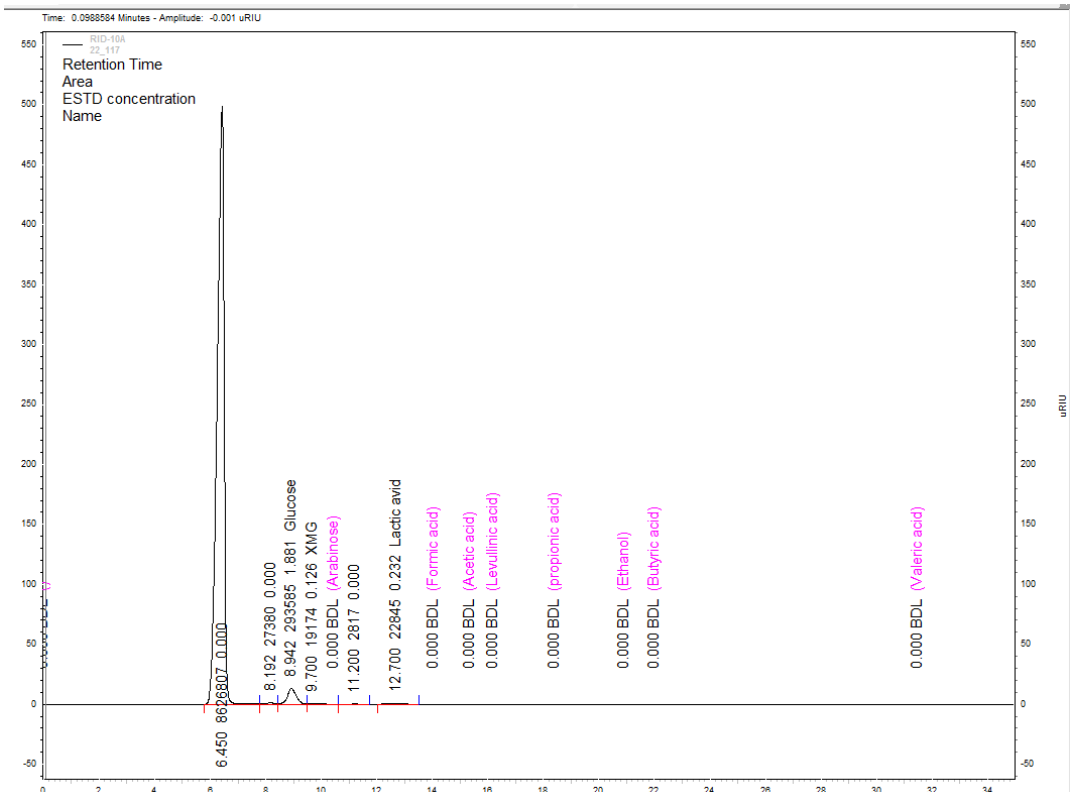

Figure 20 HPLC Trace

Chicken Serum Optimization

This experiment was done using PRA-240 WT cells at small scale in 75 mL T-flasks in triplicate for each level of CS. The CS levels tested in the flasks were: 0, 0.1, 1, 5,10, 20% v/v CS.

On Day 1 under the microscope, in the serum free flasks, the cells appeared more stationary and not as suspended in the medium as normal. 0.1 and 1% CS flasks had more growth compared to serum free. 5, 10 and 20% CS flasks all had normal growth. On Day 2, it did not appear as if there was any growth happening in the serum free flasks. The rest of the concentrations appeared to have cells growing and happy. On Day 3, the serum free cells finally started to grow and there are no noticeable differences between the flasks. On Day 4, the cells in serum free flasks seemed to be clumping together more so than normal while the rest of the concentrations appear to be normal. By Day 9, all the cells appeared to be done growing with no noticeable differences between triplicate flasks.

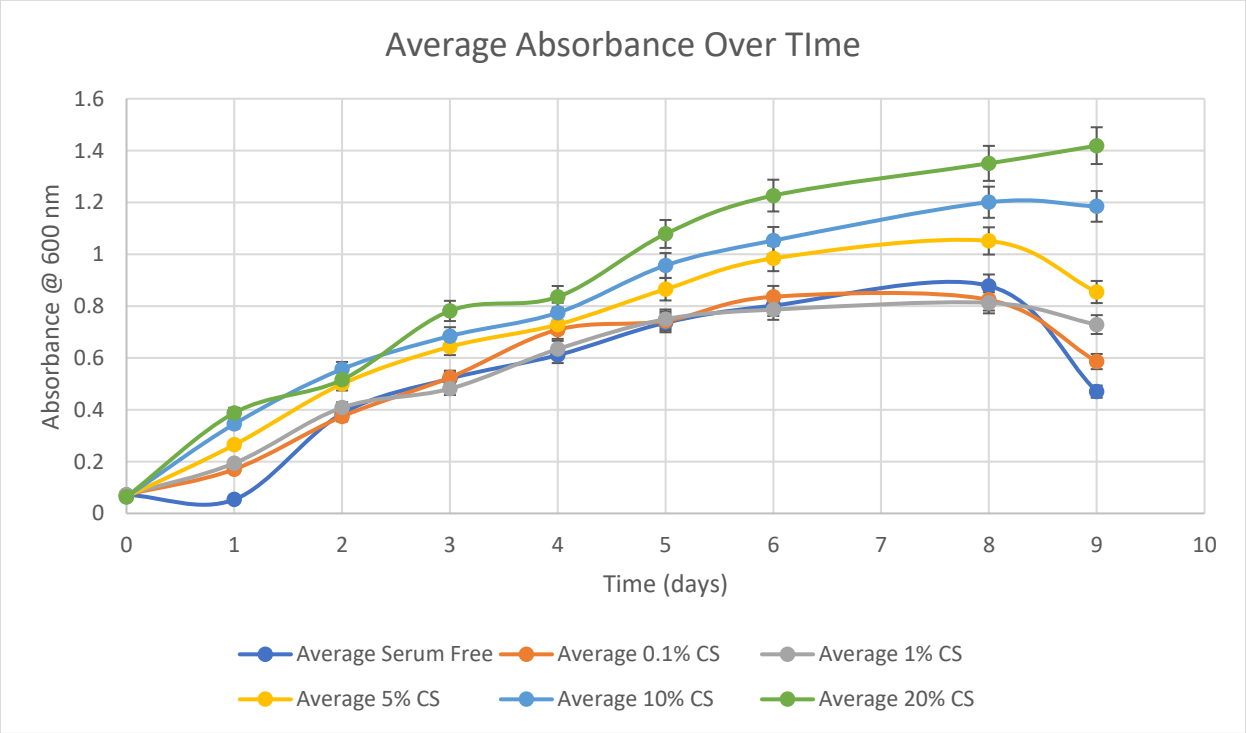

Figure 21 Growth Curve

Table 4 Grouping Information for Figure 21 Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
20	27	0.8510	A	
10	27	0.7523	A	B
5	27	0.6557	A	B
1	27	0.5414		B
0.1	27	0.5371		B
SF	27	0.5030		B

Means that do not share a letter are significantly different.

Figure 21 gives the absorbance data for each concentration over time. A One-Way ANOVA done over the curves gives a P-value of 0.00058 indicating a statistical difference between the different concentrations. Table 4 gives the more detailed Tukey method for grouping the curves together. Statistically speaking, there is not a difference between 5, 10 and 20%. 5% CS is the minimum concentration needed for abundant growth. While there was growth at the lower levels of concentration, there's enough of a difference that it's not worth using less than 5% CS in the media.

Figure 22 shows how the glucose concentration changes for the different concentrations of chicken serum media and how the glucose concentration changes over time. The serum free curve shows very little change in the concentration of glucose. This supports the slower growth curve in Figure 21. The cells ate less and were lacking nutrients necessary to achieve full growth. For the rest of the curves, the glucose concentration decreases over time. Although the glucose concentration does not approach zero. This suggests that glucose is not the limiting nutrient in the media for optimized growth. The limiting

nutrient is likely a molecule in chicken serum because growth increases as the percentage of chicken serum increases.

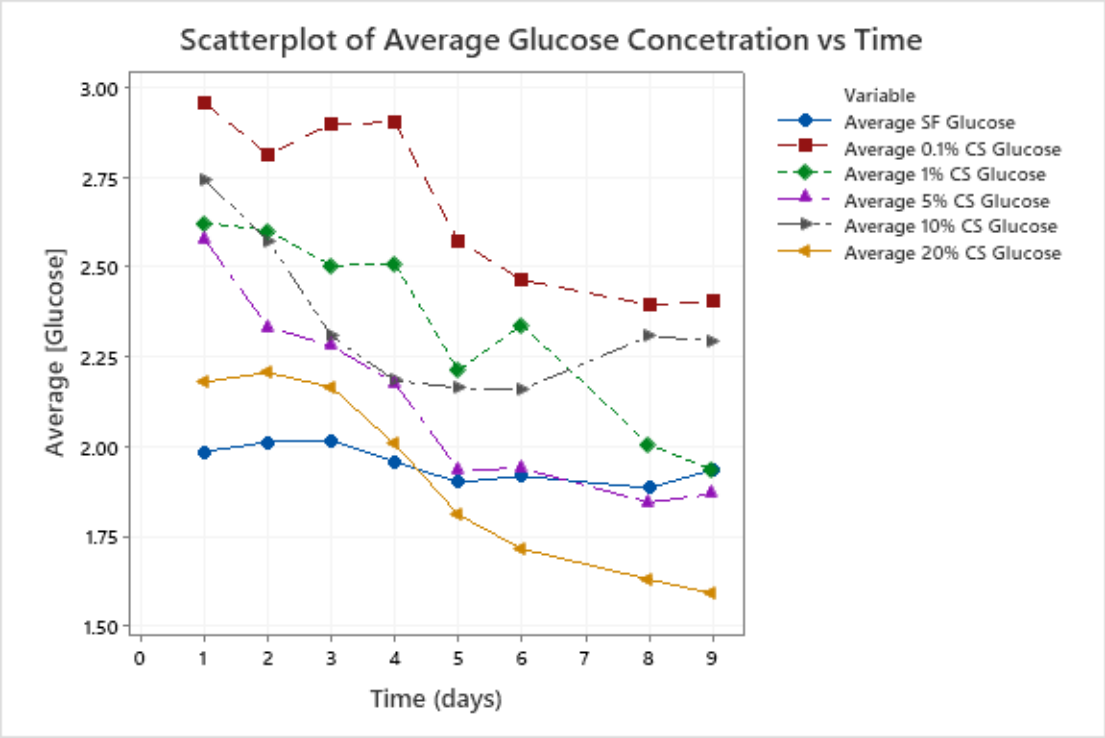

Figure 22 Glucose Concentration

Experiment 7: Comparing Cell Types in FBS Media

This experiment was to switch gears from media and try to grow genetically modified organisms in the bioreactor compared to wild type organisms. Vessels 1 and 3 contain PRA-240 WT cells in FBS media and vessels 2 and 4 contain PRA-393 GFP cells in FBS media. PRA-393 GFP cells have an inserted plasmid attached to PmMOE gene with a GFP tag for expression (Fernández-Robledo et al., 2008).

Figure 23 displays the absorbance data at 600nm for each vessel. All four redox graphs, in Figure 24, display the characteristic shape of a run free of contamination. Vessel 2 has a few areas where the redox dipped negative, however when looking at the cells under the microscope there were no visible signs of contamination. When comparing all the vessels under the microscope, there were no noticeable differences between all four bioreactors.

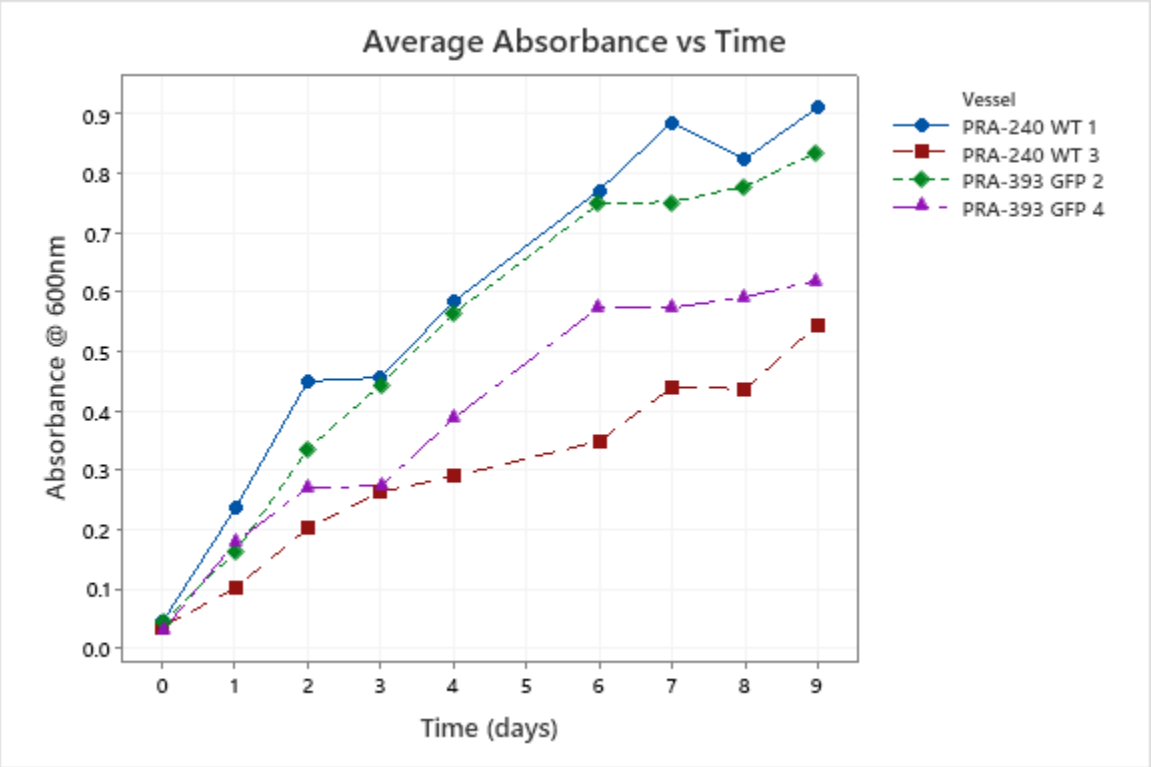

Figure 23 Growth Curve PRA-240 WT vs PRA-393 GFP in FBS media

Table 5 Grouping Information for Figure 23 Using Tukey Method and 95% Confidence

Vessel	N	Mean	Grouping	
1	18	0.5741	A	
2	18	0.5187	A	
4	18	0.3892	A	B
3	18	0.2964		B

Means that do not share a letter are significantly different.

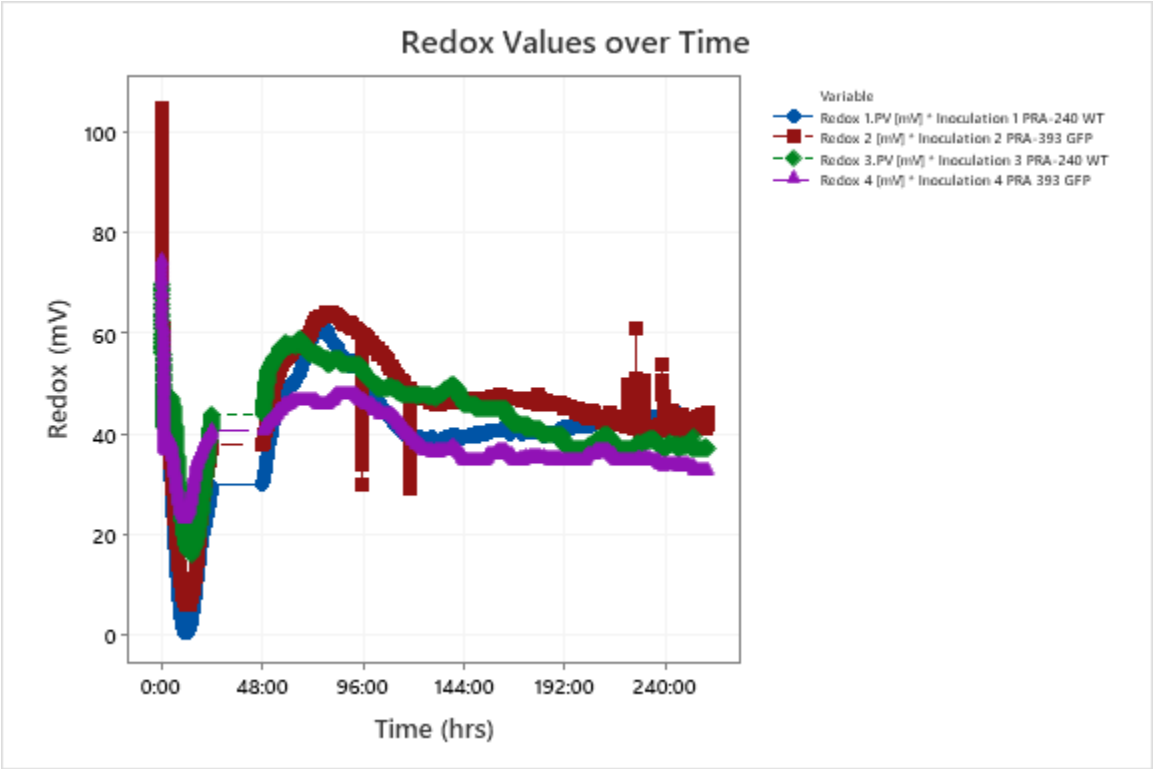

Figure 24 Redox Curve

This experiment confirmed that the rate of aeration is important to the growth of *Perkinsus*. Vessel 3 had a clogged air filter, which did not allow proper ventilation for the first 4 days of the experiment. On day 4, the air filter was replaced and the growth normalized to an extent. A One-Way ANOVA over the absorbance curves gives a P-Value of 0.004. Table 5 gives the breakdown of how the curves are statistically significant from each other.

Experiment 8: Comparing PRA-393 GFP Growth in FBS Media with Growth in CS Media

This experiment compares PRA-393 GFP cells with FBS media and CS media. Vessels 1 and 3 contain FBS media. Vessels 2 and 4 contain CS media. Under the microscope, there are no noticeable differences between cells from Day 1 to Day 3. On Day 3, all the cells appear to be in the growth phase. On day 4, Vessel 2 had visible bacterial contamination. The bacteria appeared to be taking over the culture. On Day 7, the University of Maine had a power failure in Jenness Hall. The computer running the bioreactors shut down and the system stopped recording pH, the temperature and the redox. When the power came back on, the system seemed to still be holding at the correct temperature, and the experiment ran for one more day without recording the temperature, pH and the redox. On Day 8, there were no visible signs of stress in the cells in vessels 1, 3 and 4. In Vessel 2, *Perkinsus* appears to have started growing again, taking the culture back from the bacteria. However, the culture is still visibly infected.

The absorbance data at 600 nm is given in Figure 25. A One-Way ANOVA done over the whole curve gives a P-value of 0.093 indicating that there isn't a statistical difference in the growth rate. However, if you isolate the part of the curve after the contamination happened, a One-Way ANOVA gives a P-value of 0.000000003 and the grouping information is listed in Table 7. The growth in Vessel 2 is statistically different from the other vessels.

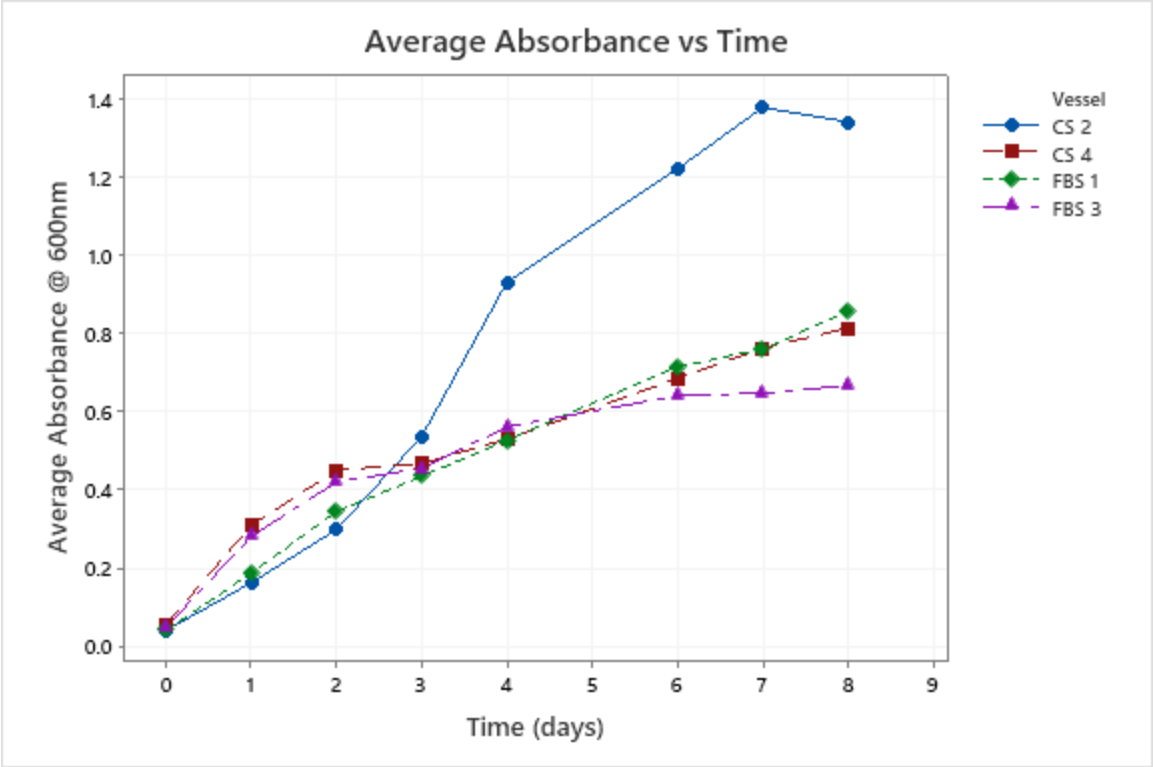

Figure 25 Growth Curve PRA-393 GFP cells grown in FBS and CS media

Table 6 Grouping Information for the whole curve for Figure 25 Using the Tukey Method and 95% Confidence

Vessel	N	Mean	Grouping
2	16	0.739	A
4	16	0.5104	A
1	16	0.4857	A
3	16	0.4657	A

Means that do not share a letter are significantly different.

Table 7 Grouping Information Day 4 and after for Figure 25 Using the Tukey Method and 95% Confidence

Vessel_	N	Mean	Grouping	
2	8	1.2183	A	
1	8	0.7190		B
4	8	0.6996		B
3	8	0.6298		B

Means that do not share a letter are significantly different.

Figure 26 gives the Redox graph for Experiment 8. Vessels 1, 3 and 4 are all starting to display the characteristic shape for the growth of *Perkinsus*. Vessel 2 starts to display the shape, but between day 3 and day 4 starts to go negative indicating the contamination.

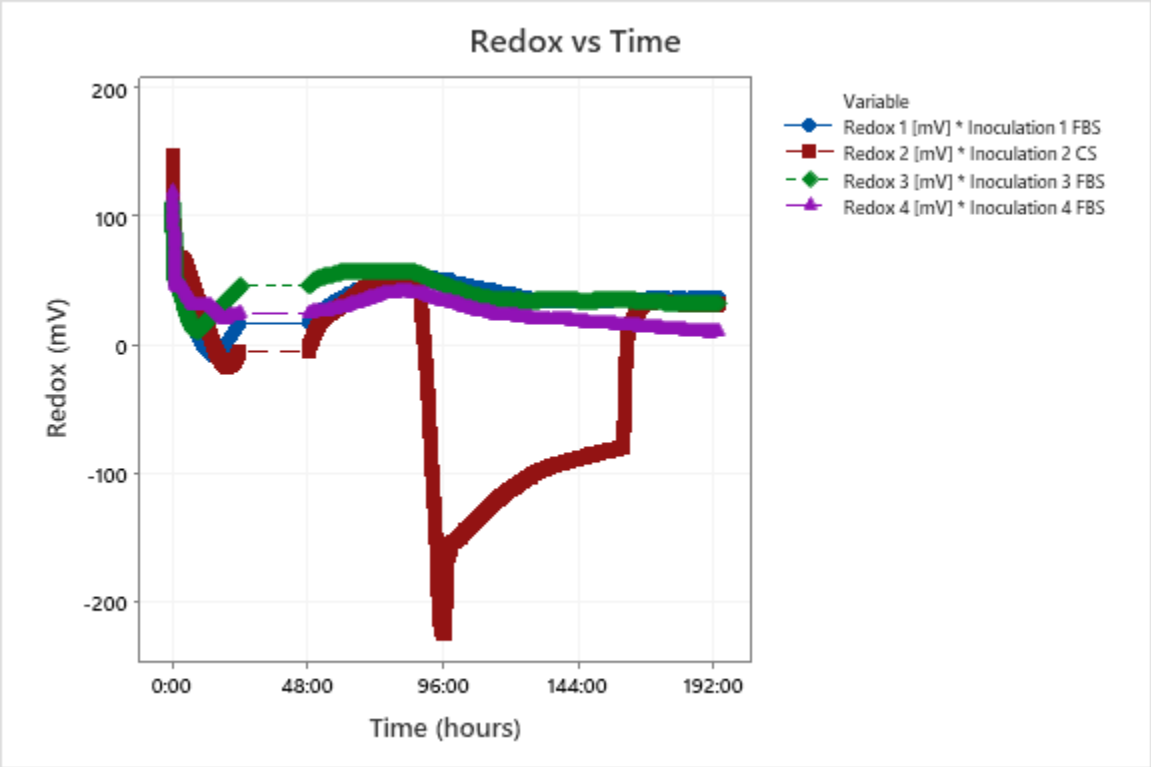

Figure 26 Redox Curve

Figure 27 shows how the glucose concentration changes over time. Vessels 1, 3, and 4 follow the same trend as previous experiments. The curves start around 2 g/L and drop to around 1.5 g/L. Vessel 2 starts at approximately 2 g/L but then drops, over time, to almost zero. Vessel 2 was contaminated with bacteria, While *Perkinsus* eats the glucose slowly, bacteria eat consume glucose at a much faster rate. This is another indication that Vessel 2 was contaminated.

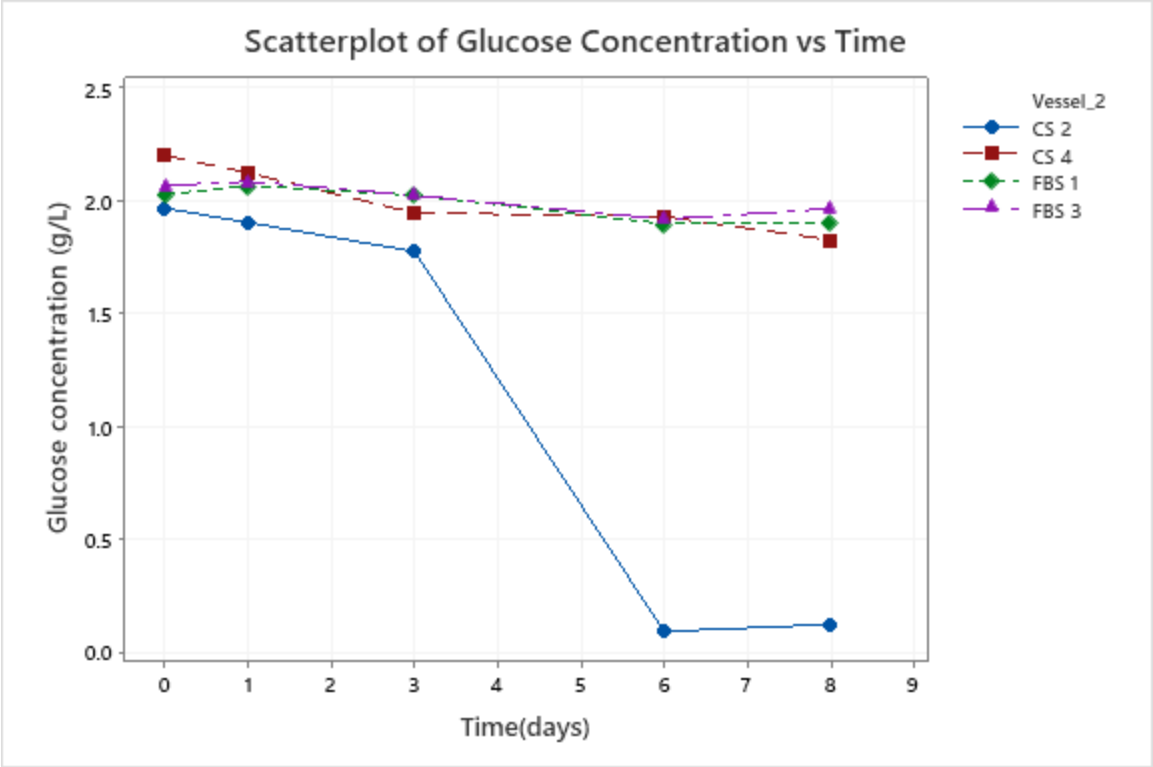

Figure 27 Glucose Concentration vs Time

CHAPTER 4

MODELING

Process Description

The four different flow sheet configurations were simulated with SuperPro Designer. While the pure components database is extensive, five new compounds had to be added to create this model (FBS Media, CS Media, coating, stabilizers, and flavoring). For ease of modeling, all five of the compounds were thermodynamically based on water.

Model 1

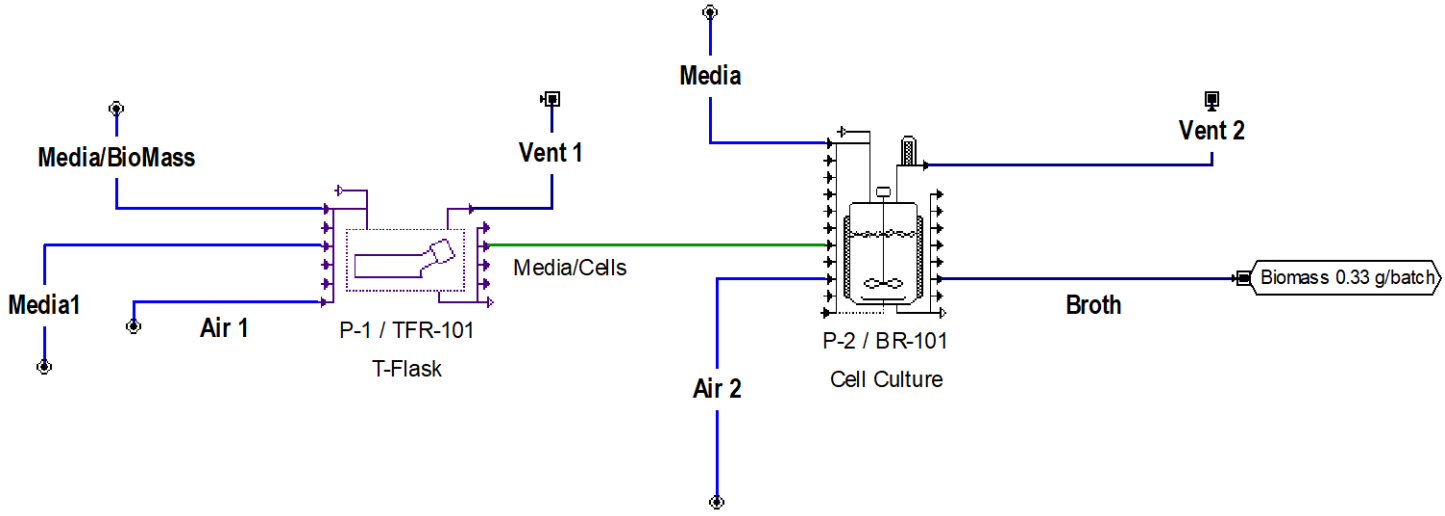

Figure 28 Experimental Small-Scale Model

Figure 28 shows the model of what was done experimentally in the lab. In the stream labeled Media1, 20 mL of media (FBS media or CS media) enter into P-1/TRF-101 with the composition of: 4.0758% FBS or CS media and 95.9242% water. In the stream labeled Media/Biomass, 5 mL enter into P-1/TRF-101 with the composition of 0.5% biomass, 4.5% FBS or CS Media, and 95% water. P-1/TRF-101 is then allowed to ferment for 8 days before the entire contents of the flask are transferred to P-2/BR-101. In the stream labeled Media, 200 mL of media (FBS or CS media) are transferred into P-2/BR-101 with the same composition as the stream of Media1. P-2/BR-101 is then allowed to ferment for 8 days before

transferring the contents out of the bioreactor. The conversion factor is set to 99% for both P-1/TRF-101 and P-2/BR-101 and the reaction is listed in Equation 1.

$$0.83M\ FBS(or\ CS)Media + 1M\ O_2 + 1M\ N_2 \rightarrow$$
$$0.02M\ CH_{1.8}O_{0.5}N_{0.2} + 0.57M\ CO_2 + 0.28M\ C_3H_6O_3 + 1.36M\ H_20 \quad (1)$$

Model 2

Figure 29 shows a theoretical process of going from small scale growth to large scale (industrial) growth. The first step, is to make media (FBS or CS) in P-2/V-101. Once the media is well mixed, 20 mL pass through a sterilization filter, P-3/DE-101 before entering P-1/TRF-101. Using stream S-106, 5 mL of media and biomass with the composition of 1% biomass, 4% FBS or CS media, and 95% water enter into P-1/TRF-101. P-1/TRF-101 is then allowed to ferment for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. Using stream S-107, the media and biomass are transferred into P-4/DBS-101.

P-6/V-103 mixes 200 mL of media (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-7-102, before entering P-4/DBS-101. P-4/DBS-101 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-5/AF-101 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-114, the entire contents of P-4/DBS-101 is transferred to P-8/DBS-102.

P-9/V-103 mixes 4 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-10/DE-103 before entering P-8/DBS-102. P-8/DBS-102 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-11/AF-102 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-123, the entire contents of P-8/DBS-102 is transferred to P-13/DBS-103.

P-12/V-104 mixes 80 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-15/DE-104 before entering P-13/DBS-103. P-8/DBS-102 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-14/AF-103 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-128, the entire contents of P-13/DBS-103 is transferred to P-16/DBS-104.

P-18/V-105 mixes 1600 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-19/DE-105 before entering P-16/DBS-104. P-16/DBS-104 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-17/AF-104 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-132, the entire contents of P-16/DBS-104 is transferred to P-21/DC-101 for centrifugation.

P-21/DC-101 separates the biomass from the media. The media leaves via stream S-139 and is then heat sterilized in P-22/ST-101, before being disposed properly. The biomass exits via stream S-138 to be freeze dried in P-20/FDR-101 with an initial loss on drying (LOD) of 4.57% and a final LOD of 0.01%.

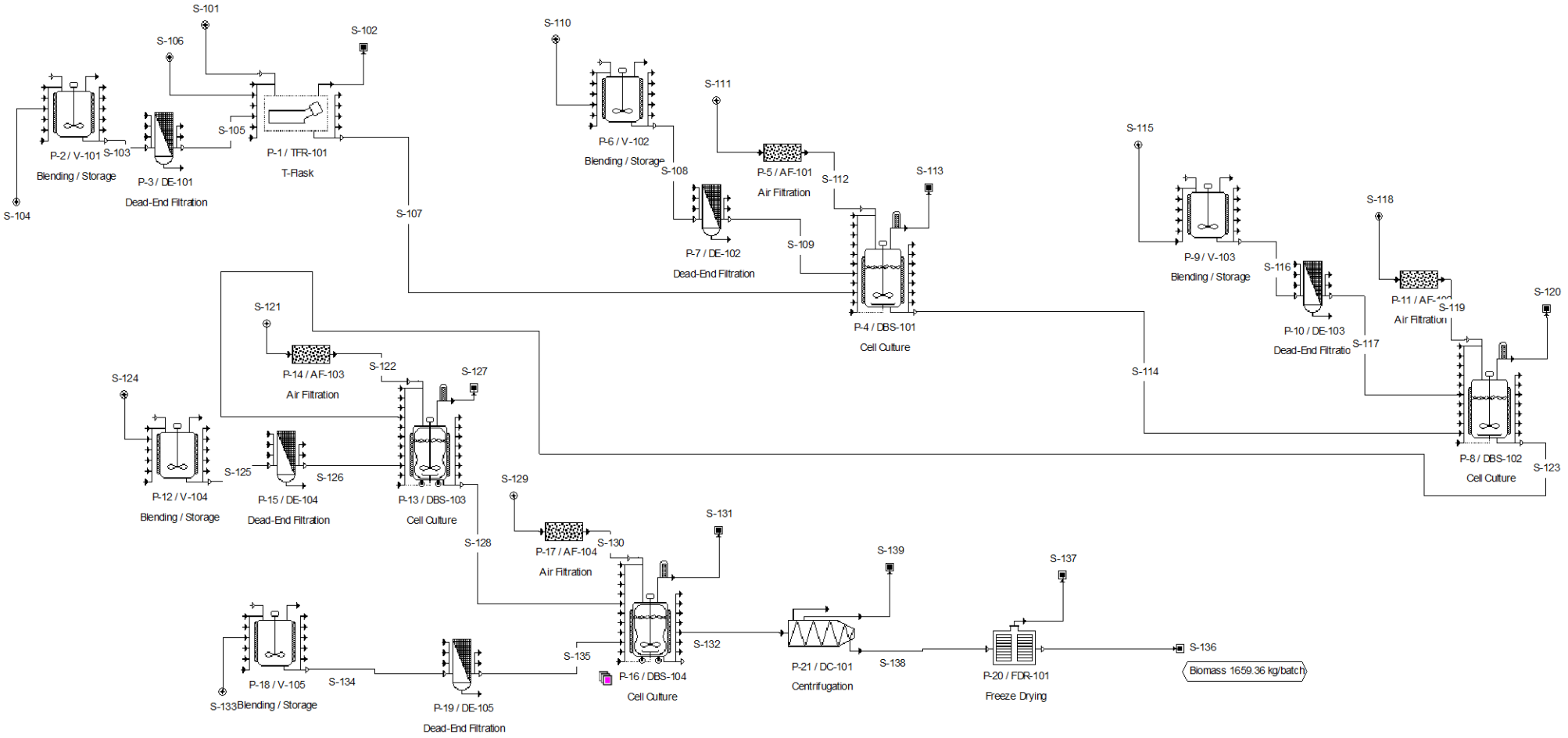

Figure 29 Industrial Scale Up Model

Model 3

Figure 30 shows a theoretical process of going from small scale growth to large scale (industrial) growth with the added factor of turning the biomass into tablets for human consumption. The first step, is to make media (FBS or CS) in P-2/V-101. Once the media is well mixed, 20 mL pass through a sterilization filter, P-3/DE-101 before entering P-1/TRF-101. Using stream S-106, 5 mL of media and biomass with the composition of 1% biomass, 4% FBS or CS media, and 95% water enter into P-1/TRF-101. P-1/TRF-101 is then allowed to ferment for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. Using stream S-107, the media and biomass are transferred into P-4/DBS-101.

P-6/V-103 mixes 200 mL of media (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-7-102, before entering P-4/DBS-101. P-4/DBS-101 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-5/AF-101 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-114, the entire contents of P-4/DBS-101 is transferred to P-8/DBS-102.

P-9/V-103 mixes 4 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-10/DE-103 before entering P-8/DBS-102. P-8/DBS-102 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-11/AF-102 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-123, the entire contents of P-8/DBS-102 is transferred to P-13/DBS-103.

P-12/V-104 mixes 80 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-15/DE-104 before entering P-13/DBS-103. P-8/DBS-102 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-14/AF-103 is an air filter which reduces the chances of bacterial contamination

during fermentation. Using stream S-128, the entire contents of P-13/DBS-103 is transferred to P-16/DBS-104.

P-18/V-105 mixes 1600 L of (FBS or CS) with a composition of 4.0758% FBS or CS media and 95.9242% water and then passes the media through a sterilization filter, P-19/DE-105 before entering P-16/DBS-104. P-16/DBS-104 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-17/AF-104 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-132, the entire contents of P-16/DBS-104 is transferred to P-21/DC-101 for centrifugation.

P-21/DC-101 separates the biomass from the media. The media leaves via stream S-139 and is then heat sterilized in P-30/ST-101, before being disposed properly. The biomass exits via stream S-138 to be freeze dried in P-20/FDR-101 with an initial LOD of 4.57% and a final LOD of 0.01%.

After being freeze dried, the biomass enters P-22/NM-101 for nano-milling. The biomass then enters P-23/V-106 for the addition of stabilizers, flavoring, and water. After being mixed together, the mixture then is sent for granulation in P-24/GRN-101 until the final LOD is 5%. After granulation, the mixture is sent to a storage contain, P-25/V-107. From the storage container, the granulated mixture is sent to P-26/TP-101 to be turned into 1-gram tablets. The tablets are then sent to a storage container, P-27/DB-101.

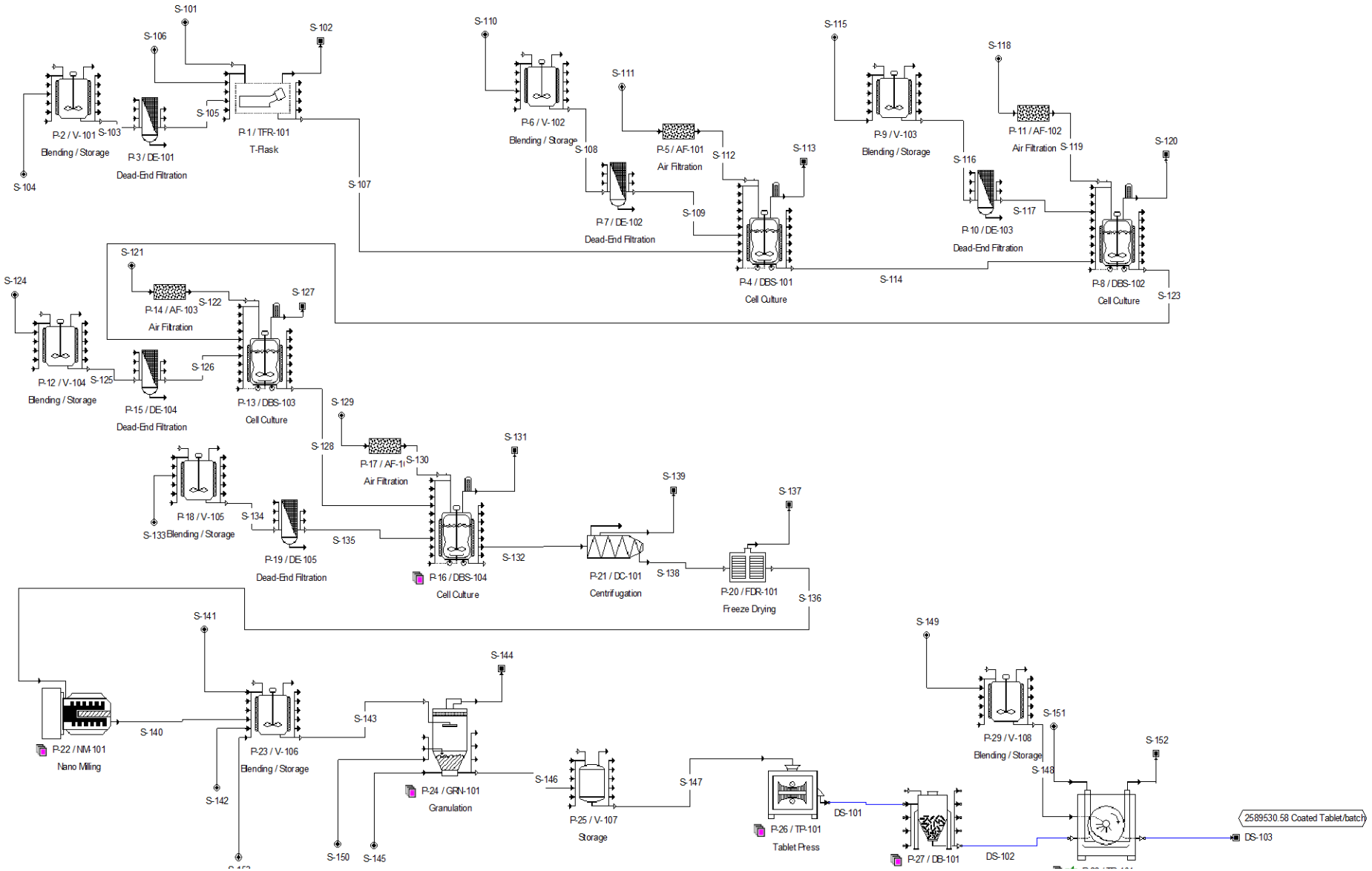

Figure 30 Industrial Scale and Creating Tablets Model

From P-27/DB-101, the tablets then enter P-28/TB-101 to receive the coating around the tablet. P-28/TB-101 goes through 4 cycles of adding the coating to the tablets. The coating solution is pulled from P-29/V-108 into P-28/TB-10.

Model 4

After spending time looking at Model 3, there are some drawbacks to the way the process was modeled. For instance, in an industrial setting the media would not be made before each step as it was in Model 3. Model 4 seeks to solve this problem by making media in one bigger batch and then sending the media to each bioreactor as needed, which is displayed in Figure 31.

P-3/V-101 mixes together 2000 L of media with the mass composition of 4.0758% FBS or CS media and 95.9242% water. The media is then passed through a sterilization filter, P-5/DE-101 before entering P-4/V-102 for storage. This step cycles independently of the main recipe so that the media can be made without reference to whether or not cells are being grown.

P-1/TRF-101 transfers in 20 mL of media from P-4/V-102 and then 5 mL of biomass and media with the mass composition of 0.5% biomass, 4.5% media, and 95% water. P-1/TRF-101 is then allowed to ferment for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. Using stream S-104, the media and biomass are transferred into P-2/DBS-101.

P-2/DBS-101 transfers in 200 mL of media from P-4/V-102 and then the contents of stream S-104. P-2/DBS-101 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-12/AF-101 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-108, the entire contents of P-2/DBS-101 is transferred to P-6/DBS-102.

P-6/DBS-102 transfers in 4L of media from P-4/V-102 and then the contents of stream S-108. P-6/DBS-102 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in

Equation 1. P-13/AF-102 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-116, the entire contents of P-6/DBS-102 is transferred to P-8/DBS-104.

P-8/DBS-104 transfers in 80L of media from P-4/V-102 and then the contents of stream S-116. P-8/DBS-104 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-14/AF-103 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-124, the entire contents of P-8/DBS-104 is transferred to P-7/DBS-103.

P-7/DBS-103 transfers in 1600L of media from P-4/V-102 and then the contents of stream S-124. P-7/DBS-103 then ferments for 8 days with a conversion factor set to 99% using the reaction listed in Equation 1. P-15/AF-104 is an air filter which reduces the chances of bacterial contamination during fermentation. Using stream S-125, the entire contents of P-7/DBS-103 is transferred to P-9/DS-101 for centrifugation.

P-9/DS-101 separates the biomass from the media. The media leaves via stream S-139 and is then heat sterilized in P-16/ST-101, before being disposed properly. The biomass exits via stream S-126 to be freeze dried in P-10/FDR-101 with an initial LOD of 4.57% and a final LOD of 0.01%.

After being freeze dried, the biomass enters P-11/NM-101 for nano-milling. The biomass then enters P-23/V-106 for the addition of stabilizers, flavoring, and water. After being mixed together, the mixture then is sent for granulation in P-24/GRN-101 until the final LOD is 5%. After granulation, the mixture is sent to a storage contain, P-25/V-107. From the storage container, the granulated mixture is sent to P-26/TP-101 to be turned into 1-gram tablets. The tablets are then sent to a storage container, P-27/DB-101.

From P-27/DB-101, the tablets then enter P-28/TB-101 to receive the coating around the tablet. P-28/TB-101 goes through 4 cycles of adding the coating to the tablets. The coating solution is pulled from P-29/V-108 into P-28/TB-10.

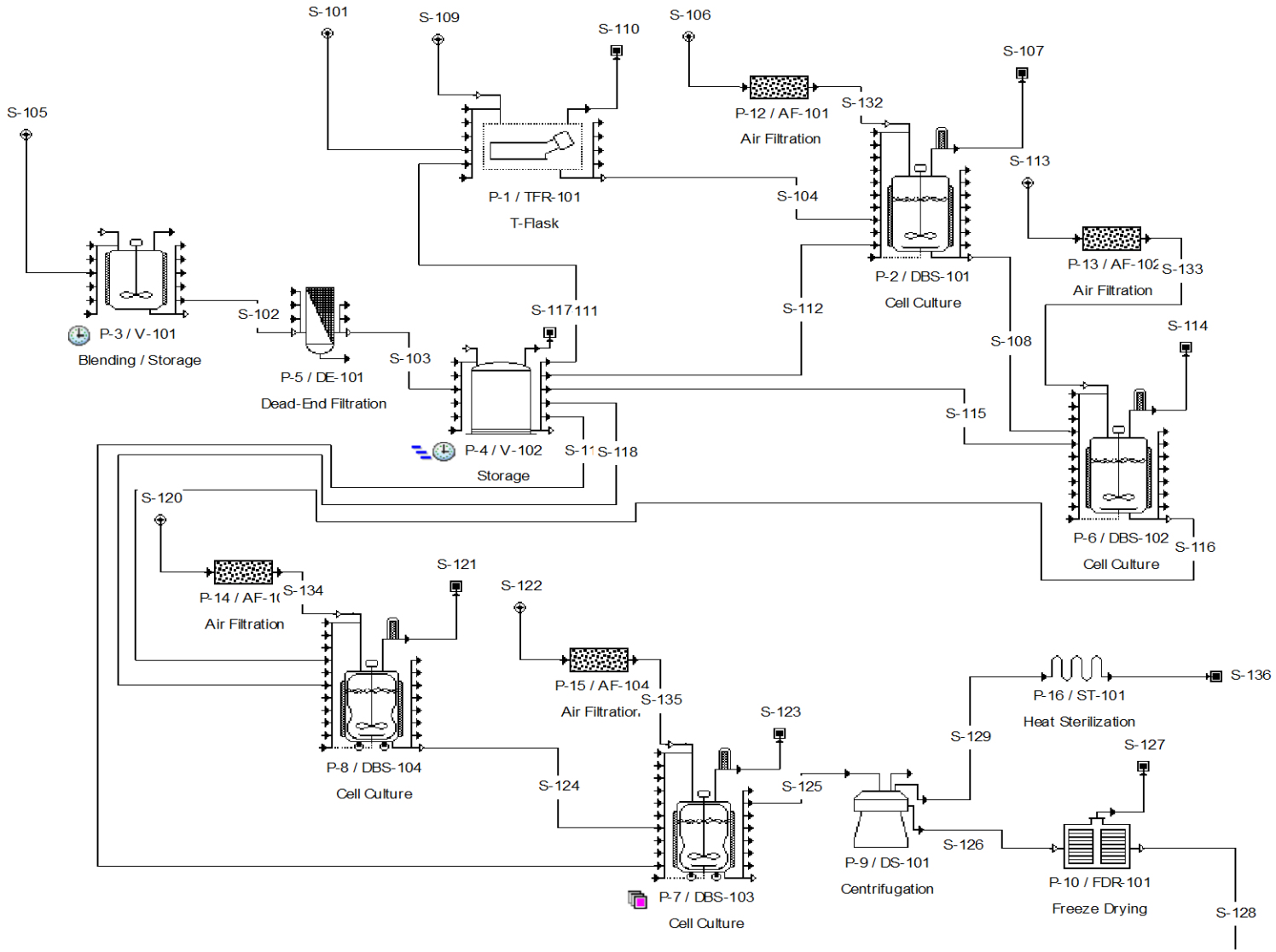

Figure 31 Realistic Industrial Scale Model

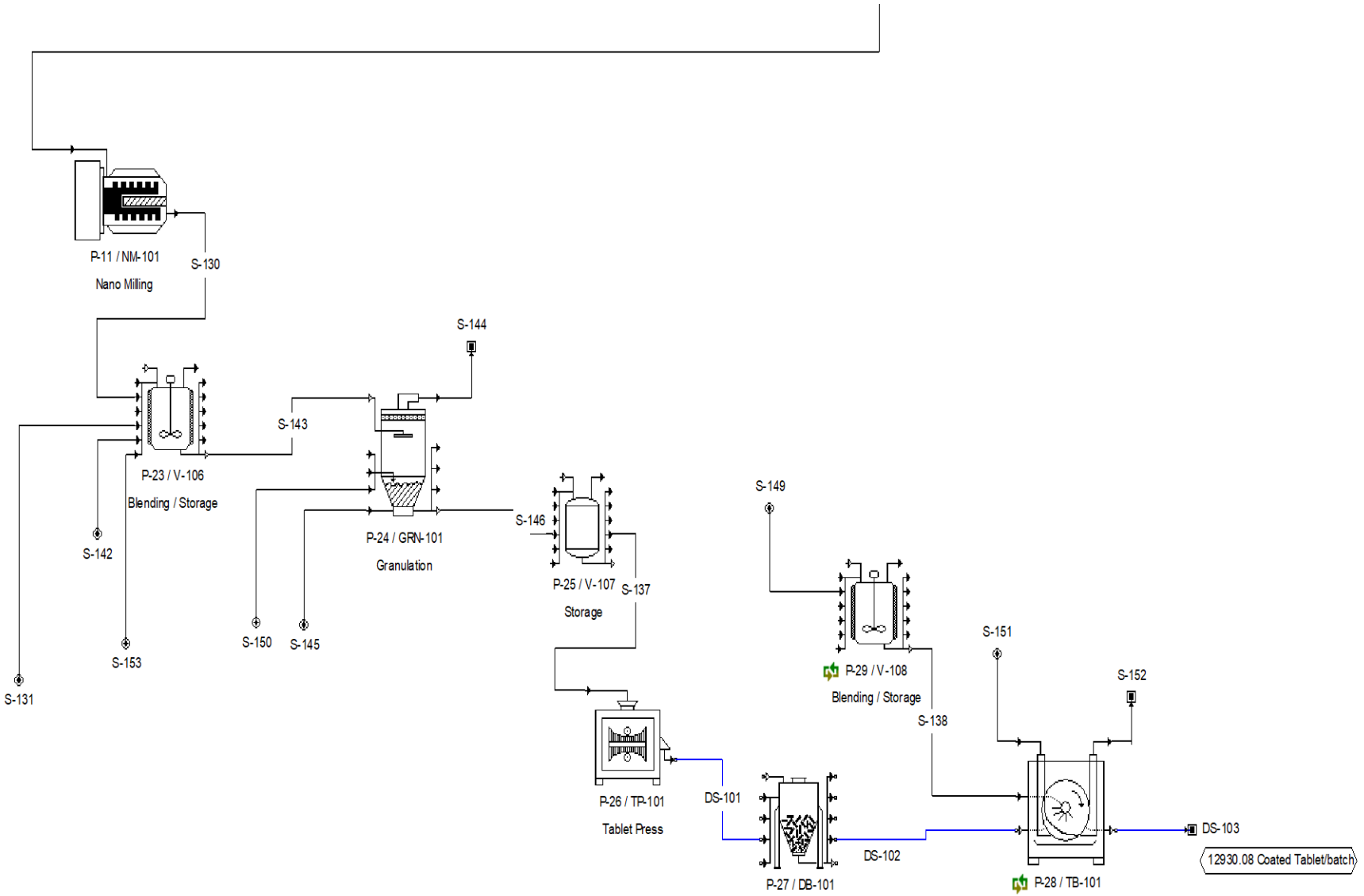

Figure 31 Continued

Economic Analysis

Calculations

Across all the process models, the goal was to compare different scenarios and to determine which would be the most cost-effective-process. The different sensitivity variables included: Media Type (FBS vs CS), Bioreactor types (all disposable, all reusable and sterilizable, and 50/50 split with the first two being reusable and the second two being disposable), and different failure rates (10%, 25%, 50%) of the product.

The economic analysis embedded in SuperPro Designer employs a capital budgeting approach to developing an estimate for overall capital and operating costs. The basis of the capital cost is the purchase cost of all the major equipment needed to fully equip a manufacturing plant. SuperPro deploys a database that contains major equipment purchase costs and then applies multiplier factors to include associated minor equipment and other capital costs. Operating costs are determined from consumable materials and supplies, labor estimates, utilities, wastes and disposals, and other associated fixed operating costs. The structure of these estimates is outlined below.

$$Capital\ Cost = Direct\ Fixed\ Capital\ (DFC) + Working\ Capital\ (WC) + Start\ up\ Costs \quad (2)$$

$$DFC = Direct\ Cost\ (DC) + Indirect\ Costs\ (IC) + Other\ Costs\ (OC) \quad (3)$$

$$DC = Equipment\ Cost(PC) + instalation + A + B + C + D + E + F + G \quad (4)$$

In which,

$$PC = Listed\ Equipment\ Purchase\ Cost\ (LEP) + Unlisted\ Equipment\ Purchase\ Cost\ (ULEPC) \quad (5)$$

and items A through G are listed in Table 8, below.

$$Operating\ Cost(per\ year) = materials + consumables + labor\ dependent + utlities + waste\ treatment + facility\ dependent + laboratory/\ QC/QA + transportion\ + miscellaneous\ + {advertising}/{selling} + running\ royalties\ + failed\ product\ disposal\ costs \quad (6)$$

$$Unit\ Production\ Cost = \frac{Operating\ Cost\ per\ year}{kg\ or\ tablets\ per\ batch * baches\ per\ year} \quad (7)$$

$$\frac{Capital\ Cost}{kg\ or\ tablet} = \frac{Capital\ Cost}{\#\ of\ kg\ or\ tablet\ \ per\ batch(\#\ of\ batches\ per\ year) * (length\ of\ project\ in\ years)} \quad (8)$$

Table 8 Cost Calculations

Direct Cost (DC)	
Piping(A)	0.35xPC
Instrumentation(B)	.40xPC
Insulation [C]	0.03xPC
Electrical Facilities [D]	0.1xPC
Buildings [E]	0.45xPC
Yard Improvement (F)	0.15xPC
Auxiliary Facilities (G)	0.4xPC
Indirect Cost (IC)	
Engineering	0.25xDC
Construction	0.35xDC
Other Cost (OC)	
Contractor's fee	0.5 x(DC+IC)
Contingency	0.1 x (DC+ IC)

Listed Equipment Purchase Costs

The purchase cost of the major equipment is based on the operating units in the process design, their size, operating conditions and the built-in cost model in Super Pro.

Model 1

Model 1 was used as a starting point to gain familiarity with the simulation software and develop a basis from which to build the rest of the models. An economic analysis was not done on Model 1 because it was not an industrial scale model.

Model 2

Figure 32 shows the total cost for the major equipment in Model 2. The failure rate and media type do not play into major equipment costs. The only factor that plays into the major equipment costs is the bioreactor type. Reusable/sterilizable bioreactors cost a total of $4.3 million; systems that host disposable bioreactors cost a total of $3.9 million; 50/50 split cost a total of $3.6 million. A further breakdown of equipment for this model is listed in Table 9.

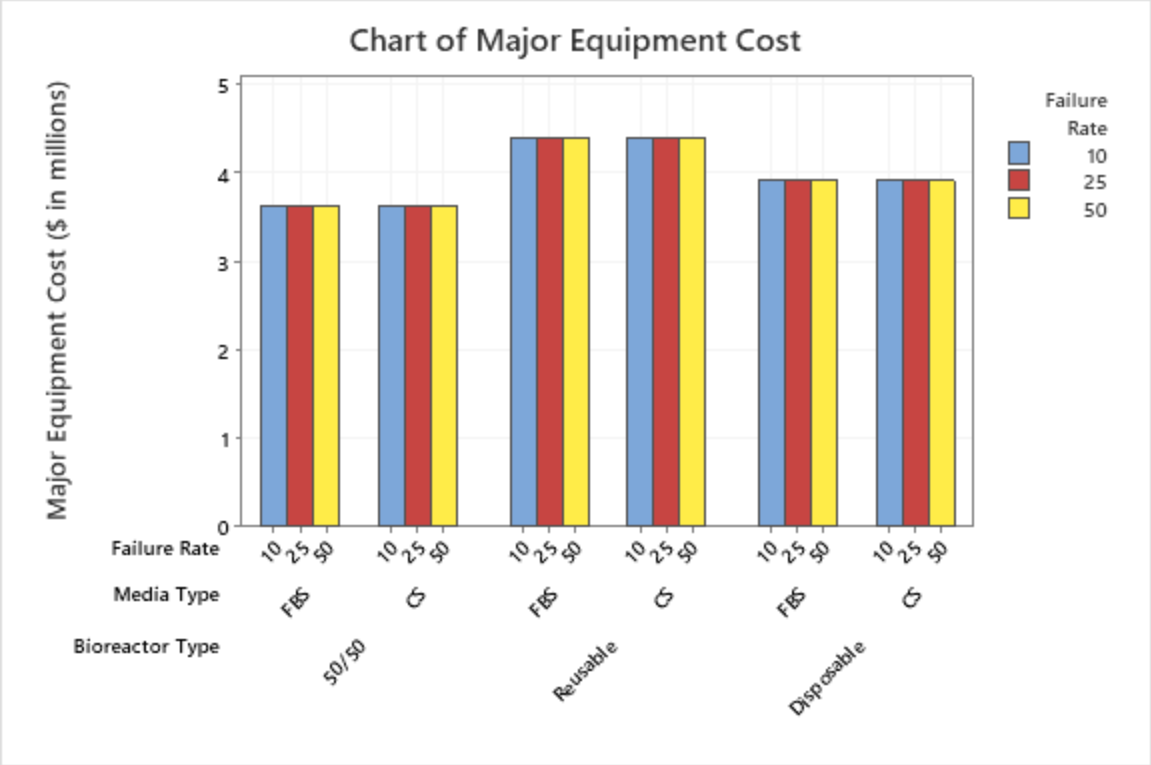

Figure 32 Major Equipment for Model 2

Table 9 Breakdown of Major Equipment Model 2

Major Equipment	Unit Cost ($)	Number of Units
Common Equipment		
Blending Tank (0.02L - 90L)	147,000	4
Dead-End Filter	41,000	5
Air Filter	7,000	4
Blending Tank (1777L)	208,000	1
Decanter Centrifuge	262,000	1
Freeze Dryer	102,000	1
Heat Sterilizer	190,000	1
Disposable Scenario		
Disposable Bioreactor V= 700L	221,000	7
50/50 Split		
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1

Table 9 Continued

Disposable Bioreactor V= 700L	221,000	5
Reusable		
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1
Bioreactor V = 128.46L	464,000	1
Bioreactor V = 2568.83L	1,260,000	1

Model 3

Figure 33 shows the total cost for the major equipment in Model 2. The failure rate and media type do not play into major equipment costs. The only factor that plays into the major equipment costs is the bioreactor type. Reusable/sterilizable bioreactors cost a total of $6.2 million; disposable bioreactors cost a total of $5.8 million; 50/50 split cost a total of $5.5 million. A further breakdown of equipment for

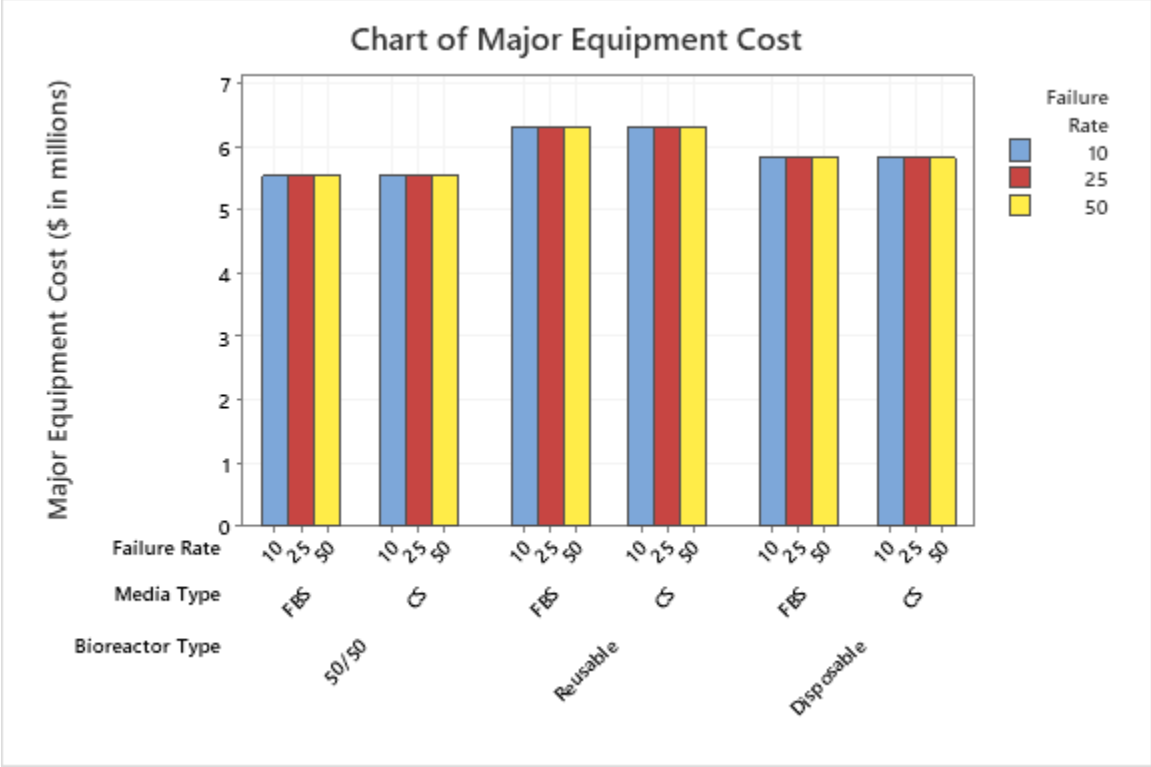

Figure 33 Equipment Costs for Model 3

this model is listed in Table 3. These numbers go up compared to Model 2 because Model 3 includes the downstream process of making tablets out of the biomass, which includes more equipment.

Table 10 Breakdown of Major Equipment for Model 3

Major Equipment	Unit Cost ($)	Number of Units
Common Equipment		
Blending Tank (0.02L - 90L)	147,000	5
Dead-End Filter	41,000	5
Air Filter	7,000	4
Blending Tank (1777L)	208,000	1
Decanter Centrifuge	262,000	1
Freeze Dryer	102,000	1
Heat Sterilizer	190,000	1
Nano Mill	241,000	1
Granulator	306,000	1
Receiver Tank	51,000	1
Tablet Press	225,000	1
Discrete Bin	54,000	1
Tablet Coater	345,000	1
Disposable		
Disposable Bioreactor V= 700L	221,000	7
50/50 Split		
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1
Disposable Bioreactor V= 700L	221,000	5
Reusable		
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1
Bioreactor V = 128.46L	464,000	1
Bioreactor V = 2568.83L	1,260,000	1

Model 4

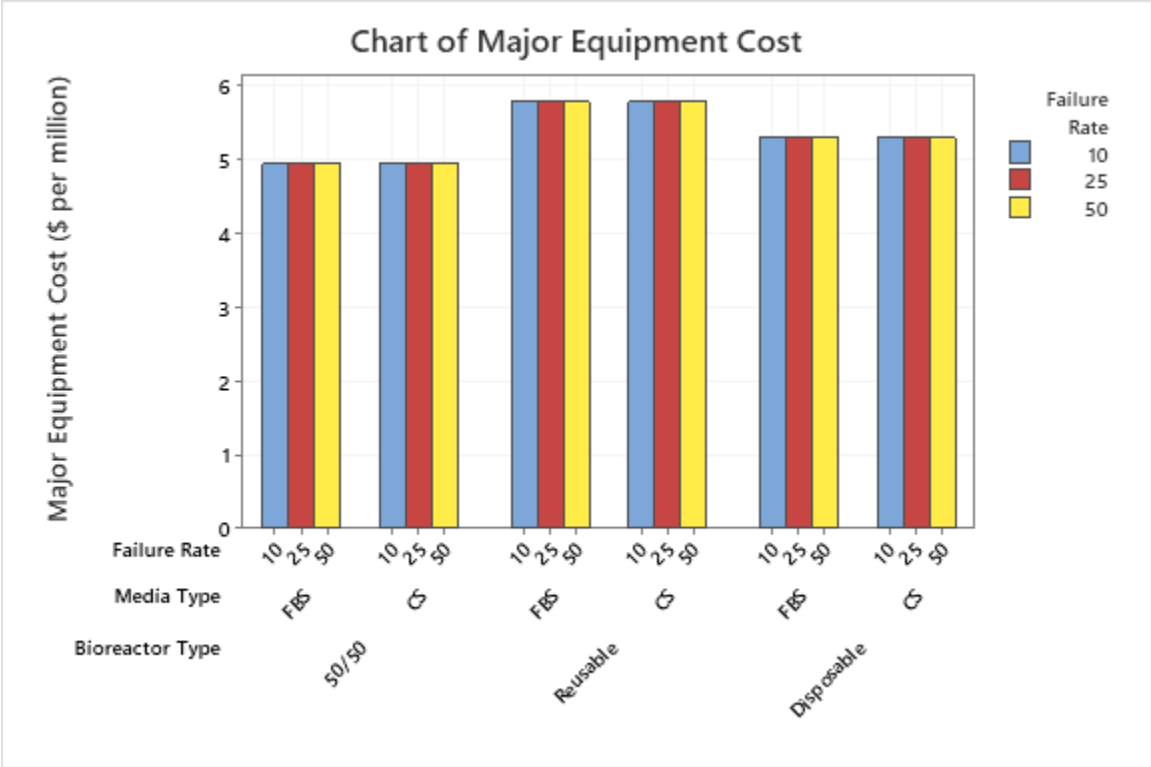

Figure 34 Equipment Costs for Model 4

Figure 34 shows the total cost for the major equipment in Model 2. The failure rate and media type do not play into major equipment costs. The only factor that plays into the major equipment costs is the bioreactor type. Reusable/sterilizable bioreactors cost a total of $5.7 million; disposable bioreactors cost a total of $5.2 million; 50/50 split cost a total of $4.9 million. A further breakdown of equipment for this model is listed in Table 11. These numbers go down because unnecessary equipment (blending tanks and sterilization filters) was eliminated from Model 3 to make Model 4.

Table 11 Breakdown of Major Equipment for Model 4

Major Equipment	Unit Cost ($)	Number of Units
Common Equipment		
Flat Bottom Tank V = 6883.08	31,000	2
Blending Tank V = 2224.76L	215,000	1
Dead End Filter	41,000	1
Disk Stack Centrifuge	219,000	1
Freeze Dryer	102,000	1
Blending Tank V = 35.93L	147,000	2
Granulator	306,000	1
Nano Mill	241,000	1
Receiver Tank	51,000	1
Tablet Press	225,000	1
Discrete Bin	54,000	1
Tablet Coater	345,000	1
Air Filter	7,000	4
Heat Sterilizer	190,000	1
Disposable		
Disposable Bioreactor V= 700L	221,000	7
50/50 Split		
Disposable Bioreactor V= 700L	221,000	5
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1
Reusable		
Bioreactor V = 0.34L	106,000	1
Bioreactor V = 6.44L	106,000	1
Bioreactor V = 128.46L	464,000	1
Bioreactor V = 2568.83L	1,260,000	1

Direct Fixed Capital

The Direct Fixed Capital (DFC) refers to the fixed assets of an investment, such as plant and equipment. There are direct and indirect costs associated with the DFC. How this number is calculated is shown in Equations 2 & 3 and Table 8.

Models 2, 3, 4

The DFC is not dependent on media type or the failure rate which can be seen in Figure 35. (Figure 35 displays data from Model 4. Figures for the other models can be found in Appendix B). Neither number contributes to how the DFC is calculated. The lowest DFC for Model 2 is $24.06 million for the 50/50 split bioreactor scenario, which correlates to the lowest scenario for Capital Cost. The DFC does increase from Model 2 because there is more equipment needed in this model. With more equipment, a bigger plant would also be necessary. The lowest DFC for Model 3 is $37.2 million for the 50/50 bioreactor split. The

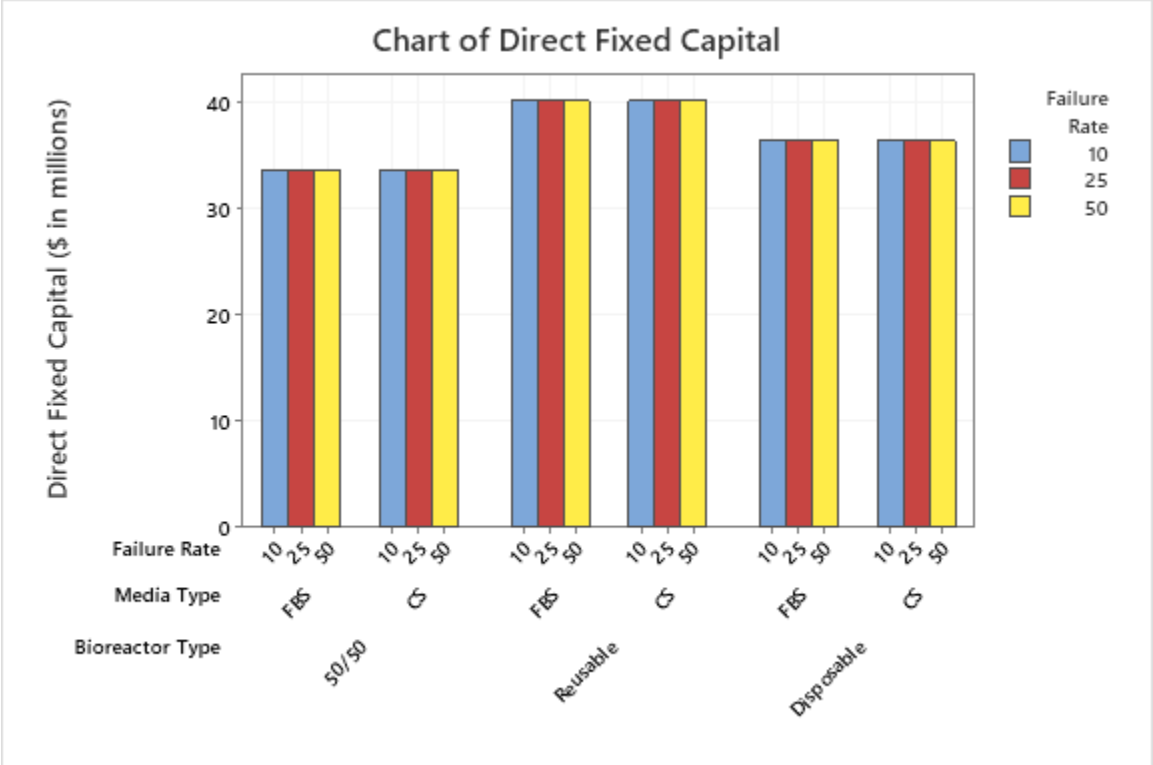

Figure 35 Direct Fixed Capital for Model 4

lowest DFC for Model 4 is 50/50 bioreactors with $33.7 million. This number is lower than Model 3 because this model eliminates unnecessary equipment while still keeping the output as high as possible.

Capital Cost

The Total Capital Investment or Capital Costs refers to the fixed costs that are associated with a process. This is based on the Major Equipment Costs and is calculated as the sum of the following cost items over all sections of a process. Equation 2 shows how this number is calculated.

Models 2, 3 and 4

The next factor looked at was the Capital Cost of the models and how they changed with the different variables (media type, bio reactor type and failure rate). All three models showed the same trends. They type of media (FBS or CS) had a slight effect on the Capital Cost with CS media being slightly lower than the cost with FBS media. The biggest effect on Capital Cost was the bioreactor types. Figure 36

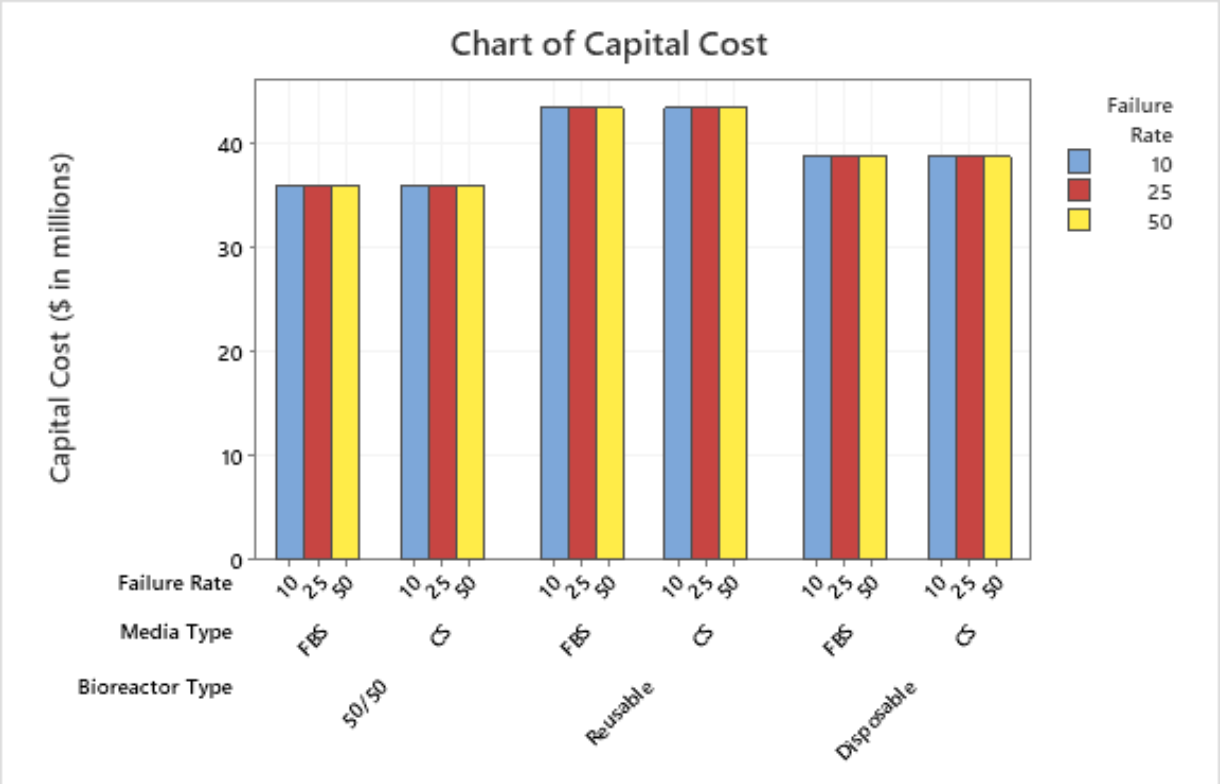

Figure 36 Capital Cost for Model 4

shows how the capital cost changes for Model 4. Figures for Models 2 and 3 can be found in Appendix B. Table 12 lists the capital costs for each model. The Capital Costs increase from lowest $26.3 million in Model 2 to $39.7million in Model 3 because there are more process steps which requires more equipment. Model 4 decreases the Capital Cost to $35.9 million.

Table 12 Capital Costs for each Model

Bioreactor Type	Media Type	Capital Cost (in millions)
Model 2		
50/50	FBS	$26.4
	CS	$26.3
Reusable	FBS	$33.2
	CS	$33.1
Disposable	FBS	$28.6
	CS	$28.6
Model 3		
50/50	FBS	$39.7
	CS	$39.7
Reusable	FBS	$46.6
	CS	$46.6
Disposable	FBS	$42.0
	CS	$42.0
Model 4		
50/50	FBS	$36.0
	CS	$35.9
Reusable	FBS	$43.4
	CS	$43.3
Disposable	FBS	$38.8
	CS	$38.7

Capital Cost per kg or Tablet

Another way to look at the capital cost is to look at the how the capital cost changes per kg of biomass produced or tablet. Equation 8 shows how this number is calculated. All three models produce 36 batches per year with a project lifetime of 15 years. With zero product failure, Model 2 produces 2.25

kg of biomass per batch, Model 3 produces 12,902.71 tablets per batch, and Model 4 produces 12,920.06 tablets per batch. Figure 37 shows how the Capital Cost changes with the product failure rate for Model 2. As the failure rate increases, the amount of capital cost charged to each kilogram of biomass produced increases. The lowest, for Model 2, is $24 thousand per kg of biomass produced.

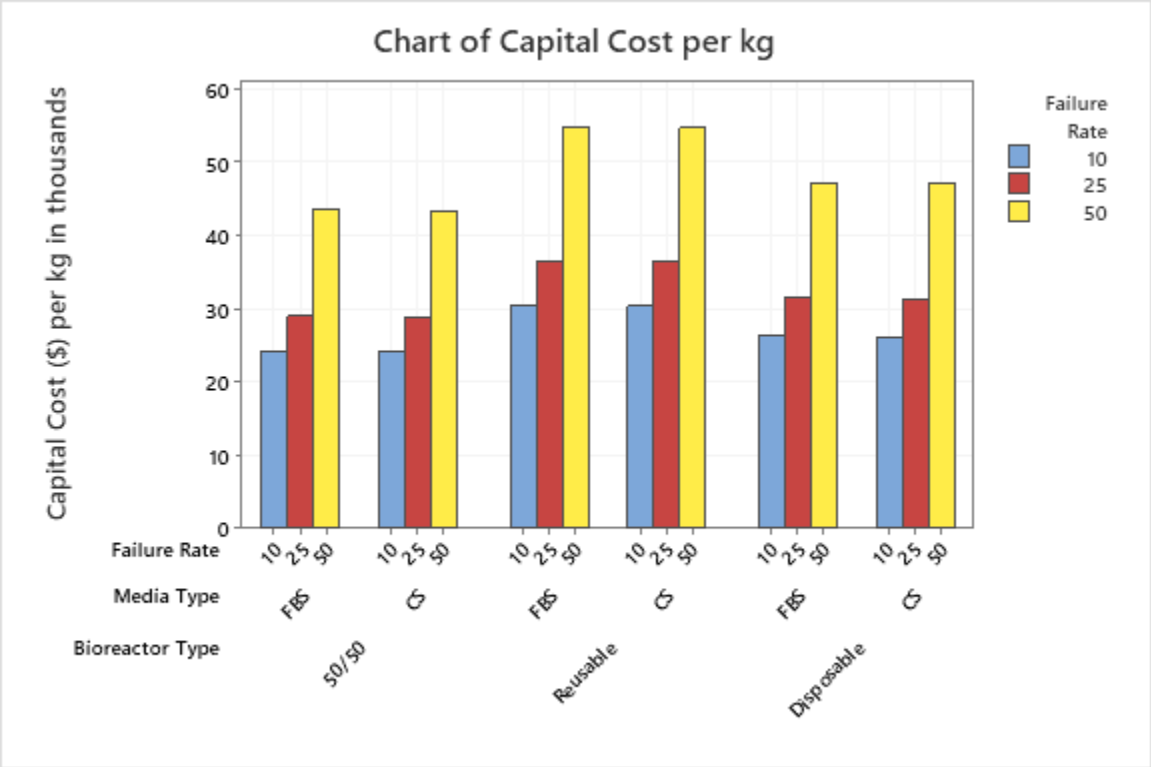

Figure 37 Capital Cost per kg Model 2

Figure 38 shows how the Capital Cost changes with the product failure rate for Model 3. The lowest for Model 3 is $6.34 per tablet.

Figure 39 shows how the capital cost changes with the product failure rate for Model 4. The lowest for Model 4 is $5.73 per tablet.

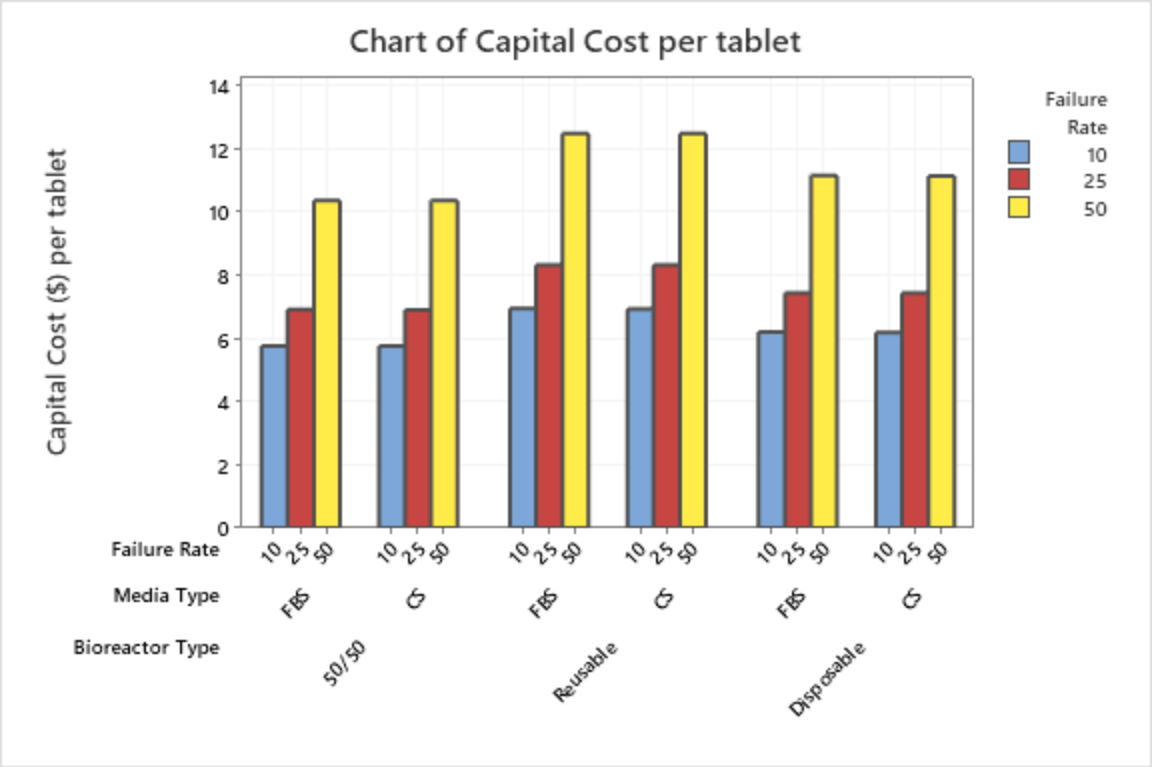

Figure 38 Capital Cost per Tablet Model 3

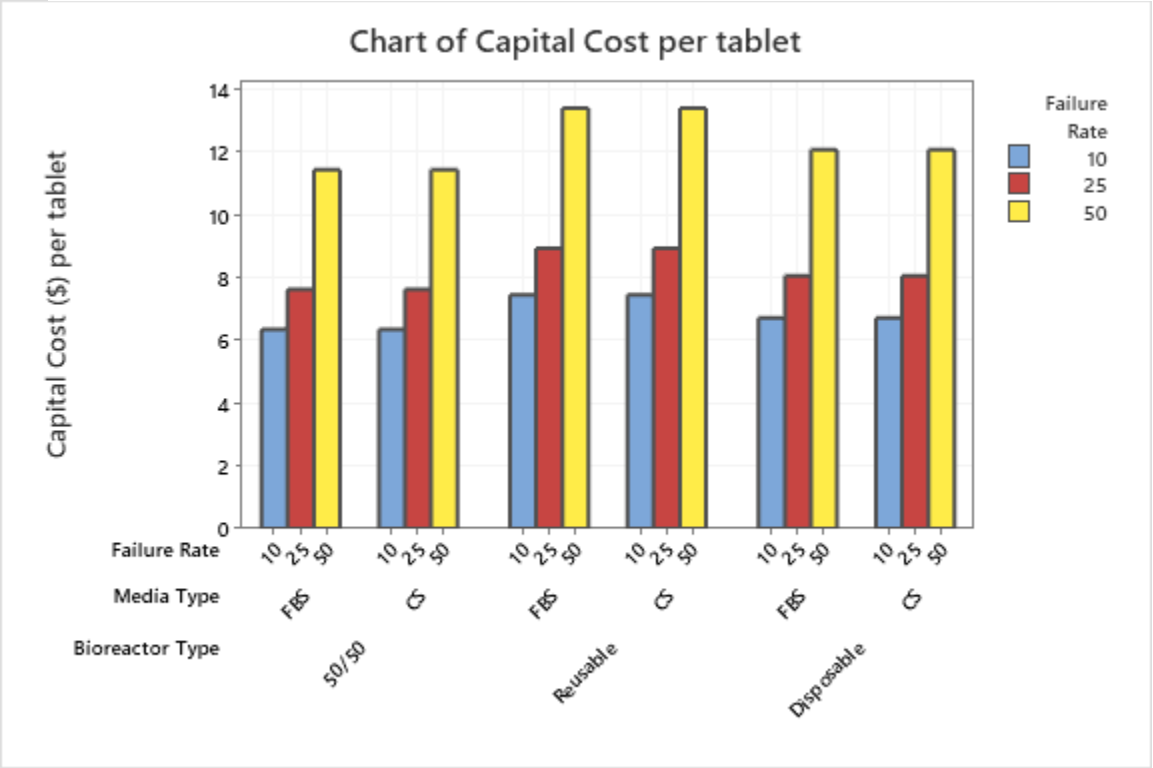

Figure 39 Capital Cost per Tablet Model 4

Operating Cost per year

The operating cost of a project includes costs that are related to the demand for a number of resources including raw materials, consumables, labor, heating/cooling utilities and power, as well as additional operational costs. Equation 6 shows how this number is calculated.

Model 2

Figure 40 displays how the Operating Cost changes with respect to the three variables: failure rate, media type and bioreactor type. Failure rate has a slight effect on the operating cost in this model. The costs increase slightly as the failure rate increases. The type of bioreactor used has a dramatic effect on the operational costs. Having all 4 bioreactors be reusable/sterilizable, increases costs by $7 million. This increase is seen because there is more cleaning involved which increases raw materials and utilities and power. Across the board, using CS media rather than FBS media reduces the operating costs because

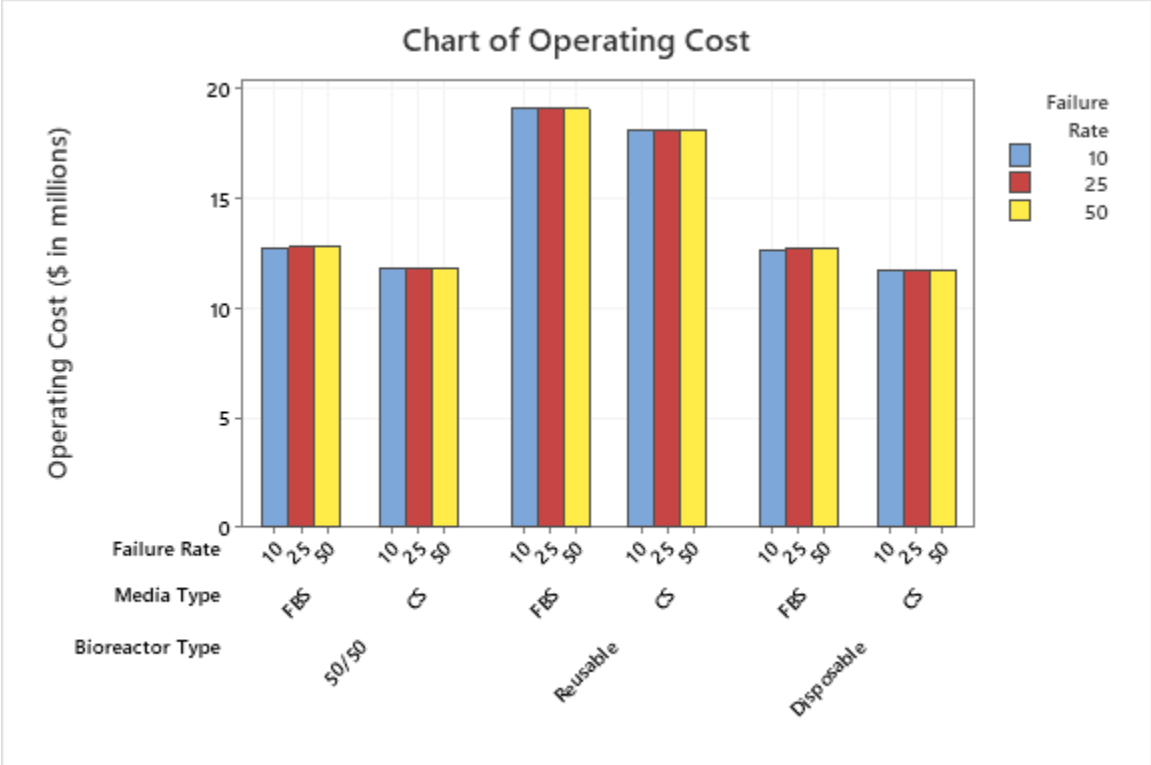

Figure 40 Operating Costs per year for Model 2

it is the less expensive option. However, there is only a slight difference in costs between completely

disposable bioreactors and the 50/50 split. Disposable bioreactors have an operating cost of $11.6 million and the 50/50 split has an operational cost of $11.7 million.

Table 13 Breakdown of Operating Costs for Model 2 The parameters used are: Disposable bioreactors, 50% failure rate, FBS media

Items	$	%
Raw Materials	1,112,000	8.77
Labor-Dependent	4,181,000	32.96
Facility Dependent	5,007,000	39.47
Laboratory/QC/QA	627,000	4.94
Consumables	1,748,000	13.78
Waste Treatment/Disposal	1,000	0.01
Utilities	6,000	0.05
Failed Product Disposal	4,000	0.03

Model 3

Figure 41 shows how the operating costs change with respect to the three variables (failure rate, media type and bioreactor type) for Model 3. In this model, the failure rate has a more dramatic effect on

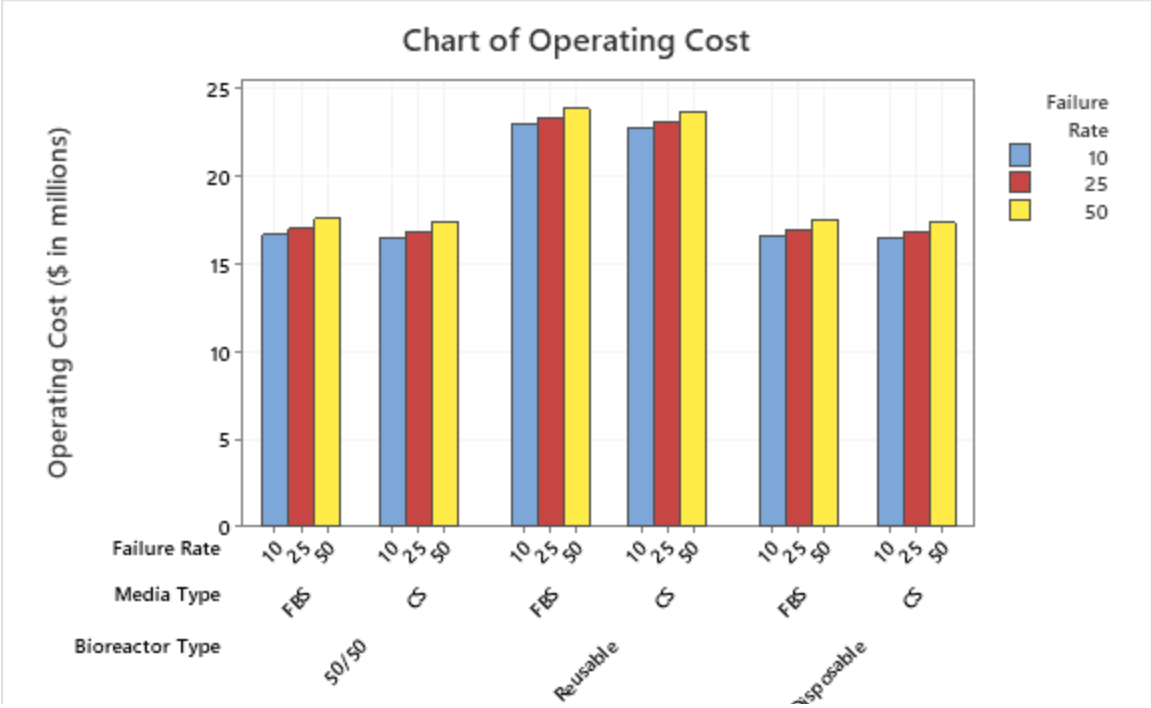

Figure 41 Operating Costs per year Model 3

the operating costs. Across the board, as the failure rate increases the operating costs increase. The difference between 10% and 50% is between 900 thousand and 1 million dollars. The type of bioreactor has the most effect on the operating cost, while the media type has a smaller effect. Reusable/sterilizable bioreactors, at their lowest, have an operating cost of $22.8 million with FBS and $22.6 million with CS. 50/50 bioreactors, at their lowest, have an operating cost $16.6 million with FBS and $16.4 million with CS. Disposable bioreactors, at their lowest, have an operating cost of $16.5 million with FBS and $16.3 million with CS.

Table 14 Breakdown of Operating Cost for Model 3: The parameters used are: 25% failure rate, FBS media, Reusable Bioreactors

Items	$	%
Raw Materials	3,476,000	14.98
Labor-Dependent	4,499,000	19.39
Facility Dependent	8,061,000	34.73
Laboratory/QC/QA	675,000	2.91
Consumables	175,000	0.75
Waste Treatment/Disposal	5,750,000	24.78
Utilities	8,000	0.03
Failed Product Disposal	564,000	2.43

Model 4

Figure 42 shows the operating costs for Model 4 and how it changes with respect to failure rate, media type, and bioreactor type. The failure rate has a similar effect on Model 4 as it did in Model 3. As the failure rate increases, the operating costs increase. The difference between 10% and 50% is between $900 thousand and $1 million across all scenarios. CS media lowers the operating costs, as opposed to FBS media, however the difference is not as dramatic as the type of bioreactor. Reusable/sterilizable bioreactors, at their lowest, have an operating cost of $21.4 million with FBS and $21.2 million with CS. 50/50 split, at their lowest, have an operating cost of $14.7 million with FBS and $14.4 million with CS. A breakdown of the operating cost at their lowest is displayed in Table 8. Disposable bioreactors, at their

lowest, have an operating cost of $14.7 million with FBS and $14.5 million with CS. As opposed to Model 3, Model 4 lowers the operating costs in all scenarios and the better option would be the 50/50 split.

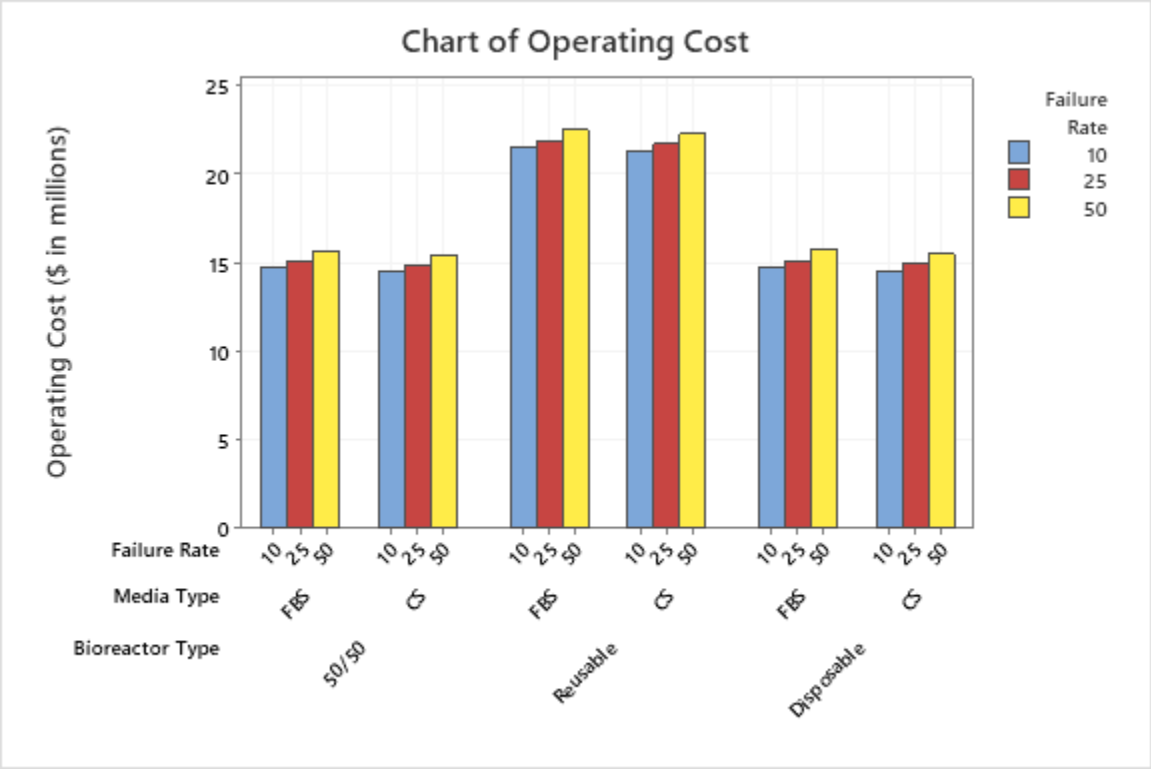

Figure 42 Operating Costs per year Model 4

Table 15 Breakdown of Lowest Operating Cost The parameters are: 50/50 bioreactors, 10% failure rate, CS media for Model 4

Items	$	%
Raw Materials	1,168,000	8.01
Labor-Dependent	4,301,000	29.50
Facility Dependent	6,356,000	43.60
Laboratory/QC/QA	645,000	4.42
Consumables	1,156,000	7.93
Waste Treatment/Disposal	713,000	4.89
Utilities	6,000	0.04
Failed Product Disposal	233,000	1.60

Unit Production Cost

The unit production cost tells us how expensive it will be to make the product. Equation 7 lists how this number is calculated.

Model 2

Model 2's goal was to create biomass. The unit production cost is based on kg of biomass. Figure 43 shows how the Unit production cost for Model 2 changes with respect to the three variables: bioreactor type, media type, and failure rate. The failure rate has the most dramatic and direct effect on the unit production cost. When the failure rate increases, so does the unit production cost. When you compare the media type, CS comes out lower than FBS every time. When you compare the bioreactor type, reusable/sterilizable bioreactors range from $267,9700 – 482,426 per kg with FBS and $254,321 - 456,858 per kg with CS. Disposable bioreactors range from $173,593- 312,557 per kg with FBS and $159,949 – 287,988 per kg with CS. 50/50 split bioreactors range from $174,372- $313,950 per kg with FBS and $160,072- $289,382 per kg. Disposable bioreactors have a slightly lower unit production cost than the 50/50 split bioreactors.

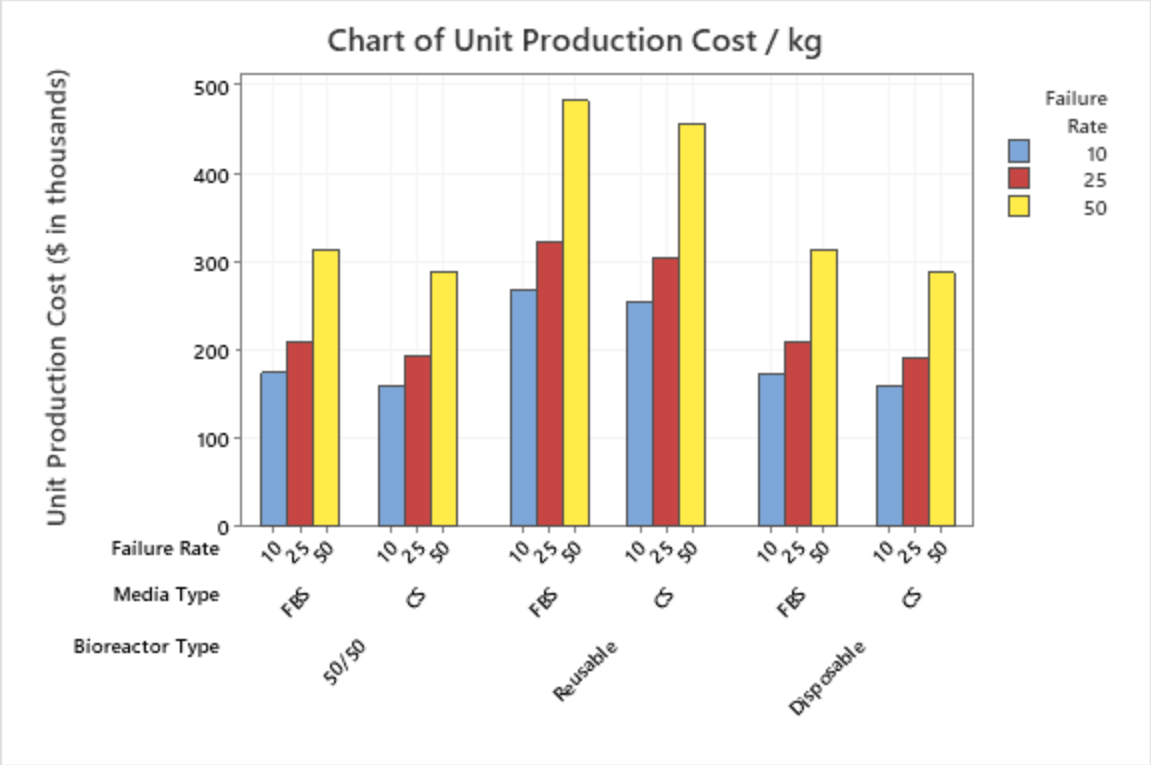

Figure 43 Unit Production Costs for Model 2

Model 3

Model 3 switches gears from Model 2 and turns the biomass into tablets. The unit production cost is now based on the cost per tablet. Figure 44 shows how the unit production cost changes for Model 3 with respect to media type, failure rate, and bioreactor type. Again, failure rate as the most dramatic and direct effect on the unit production cost. As the failure rate increases, so does the production cost. When you compare media type, CS comes out lower than FBS every time. When you compare the bioreactor type, reusable/sterilizable bioreactors range from $56.27-105.28 per tablet with FBS and $55.83-104.49 per tablet with CS. Disposable bioreactors range from $39.62-75.31 per tablet with FBS and $39.18-74.52 per tablet with CS. 50/50 split bioreactors range from $39.75-75.55 per tablet with FBS and $39.31-74.76

per tablet with CS. The lowest unit production cost occurs with disposable bioreactors and CS. However, the differences between 50/50 split and disposable bioreactors are quite small.

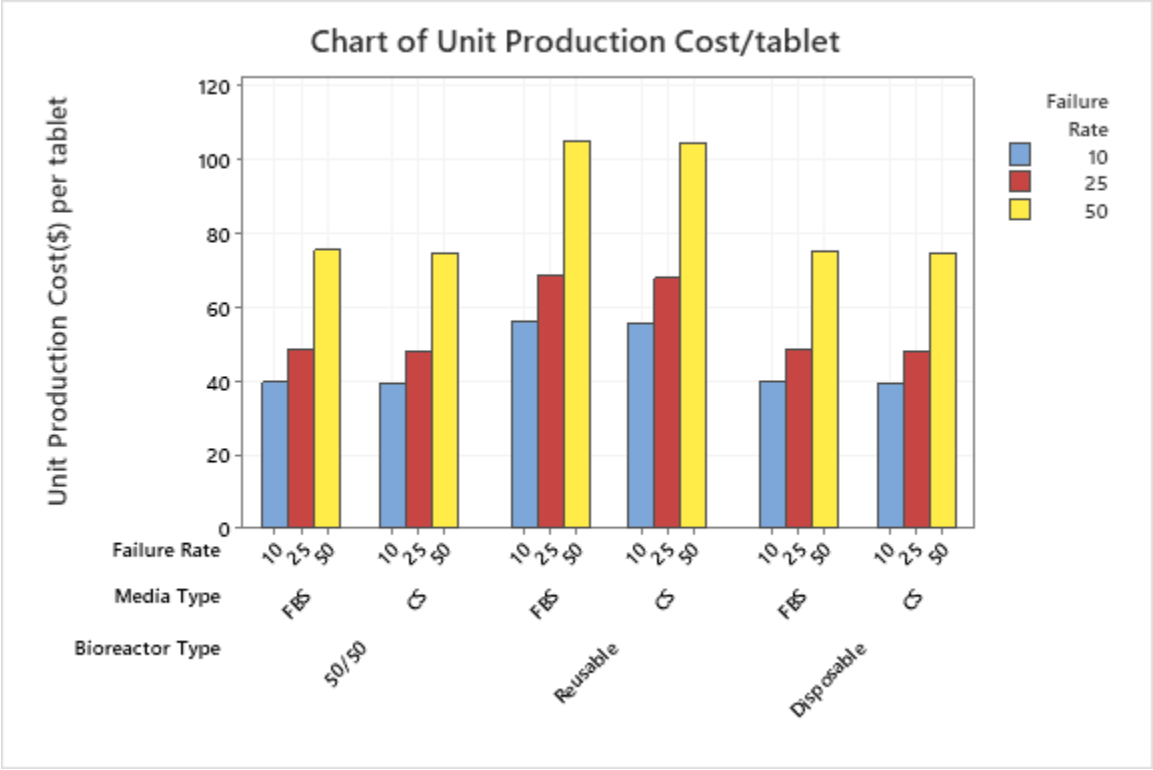

Figure 44 Unit Production Cost per tablet for Model 3

Model 4

Model 4 the unit production cost, like Model 3, is based on cost to make a tablet. Figure 45 shows how the unit production cost changes for Model 4 with respect to media type, failure rate, and bioreactor type. Failure rate has a direct relationship with the unit production cost; as the failure rate increases, so does the unit production cost. When you compare media type, CS comes out lower than FBS across failure rate and media type. When you compare bioreactor type, reusable bioreactors range from $51.31-96.36 per tablet with FBS and $50.78 – 95.40 per tablet with CS. Disposable bioreactors range from $35.18-67.32

per tablet with FBS and $34.65-66.36 per tablet with CS. 50/50 split bioreactors range from $35.10-67.19 per tablet with FBS and $34.58-66.25 per tablet with CS. This model has the lowest unit production cost with the 50/50 split scenario with CS, while disposable bioreactors are only pennies behind.

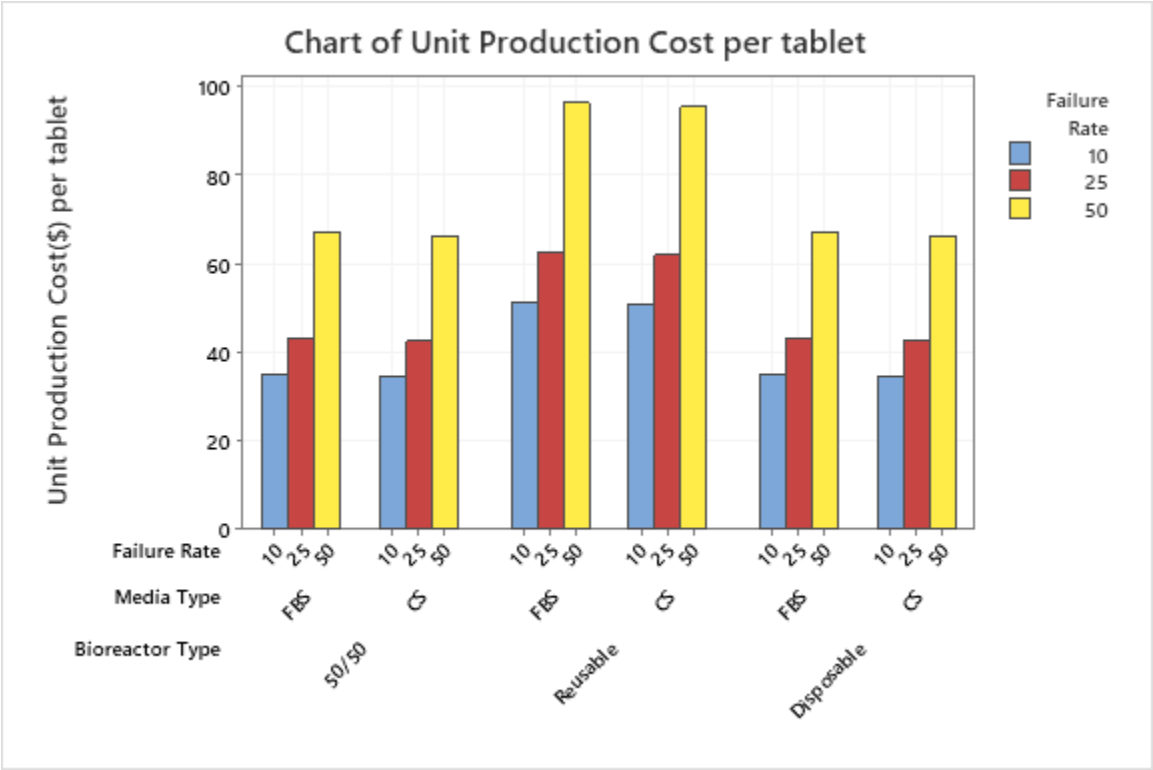

Figure 45 Unit Production cost per tablet for Model 4

Using a 10% failure rate, 50/50 bioreactors in Model 4 the unit cost is $34.58. Adding in $6 to help cover capital costs, the selling price for the tablet would be $40.58. This would give a return on investment of 12% and a payback time of 8 years.

CHAPTER 5

DISCUSSION

Experimental

The experiments started out by growing *Perkinsus marinus* at 20 mL scale in T flasks and then scaling up ten-fold into a 400 mL (220 mL working volume) bioreactor.

The growth of *Perkinsus* was tracked using the absorbance measured at 600 nm. Generally, the absorbance increased by 0.1 – 0.2 A per day. There was some fluctuation depending on where in the growth cycle the sample was taken. Towards the end of the growth, the change in absorbance would be smaller due to *Perkinsus* slowing down in growth. An unusual jump in absorbance was an indication that the cultures became contaminated, which would then be confirmed by looking at the cultures under the microscope. With improving technique, by experiment 3 the growth of *Perkinsus* in the bioreactors was completed contamination-free.

Aeration became an issue at the very beginning of growing *Perkinsus* in the bioreactors. In Experiment 1, it was discovered that the air supply line was not hooked up to the reactors. Solving this issue took a top priority and once the airline was hooked up, the air sparger was inserted directly into the medium. Immersing the sparger caused the medium to instantly start foaming. Foaming of the media was caused by the tiny air bubbles from the sparger and gave a greater risk of contamination, since the foam could propagate into the peripheral connections on the bioreactor. There was a discussion of whether or not to add anti-foaming agents to the media to try to reduce the foaming of the media. We decided this was not the way to go due to the unknown effects the foaming agents could have on the cells. Ultimately, it was decided that the air would flow over the headspace and allow the oxygen to diffuse into the

medium. In Experiment 7, it was discovered that the air flow rate directly impacted the growth rate. A clogged air filter lowered the rate of aeration in Vessel 3, while the other three vessels maintained 3.5-4 mL/s or 1.2-1.33 headspace vol/min.

Despite improved technique and operating conditions, one of the biggest issues with the bioreactors was contamination of bacteria in the cultures, even with antibiotics already included in the media. The antibiotics used in the medium are penicillin G (100 U/mL) and streptomycin sulfate (100 U/mL), which are standards in the cell culture industry. Once antibiotic-resistant bacteria enter the culture, it becomes a challenge to remove them from the nutrient rich medium. One possibility that could help with this issue would be to change or add different antibiotics to the medium.

The cause of contamination could be from multiple sources. However, the most probable cause of contamination is from the sample ports. The boundary of sterility is breached once a sample is removed or if the sample port was not sterilized for the required time, or perhaps the required temperature was not achieved. There is also the possibility that the septum was not installed correctly allowing contamination. Another possibility is that there was an integrity breach of the gas filters. Due to the nature of glass, there could also be tiny cracks in the vessels themselves which could provide an environment which would allow bacteria to grow.

After running multiple experiments, the redox curve started to show a characteristic shape for a clean, uncontaminated fermentation, which can be seen in Figures 9, 16, 22, 24. This redox response became useful as a real time indication of whether the fermentation vessels were contaminated or uncontaminated before looking at the cells under the microscope. Superimposing the growth curve over the redox curve, one can see that the injection of the inoculum leads to the redox becoming negative at the beginning of the experiment. There are a number of possibilities for the negative redox. The medium before inoculation may have a low volume of dissolved oxygen which would contribute to the negative

redox. The inoculum itself could be oxygen starved. The inoculum is grown in a T-flask which allows oxygen to enter the medium through a filter, but this is not controlled. In the bioreactors, as the cells grow and adjust to their new environment, the redox starts to turn positive. The air flow over the reactor surface enables oxygen the chance to dissolve into the medium to be used by the cells. The positive bump in the redox curve correlates to the exponential growth phase. However, this is counterintuitive to what we believe should be happening. As the cell grow, they should be using more oxygen which would correlate to the redox dropping. As cell growth slows down, the redox curve drops again, but stays positive reaching an equilibrium type state. The cells have stopped growing due to running out of nutrients or space in the reactor.

Once the redox started dipping down prominently or staying negative, which can be seen in Figures 5, 7, 12, 24, it became an indication to check the cultures for contamination. Looking at the cultures under the microscope would confirm that something else was growing in the fermentation vessel. Bacteria more often have a much quicker doubling time and use up the nutrients in the medium faster than *Perkinsus* uses them. They will also use the dissolved oxygen in the medium faster, which would contribute to the redox curve becoming negative and staying negative.

Given our observations of apparent bacterial contamination in low redox fermentations, this biotic explanation of the low redox values seems reasonable. It may be possible that other chemical processes are at work creating the low redox fermentations, but to date we have not formulated an alternate hypothesis that could explain the observed behavior.

Using the HPLC to track the glucose concentration during fermentation led to some interesting results. The glucose concentration decreased more slowly than what was expected. The concentration only approached zero during contaminated runs (see Figure 25). This leads to the assumption that glucose

is not the limiting nutrient in the growth medium. But if glucose is not the limiting nutrient, what is? Is the limiting nutrient located in the serum? And if it is located in the serum, which molecule is it?

Chicken Serum Optimization

One of the goals of this investigation was to look at all the medium ingredients to determine if there were any that could be replaced for a less expensive alternative. The most expensive ingredient per liter was FBS, which led to the question: what could replace FBS while still supplying the necessary nutrients that FBS contains? Looking through the literature, we found several possibilities that had the potential of working. Two possibilities discussed were coconut water (Shilpa, 2014) and egg yolks (Sasse, Lengwinat, Henklein, Hlinak, & Schade, 2000). There wasn't much more investigation into coconut water as a replacement beyond the initial findings. It could still be a viable option to reduce costs.

One of the issues discovered with egg yolks was that it would often cause precipitation in the medium. Instead of egg yolks in the medium, Cornet used chicken serum as a replacement for fetal bovine serum - in the growth of the bivalve primary cells, *Mytilus galloprovincialis* (Cornet, 2006). *Mytilus galloprovincialis* is a Mediterranean mussel. While there are many differences between an oysters and mussels, they are similar enough that this discovery led to more investigation into chicken serum as a possible replacement. I then uncovered a paper that grew *Crassostrea virginica*, the oyster host of *Perkinsus*, using chicken serum in the growth medium (Richards et al., 2018). This gave more evidence to the idea that chicken serum could be a replacement for fetal bovine serum for *Perkinsus* because it has been used to successfully grow its host cells.

When the original media formulation was developed, 5% v/v FBS was determined to be the optimal concentration for cell growth. Higher concentrations of FBS were inhibitory (Gauthier & Vasta, 1995). However, we were unsure if this dosage would be at the optimal concentration for CS. From the data displayed in Figure 19, we can see that 20% CS increases the growth more than 5% v/v CS does, while

lower levels of CS still showed some growth, but a smaller growth rate. This leads to the conclusion that 5% v/v CS is the minimum percentage needed to achieve abundant growth, while a higher percentage can still be used. The higher percentages will increase the cost of the medium and diminish the advantage of using CS as a substitute for FBS. It comes down to the question, which is more important: growth or cost?

Why does increasing the percentage of CS increase growth, while it's known that if the percentage of FBS is increased, it inhibits growth (Gauthier & Vasta, 1995)? Three of the major glycoproteins of FBS are fetuin, albumin, and transferrin. Fetuin enhances growth, albumin has a slight positive effect on growth, and transferrin inhibits growth (Gauthier et al., 1995). What are the differences between CS and FBS? According to a sample certificate of analysis (COA) from Sigma Aldrich (which can be found in Appendix C), the concentration of albumin for both FBS and CS is 1.6 g/dL. The certificates do not list fetuin or transferrin for either serum. Are there conformational changes in the proteins just enough that they align more closely with what *Perkinsus* needs? In terms of albumin, the structure of bovine albumin is listed on the RCSB Protein Data Bank, but the structure for chicken albumin is not listed. Fetuin is not listed for bovines or chickens. In the RCSB Protein Data Bank, transferrin structures can be found for both bovines and chickens. A structure comparison shows that diferric chicken serum transferrin (1N04) matches only a lactoferrin (1BLF) for bovines. While lactoferrins and transferrins have similar structures and functions, lactoferrins are found in milk, not serum. Currently the question of the differences between FBS and CS proteins remains as a hypothesis.

One of the differences found in the COA was the concentration of cholesterol. FBS has a concentration of 31 mg/dL while CS has a four-fold higher concentration of 130 mg/dL. Another notable difference between the two serums is the concentrations of globulins. Present in CS is alpha 1 globulin (0.16 g/dL), alpha 2 globulin (0.33 g/dL), beta globulin (0.58 g/dL), and gamma globulin (0.29 g/dL). In comparison in FBS is alpha 2 globulin (1.3 g/dL), beta globulin (0.6g/dL) and gamma globulin (0.1 g/dL). FBS does not contain alpha 1 globulin.

An important issue with serum, in general, is the batch-to-batch consistency. The above numbers are from a sample COA and may not reflect exactly with what was used during experiments. However, they do give a general idea of what some of the molecular differences are between FBS and CS.

Genetic Variants

Experiment 7 compared the growth of PRA-240 wild-type to PRA-393 GFP mutant cells in the bioreactor with FBS medium. This experiment established that the genetic variant could grow in the bioreactor and the growth curve can be seen in Figure 21.

Experiment 8 compared the growth of PRA-393 GFP mutant cells in FBS medium and CS medium. This experiment established that the genetic variants can be grown in CS medium and the growth curve can be seen in Figure 23.

Production Process Model

The model was built to expand on the knowledge gained from the experimental data and to test economic questions, like what is the optimal amount of CS to use? Lab scale production is not cost effective, however being able to scale up the process for vaccine production will make the production more cost effective.

The failure rate in the model refers to the fraction of fermentation batches that are initiated but fail to grow or fail to meet required product standards, and was modeled as a percentage of the end product. The most likely cause of failure is likely to be contamination coming from bacteria during the fermentation steps. Disposable bioreactors reduce the chance of contamination happening. With the use of disposable bioreactors, there would be fewer wasted runs. In terms of money, the most cost-effective version of Model 4 was the 50/50 split with the first two bioreactors reusable and the second two bioreactors disposable. Other scenarios with the disposable bioreactors in the different sequences were

also looked at. However, the least expensive option across the board was with the first two bioreactors reusable and the second two disposable. One possible issue with this scenario is that SuperPro prices all disposable bioreactors at $221,000 with a volume of 700 L. The bag that gets replaced after each run costs $6,220. According to our results, the high costs (per unit production) associated with high failure rates suggests that that going completely disposable might be the better option in terms final of cost.

One issue with bioreactor bags is that they have to be handled with care. Inspecting for failure rates of a one-use bag can be a challenge. Stainless steel bioreactors can be inspected for failure with a simple pressure test. In contrast, as soon as any pressure is introduced to a bag, it will inflate like a balloon. So likely the fermentation facility will depend in the quality control of the provider of the disposable reactor.

Model 4 was used to determine what the optimal amount of chicken serum would be. When all the economics were configured, Equation 1 was used. Serum was not separated out in the equation. In order to address this, the stoichiometric equation was changed to Equation 9.

$$0.83M\ CS\ Media + 0.56\ Serum + 1M\ O_2 + 1M\ N_2 \rightarrow$$
$$0.02M\ CH_{1.8}O_{0.5}N_{0.2} + 0.62M\ CO_2 + 0.31M\ C_3H_6O_3 + 1.64M\ H_20 \quad (9)$$

The media formulation was then set to 5% in the 50/50 bioreactor scenario with a 10% failure rate. The difference between a concentration of 20% and 5% CS was 15 tablets per batch. All the economics for 20% CS increased from where 5% CS is. According to the model, 5% CS is the optimal concentration.

There are some issues with the model that should be addressed. One issue is that when assessing the economics of the model, costs of materials I used were lab scale numbers. For example, the cost of FBS media per liter is $123.30 and the cost of CS media per liter is $48.00. Most likely these numbers would decrease upon scaling up. Thus, the operating costs and raw material costs may actually go down from what's been reported with the model.

Another issue with the model is what further processing will be required after *Perkinsus* has finished growing. In this model we assumed that the cells would be have to be killed before being turned into a tablet. However, it may be that the cells may need to be alive for vaccine delivery, which would greatly complicate storage, transport and raise the cost considerably for effective distribution and delivery of the vaccine. Alternatively, the therapeutic proteins might need to be extracted from the cells before being turned into a tablet. Ideally, the vaccine could be administered orally, which is why we chose to use tablets as a final product, but it's also possible that the vaccine may need to be delivered as a shot. Since the development of therapeutic proteins using Perkinsus is still far from practical implementation, details on these aspects of final drug development have been presented as one of several possible final configurations.

Another improvement that can be made to the model is that the exact chemical formula for *Perkinsus* was not used for determining cell growth, which was modeled as a stoichiometric reaction. I used the built-in biomass composition as a substitute for not knowing the exact chemical composition. I also used a very simple reaction (Equation 1) for simulating the fermentation. This reaction can be further developed to represent a more detailed and accurate approach to predicting how much biomass can be produced.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

Conclusions

It is possible to grow *Perkinsus marinus* in bioreactors. Both wild type and modified cultures were successfully grown.

Sufficient aeration can be achieved by sweeping the void space with a fresh supply of air and maintaining moderate stirring.

Redox measurements are useful for real-time detection of bacterial contamination.

Chicken serum is an effective and less expensive replacement for FBS.

Failure rates in fermentation production appear to be the most significant operational risk to cost of production.

Based on economic estimates embedded in SuperPro, cost of growing Perkinsus cell mass could cost from $143 to $287 per g.

Future Work

Determine what proteins are present in CS and which ones actually help *Perkinsus.*

Determine if CS has to undergo more vigorous testing to be used in pharmaceuticals.

HPLC- determine if there are any matrix effects; deproteinize the samples and then run through again

Accurately determine what the limiting nutrient is in the media.

Upscale even further (1L, 4L, etc.)—anticipated issues: Aeration scale up? Contamination? Move to disposable reactors?

Develop modality of vaccine delivery for proteins generated from *Perkinsus marinus.*

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Appendix A. Experimental Data

Dry Weight

Due to the small sample sizes, there is a lack of precision in the data for dry weight and percent

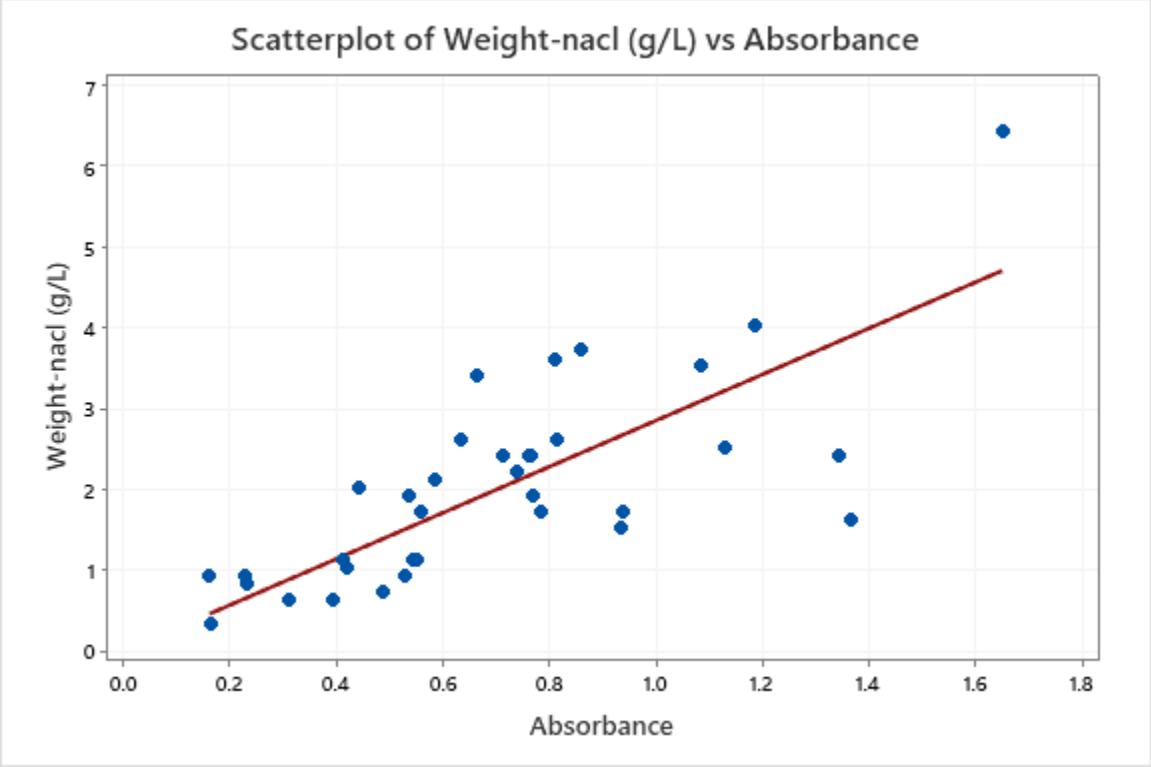

Figure 46 Scatter Plot of Dry weight vs Absorbance. Regression line equation: y = 2.956x with an r^2 = 88.3%

ash in the biomass. At this present time, no conclusions can be drawn from the data.

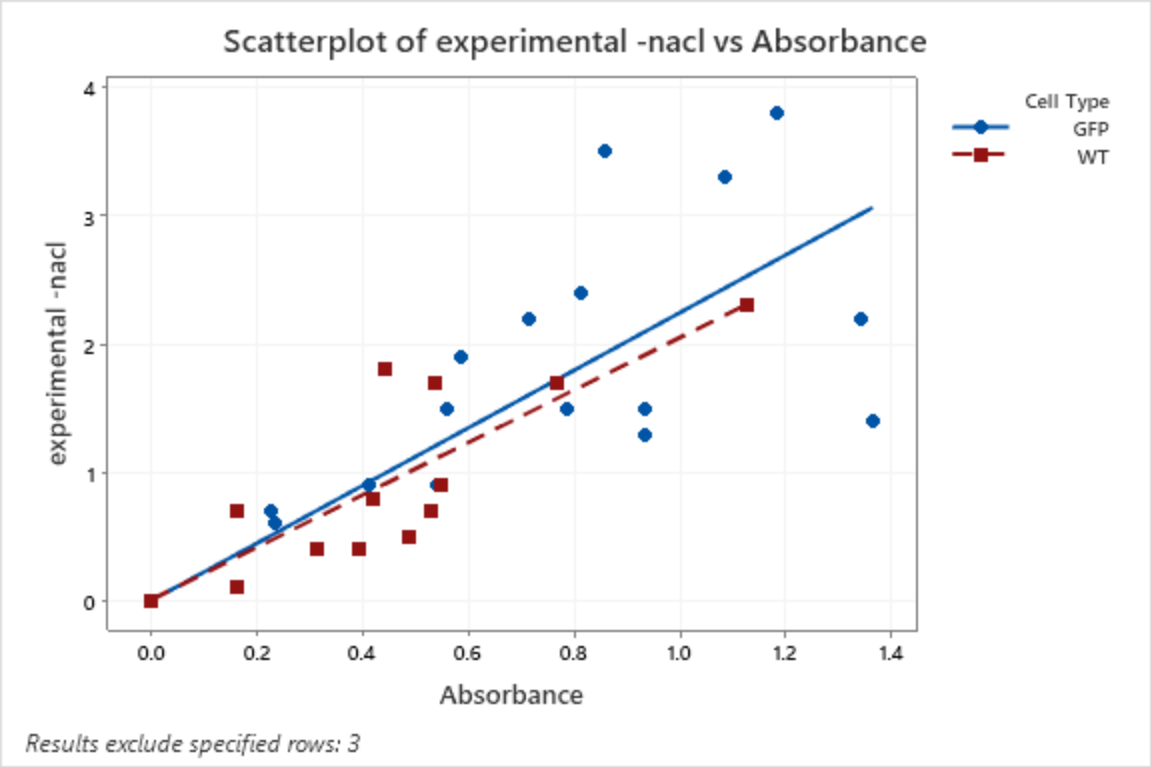

Figure 47 Scatter Plot of Dry Weight Broken Down by Cell Type. GFP regression line: y = 2.246x with r^2 = 85.2% WT regression line: y = 2.046x with r^2= 88.3%

Ash in Biomass

Table 16 Percentage of Ash in Biomass

Cell Type	% Ash
WT	41.7910
WT	44.8276
WT	0.0000
GFP	11.7647
GFP	3.1250
GFP	0.0000

Standard deviation all: 20.9

Standard deviation WT: 25.1

Standard deviation GFP: 6.1

Appendix B. Extra Model Graphs

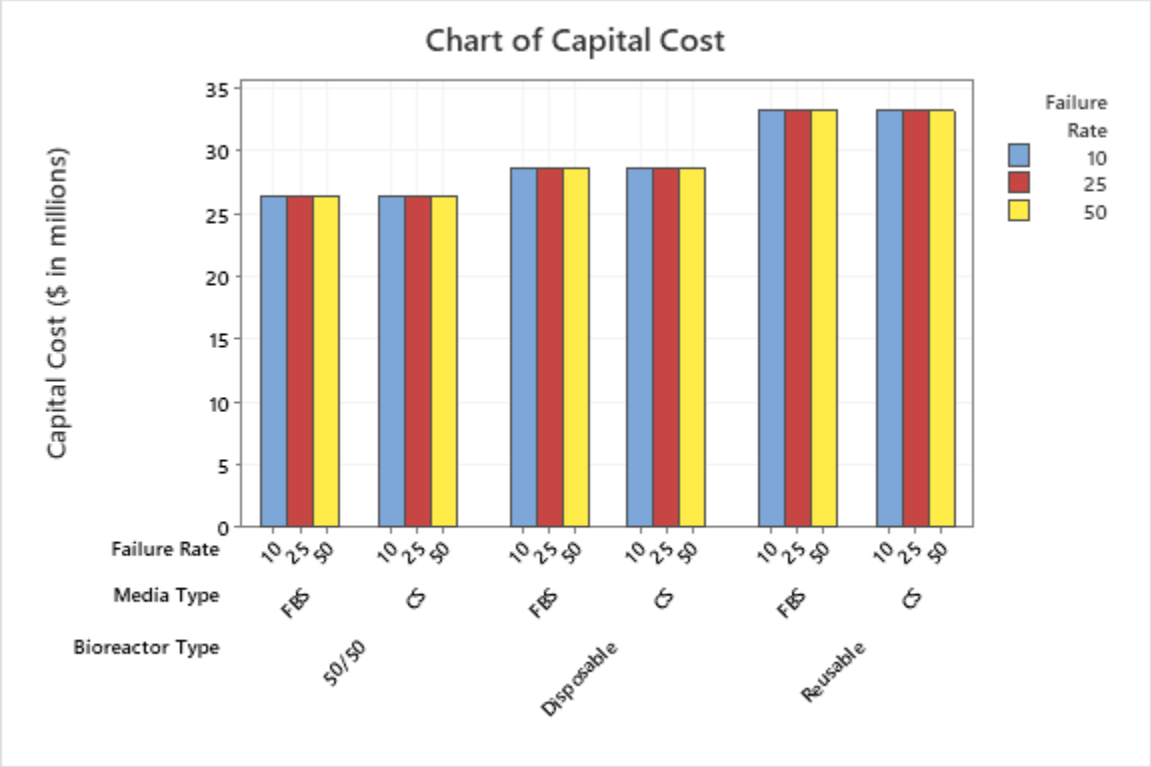

Figure 48 Capital Cost Model 2

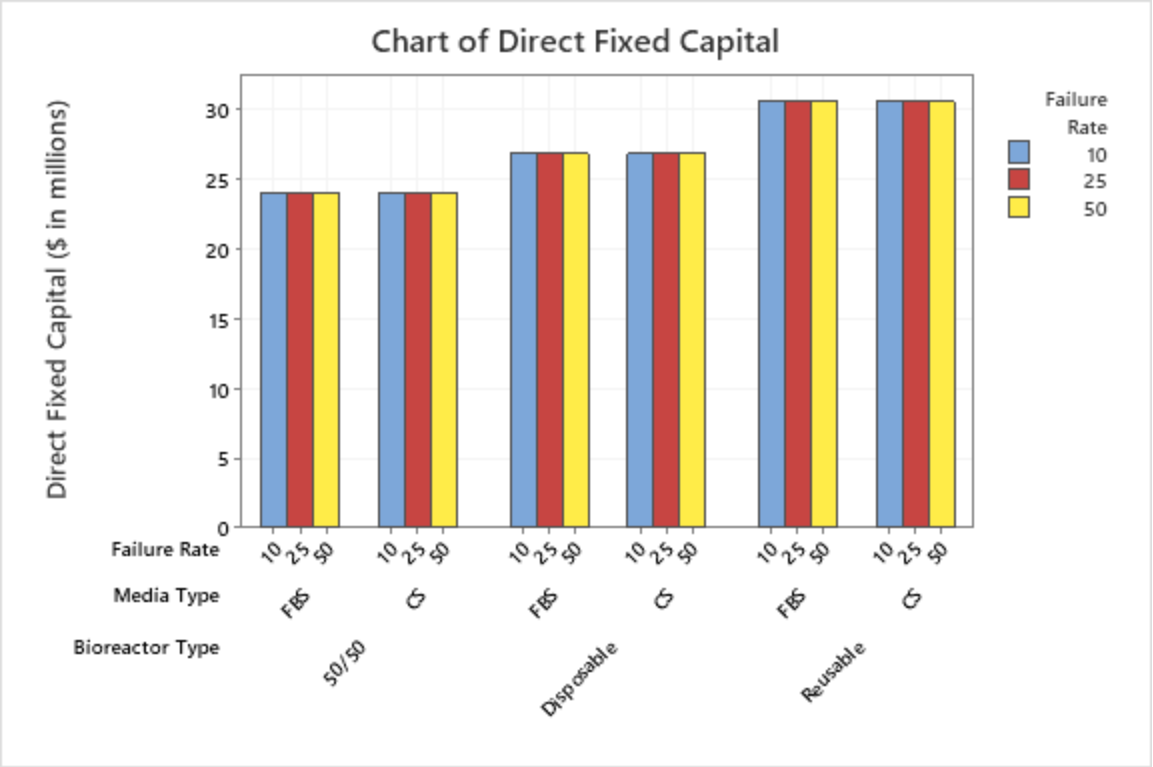

Figure 49 DFC Model 2

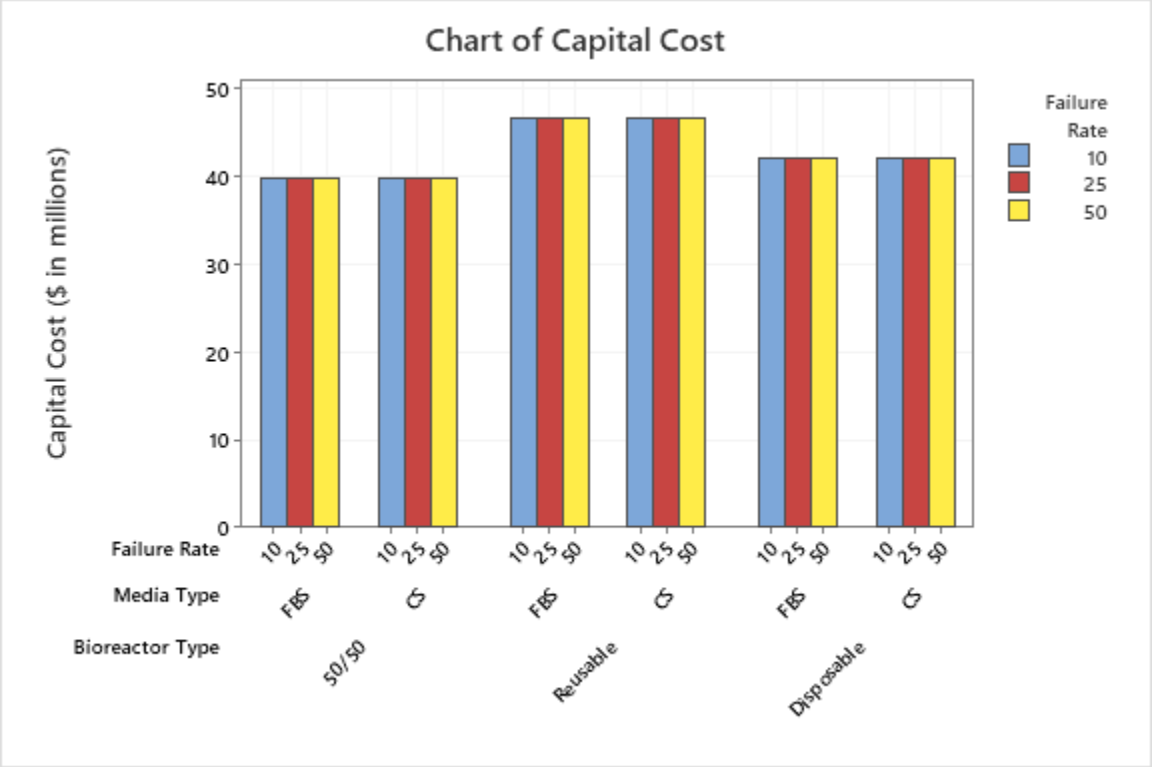

Figure 50 Capital Cost Model 3

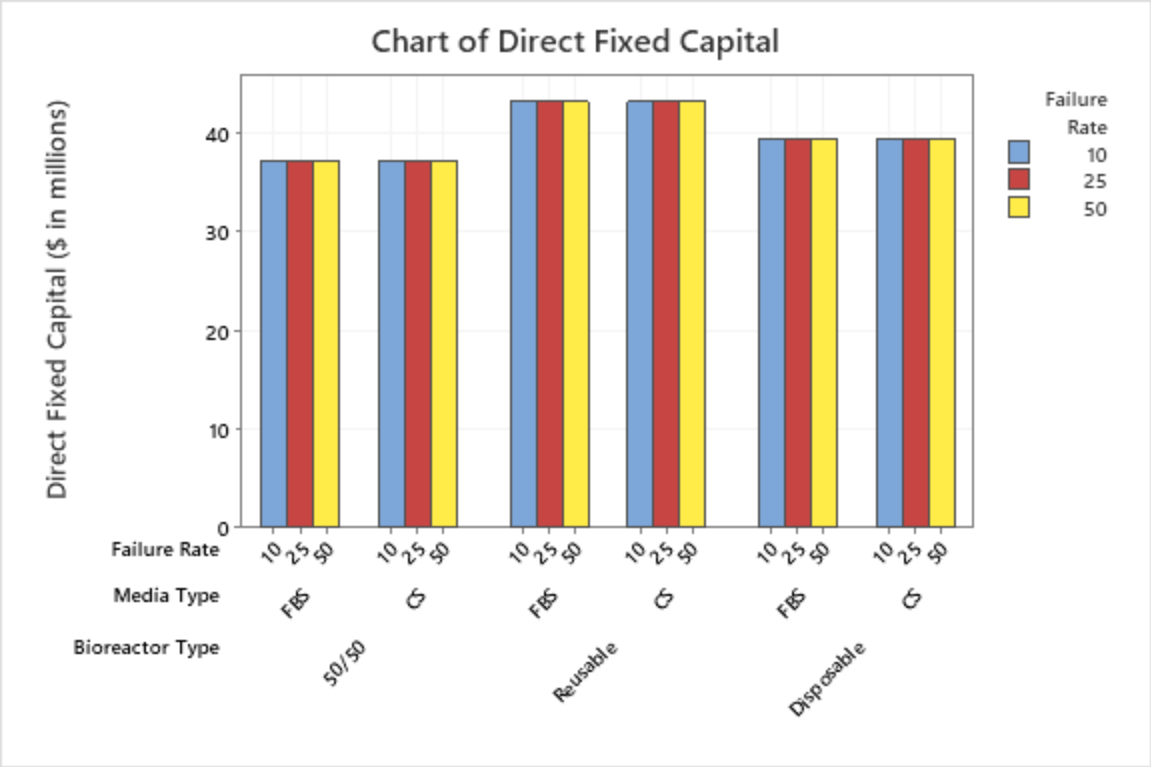

Figure 51 DFC Model 3

Appendix C. Certificate of Analyses

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Chicken Serum, USA origin, sterile-filtered, suitable for cell culture
Product Number	C5405
Product Brand	SIGMA

TEST	SPECIFICATION	LOT 12M494 RESULTS
Cell Lines	Record cell lines	Sp2/0 cell line
Sterility	No microbial growth detected	No microbial growth detected
Mycoplasma	None detected (Broth Culture)	None detected
Osmolality	285 - 340 mOsm/kg H2O	295 mOsm/kg H2O
Location of Reserve Sample	Present	3 X 100mL in QC Retain Bin #4096
Total Protein	2.0 - 4.3 g%	3.0 g%
Chemical Profile	Cholesterol - Report result (mg/dL)	130 mg/dL
	Iron - Report result (mcg/dL)	119 mcg/dL
	Glucose - Report result (mg/dL)	196 mg/dL
	Sodium - Report result (mEq/L)	137 mEq/L
	Triglyceride - Report result (mg/dL)	46 mg/dL
Electrophoretic Profile	Albumin (g/dL) - Record	1.6 g/dL
	Alpha 1 Globulin (g/dL) - Record	0.16 g/dL
	Alpha 2 Globulin (g/dL) - Record	0.33 g/dL
	Beta Globulin (g/dL) - Record	0.58 g/dL
	Gamma Globulin (g/dL) - Record	0.29 g/dL
Mycoplasma	None detected (DNA Fluorochrome Stain)	None detected
pH	7.0 - 8.2	7.8
Ouchterlony Species Identification	Satisfactory	Satisfactory
Endotoxin	<= 50 EU/mL	1 EU/mL
Hemoglobin Test	<= 60 mg%	32 mg%
Serum Performance Test	Comparable to control lot	Comparable to control lot

Country of Origin	Record country of origin	United States
Appearance	Clear straw to amber colored liquid	Clear amber colored liquid

COMMENTS

C of A comments	Country of final product processing: United States
Origin	The materials used in this product were collected in the United States.
Storage	Store at -20 C
	For R&D use only. Not for drug, household, or other uses.
Approval Date and Time	10/25/2013 11:06:42
Manufact Date	12/17/2012
Expiration Date	12/31/2016

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Fetal Bovine Serum, USA origin, sterile-filtered, suitable for cell culture, suitable for hybridoma
Product Number	F2442
Product Brand	SIGMA

TEST	SPECIFICATION	LOT 18N103 RESULTS
Endotoxin	<= 10 EU/mL	0.2 EU/mL
Mycoplasma	None detected (Broth Culture)	None detected
Osmolality	260 - 340 mOsm/kg H2O	304 mOsm/kg H2O
AVA (9CFR113.53)	Bovine Adenovirus (type 3 and 5) - ,None detected	None detected
	Bovine Parvovirus - None detected	None detected
	Blue Tongue Virus - None detected	None detected
	BVDV by FA - Tested	Tested
	Cytopathic Effect - None detected	None detected
	Hemadsorption - None detected	None detected
	Infectious Bovine Rhinotracheitis - None,detected	None detected
	Parainfluenza 3 - None detected	None detected
	Rabies Virus - None detected	None detected
	Reovirus - None detected	None detected
	Bovine Respiratory Syncytial Virus - ,None detected	None detected
	Vesicular Stomatitis Virus -,None detected	None detected
Bacteriophage Testing	Record (PFU/mL)	54 PFU/mL
Total Protein	3.0 - 4.5 g%	3.5 g%
Chemical Analysis	Cholesterol - Report result	31 mg/dL
Hormone Level	Estradiol - Report result	20.1 pg/mL
Chemical Analysis	Iron - Report result	150 mcg/dL

	Glucose - Report result	120 mg/dL
Hormone Level	Insulin - Report result	0.9 mcIU/mL
Chemical Analysis	Sodium - Report result	133 mEq/L
Hormone Level	Progesterone - Report result	<0.1 ng/mL
Sterility per current USP	Negative	Negative
Hormone Level	Testosterone - Report result	11 ng/dL
Tetracycline	Report Result	None detected
Chemical Analysis	Triglyceride - Report result	72 mg/dL
Serum Antibody Titer - BRSV	Report result	< 1:2
Serum Antibody Titer - BVDV	Report result	1:2
Serum Antibody Titer - IBR	Report result	< 1:2
Serum Antibody Titer - PI3	Report result	< 1:2
Cloning Assay	Pass	Pass
Mycoplasma	None detected (DNA Fluorochrome Stain)	None detected
Insect Cell Culture Test	Min CD 1.2X10E6, Max DT 33.5 hr	CD 3.1X10E6, DT 24.3 hr
Cell Lines	Record cell lines used	Sf9 cell line
pH	6.7 - 8.0	7.2
Bovine IgG	<= 1 mg/mL	0.09 mg/mL
Serum Performance Test	Pass	Pass
Cell Lines	Record Cell Lines Used FIO	BHK-21 cell line
Hemoglobin	<= 20 mg%	17 mg%
Electrophoric Profile	Albumin - Report Result	1.6 g/dL
	Alpha 1 Globulin - Report result	0.0 g/dL
	Alpha 2 Globulin - Report result	1.3 g/dL
	Beta Globulin - Report result	0.6 g/dL
	Gamma Globulin - Report result	0.1 g/dL
Country of Origin	Record	United States
Appearance	Clear straw to amber colored liquid	Clear amber colored liquid

COMMENTS

C of A comments	Country of final product processing: United States
Intended Use	For R&D use only. Not for drug, household, or other uses.
Origin	The material used in this product was collected in the United States. Animals used for collection of serum were USDA inspected and acceptable for slaughter.
Storage	Store at -20 C
Approval Date and Time	1/25/2019 14:37:28
Manufact Date	12/11/2018
Expiration Date	12/31/2023

Appendix D. DAS-GIP System How To

Step 1: Turn everything on

a) There are 3 switches behind the monitors for DAS-GIP

b) The computer and computer monitor

1. The password to turn on the computer is 06maine08

2. The password for DAS-GIP is dasgip.

Step 2: Once DAS-GIP is open, Click on File and then New Work Flow

Step 3: The New Work Flow Box should pop open with two tabs

Tab 1: Based on Template

a) Select the type of control system you want for your experiment: pH control acid/base, pH control acid, pH control base or pH-measurement; all have temperature control

b) Select the number of reactors you plan to use for the experiment (1-4)

1. If you plan to use less than 4 reactors unplug the pH sensors pH4RD4 that won't be in use. Otherwise, you will get an error message when you calibrate the pH sensors

Tab 2: Based on Previous Work flow

a) Click on your previous experiment

b) if you want an exact copy, check the box, if not then select the number of reactors

Step 4: Open the work Flow

a) Decide which calibration procedures need to be done: pH, pump, CIP, temperature

1. pH calibration is always check marked

2. pump and CIP only need to be done if the pumps and feed lines are being used in the work flow

3. Temperature calibration should be done if you think something is wrong with the temp probes

b) Click Finish

c) A box will ask if you want to save changes, click yes

Step 5: Set up the requirements of the experiment

a) Set the time of the experiment. It's preset for 24 hours but can be lengthened or shortened depending on what your needs are

b) Set the Temperature for each reactor individually

1. Double Click on the box displaying the temperature (it's preset to 37°C)
2. In the Tab labeled Profile Set up 1, T click on Data then enter your desired temperature
3. Repeat for all the reactors that need a temperature change

c) Change the Rate of stirring individually

1. Double click on the box displaying the stirring (it's preset to 400rpm)
2. In the Tab labeled Profile Set up 1, N click on Data and enter the desired stir rate
3. Repeat for all reactors

d) If you are adding things via the feed tubes, use the Substrate A and B boxes to control what is being added to the reactors; how it's added, what the triggers are, etc.

e) Once everything is organized click Finish

f) A box will pop up asking if you want to save, click OK

Step 6: pH Calibration

a) A box will pop up asking if you want to start the pH calibration. Click Yes

b) The preset buffer solutions are 7 and 4, but can be changed if needed

1. Immerse ALL the pH sensors into the 1st buffer [generally pH 7]
2. Wait until all the readings are stable
3. Click Start; yellow rings indicate calibration in progress
4. Wait until all rings are blue and message reads “Ready 1”
5. Rinse off all pH sensors with DI water and pat dry
6. Immerse ALL the pH sensors into the 2nd buffer [generally pH 4]
7. Wait until all the readings are stable
8. Click Start
9. Wait until all rings change from yellow to green and message reads “Ready 2”
10. Recheck calibration by putting the sensors back into first buffer

c) Click Finish to Leave

d) A box will pop up if asking if you are finished, click yes

Step 7: Autoclave

a) A box will pop up asking if you want to continue with procedure control. Click NO.

b) Prepare all vessels for the autoclave

1. Cover all sensors with caps
2. Cover all filters with aluminum foil
3. Place the magnetic stir bars in the vessel far away from the housing [the magnet is very strong and will break the vessel]
4. Make sure all entrances are sealed
5. Autoclave the vessels with/without medium [depends on the medium you are using]. Generally, the vessels are autoclaved at 121°C for 1 hour, but that depends on your experiment

6. Let the vessels cool down completely before use [I let them cool down over night]

Step 8: Prepare the system

a) Once vessels are cooled down; add the medium to the vessel if you have not done so already
b) Inoculate the vessels under a clean hood
c) Make sure everything is turned on
d) Hook up Each Vessel one by one
    1. Insert the grounding line [green and yellow] into the top of the vessel
    2. Hook up the Redox [solid color] and pH [grey stripe above the solid color] probes; the colors should match each other
    3. Hook up the water to the condenser [red line goes on top; blue line goes on bottom]
    4. Insert the Air line into the air filter
    5. Turn on the air; the valve is located in bay B
    6. At the back of the housing, open the water lines for each vessel
    7. Repeat for all Vessels
    8. Turn on the Water

Step 9: Start the Work Flow

a) Open DAS-GIP control
b) Under Waiting; you'll find your work flow
c) Right Click on your work Flow and Select Run
d) The control page will pop open
e) You can trigger each even individually or all at once (I tended to trigger each even individually)
    1. Turn on temperature control by clicking the X next to T
    2. Turn on the stir bar by clicking the X next to SC

3. If using, trigger A and B by clicking on the X next to them
4. Trigger Inoculation Run Time by clicking the X next to Ino.

f) Once everything is triggered you can toggle between values to keep an eye on things, but for the most part you can leave it alone. The computer knows what it's doing

g) Take samples as often as you need to depending on your experiment

Step 10: Finish the work Flow

a) Once the experiment is complete click FINISH in the top right corner

b) A box will pop up asking if you are sure, click yes

c) Your work flow will move from Waiting to Finished

d) Right Click on the work flow and click on Export (ZIP)

1. The file will be exported to a file on the desktop labeled "DASGIP Export"
2. You can move the file to a different location or leave it
3. There will be 3 different files in the zip file
    a. [your work flow].control will have all the information from the experiment
    b. [your work flow] just gives basic information about the work flow
    c. [your work flow].pH Calibration gives the calibration information
4. Export the Zip File and Open [your work flow].Control
    a. Once the file is open it will give you the option to create graphs
    b. All the data will be separated by having each reactor in different tab
    c. When you save the file, save it as a Microsoft Excel Worksheet so that it will be compatible with newer versions of Excel
    d. Use an USB drive to pull your data off the computer

Step 11: Clean Up

a) Dispose of everything properly (biohazards do not go down the drain)

b) Clean out the vessels

c) Unhook everything, store probes properly

d) Turn off DASGIP

e) Turn off the Computer

Appendix E. Model Information

Table 17 Model 2 All Disposable Bioreactors Info

Label	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1

Table 17 Continued 1

P-5/AF 101	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4
	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1

Table 17 Continued 2

P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal Solids Concentration in Stream = 1000g/L	Relative to end of Transfer-Out-1 in P16
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21
P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01 FBS, Lactic Acid, and Water set to be volatile	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH(2M)	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 18 Model 2 50/50 Split bioreactors Info

Label	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 18 Continued 1

P-5/AF 101	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4
	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water	Relative to Start of Agitate-1

Table 18 Continued 2

		Time = 8 days	
	Cool-1	Final Temp = 20° C	Relative to End of Ferment-1
		Cooling Agent = Chilled Water	
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C	Relative to End of Transfer-In-2
		Heat Agent = Hot Water	
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C	Relative to Start of Agitate-1
		Heat Transfer Agent = Chilled Water	
		Time = 8 days	
	Cool-1	Final Temp = 20° C	Relative to End of Ferment-1
		Cooling Agent = Chilled Water	
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal	Relative to end of Transfer-Out-1 in P16
		Solids Concentration in Stream = 1000g/L	
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21
P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01	Relative to end of Transfer-In-1
		FBS, Lactic Acid, and Water set to be volatile	
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH(2M)	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 19 Model 2 All Reusable Info

Label	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 19 Continued 1

P-5/AF 101	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4
	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1

Table 19 Continued 2

	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste disposable = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal Solids Concentration in Stream = 1000g/L	Relative to end of Transfer-Out-1 in P16
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21

Table 19 Continued 3

P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01 FBS, Lactic Acid, and Water set to be volatile	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH(2M)	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 20 Model 3 All Disposable Info

Label in Model	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-5/AF 101	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4

Table 20 Continued 1

	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1

Table 20 Continued 2

	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal Solids Concentration in Stream = 1000g/L	Relative to end of Transfer-Out-1 in P16
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21
P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01 FBS, Lactic Acid, and Water set to be volatile	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH(2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-22/NM-101	Nano-Mill-1	Default Settings	Relative to End of Transfer-Out-1 in P-20
	CIP-1	Default Settings	
	SIP-1	Default Settings	Relative to End of CIP
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P22
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3

Table 20 Continued 3

	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP-1	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1
P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28
P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

Table 21 Model 3 50/50 Info

Label	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 21 Continued 1

P-5/AF 101	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4
	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1

Table 21 Continued 2

	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal Solids Concentration in Stream = 1000g/L	Relative to end of Transfer-Out-1 in P16
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21
P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01 FBS, Lactic Acid, and Water set to be volatile	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH(2M)	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 21 Continued 3

P-22/NM-101	Nano-Mill-1	Default Settings	Relative to End of Transfer-Out-1 in P-20
	CIP-1	Default Settings	
	SIP-1	Default Settings	Relative to End of CIP
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P22
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3
	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP-1	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1
P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28
P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

Table 22 Model 3 Reusable Info

Label	Operations	Changes to Default	Scheduling Start Times
P-2/V-101	Charge-1	Volume = 20 mL	Beginning of batch
	Agitate-1	Default Settings	Relative to end of charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of agitate-1
P-3/DE-101	Filter-1	Default settings	Relative to end of transfer out-1 in P-2
P-1/ TRF-101	Transfer-In-1	Default settings	Relative to end of Filter-1 in P-3
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Charge-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Cooled Water Time = 8 days	Relative to end of Heat-1
	Cool-1	Final Temp = 20° C Cooling Agent = Cooled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
P-6/V-102	Charge-1	V = 200 mL	Relative to end of Cool-1 in P-1
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate -1
P-7/ DE-102	Filter-1	Default settings	Relative to end of Transfer-Out-1 in P-6
P-4 / DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment in P-1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to end of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to end of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1

Table 22 Continued 1

P-5/AF101	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-4/DBS-101
P-9/V103	Charge-1	V = 4L	Relative to end of Cool-1 in P-4
	Agitate-1	Default Settings	Relative to end of Charge 1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-10/DE-103	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-9
P-8/ DBS 102	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-10
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-11/AF-102	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-8/DBS-102
P-12/V-104	Charge-1	V = 80L	Relative to end of Cool-1 in P-8
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-15/DE-104	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-12
P-13/DBS-103	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-15
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1

Table 22 Continued 2

	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-14/AF-103	Filter-1	Default	Set by Master-Slave Relationship Operation: Ferment-1 in P-13/DBS-103
P-18/V-105	Charge-1	V = 1600L	Relative to end of Cool-1 in P-13
	Agitate-1	Default	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
P-19/DE105	Filter-1	Default Settings	Relative to end of Transfer-Out-1 in P-18
P-16/DBS 104	Transfer-In-1	Default Settings	Relative to End of Filter-1 in P-19
	Transfer-In-2	Default Settings	Relative to End of Transfer-In-1
	Heat-1	Final Temp = 28° C Heat Agent = Hot Water	Relative to End of Transfer-In-2
	Agitate-1	Agitation Time = 8 days	Relative to End of Heat-1
	Ferment-1	Final Temp = 28° C Heat Transfer Agent = Chilled Water Time = 8 days	Relative to Start of Agitate-1
	Cool-1	Final Temp = 20° C Cooling Agent = Chilled Water	Relative to End of Ferment-1
	Transfer-Out-1	100% of vessel contents	Relative to End of Cool-1
	CIP-1	NaOH(2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-17/AF104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation: Ferment-1 in P-16/DBS-104
P-21/DC-101	Centrifuge-1	Equipment based on Solids Removal Solids Concentration in Stream = 1000g/L	Relative to end of Transfer-Out-1 in P16
	SIP-1 (steam in place)	Default Settings	
P-30/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-21
P-20/ FDR-101	Transfer-In-1	Default Settings	Relative to end of Centrifuge-1 in P-21
	Dry-1	Final LOD = 0.01	Relative to end of Transfer-In-1

Table 22 Continued 3

		FBS, Lactic Acid and Water set to be volatile	
	Transfer-Out-1	100% of vessel contents	Relative to end of Dry-1
	CIP-1	Uses NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-22/NM-101	Nano-Mill-1	Default Settings	Relative to End of Transfer-Out-1 in P-20
	CIP-1	Default Settings	
	SIP-1	Default Settings	Relative to End of CIP
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P22
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3
	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP-1	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1
P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28

P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

Table 23 Model 4 Disposable Info

Label	Operations	Changes to Default	Scheduling Start Times
P-3/V-101*	Charge-1	V = 2000 L	Relative to Beginning of Batch
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default Settings	Relative to end of Transfer-Out-1
P-5/DE-101	Filter-1	Default Settings	Relative to end of Transfer-Out-1
P-4/V-102*	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Transfer-Out-1	Set Volume = 20 mL	Relative to end of Transfer-In-1
	Store-1	Set by Master-Slave Relationship Operation Ferment-1 in P-1	Relative to end of Transfer-Out-1
	Transfer-Out-2	Set Volume = 200 mL	Relative to end of Ferment-1 in P-1
	Store-2	Set by Master-Slave Relationship Operation Ferment-1 in P-2	Relative to end of Transfer-Out-2
	Transfer-Out-3	Set Volume = 4 L	Relative to end of Store-2
	Store-3	Set by Master-Slave Relationship Operation Ferment-1 in P-6	Relative to end of Transfer-Out-3
	Transfer-Out-4	Set Volume = 80 L	Relative to end of Ferment-1 in P-7
	Store-4	Set by Master-Slave Relationship Operation Ferment-1 in P-8	Relative to end of Transfer-Out-4
	Transfer-Out-5	Set Volume = 1600L	Relative to end of Store-4
	SIP	Default Settings	Relative to end of Transfer-Out-5
P-1/TRF-101	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-2/ DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1

Table 23 Continued 1

P-12/ AF-101	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-2
P-6/ DBS-102	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-2
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-13/AF-102	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-6
P-8/ DBS-104	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-6
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-14/ AF-103	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-8
P-7/ DBS-103	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-8
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-15/AF-104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-7
P-9/DS-101	Centrifuge-1	Solids Removal Solids Concentration = 1000 g/L	Relative to end of Transfer-Out in P-7
	SIP-1	Default Settings	Relative to end of Centrifuge-1
P-16/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-9
P-10/ FDR-101	Dry-1	Final LOD = 0.01% FBS Media, Lactic Acid and Water are volatile	Relative to end of Centrifuge-1 in P-9
	SIP-1	Default settings	Relative to end of Dry-1

Table 23 Continued 2

P-11/ NM-101	Nano-Mill-1	Default Settings	Relative to end of Dry-1 in P-10
	SIP-1	Default Settings	Relative to end of Nano-Mill-1
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P-11
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3
	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1
P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28
P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

Table 24 Model 4 50/50 Info

Label	Operations	Changes to Default	Scheduling Start Times
P-3/V-101*	Charge-1	V = 2000 L	Relative to Beginning of Batch
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default Settings	Relative to end of Transfer-Out-1
P-5/DE-101	Filter-1	Default Settings	Relative to end of Transfer-Out-1
P-4/V-102*	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Transfer-Out-1	Set Volume = 20 mL	Relative to end of Transfer-In-1
	Store-1	Set by Master-Slave Relationship Operation Ferment-1 in P-1	Relative to end of Transfer-Out-1
	Transfer-Out-2	Set Volume = 200 mL	Relative to end of Ferment-1 in P-1
	Store-2	Set by Master-Slave Relationship Operation Ferment-1 in P-2	Relative to end of Transfer-Out-2
	Transfer-Out-3	Set Volume = 4 L	Relative to end of Store-2
	Store-3	Set by Master-Slave Relationship Operation Ferment-1 in P-6	Relative to end of Transfer-Out-3
	Transfer-Out-4	Set Volume = 80 L	Relative to end of Ferment-1 in P-7
	Store-4	Set by Master-Slave Relationship Operation Ferment-1 in P-8	Relative to end of Transfer-Out-4
	Transfer-Out-5	Set Volume = 1600L	Relative to end of Store-4
	SIP	Default Settings	Relative to end of Transfer-Out-5
P-1/TRF-101	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-2/ DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1

Table 24 Continued 1

	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-12/ AF-101	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-2
P-6/ DBS-102	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-2
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-13/AF-102	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-6
P-8/ DBS-104	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-6
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-14/ AF-103	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-8
P-7/ DBS-103	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-8
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-15/AF-104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-7
P-9/DS-101	Centrifuge-1	Solids Removal Solids Concentration = 1000 g/L	Relative to end of Transfer-Out in P-7
	SIP-1	Default Settings	Relative to end of Centrifuge-1
P-16/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-9

Table 24 Continued 2

P-10/ FDR-101	Dry-1	Final LOD = 0.01% FBS Media, Lactic Acid and Water are volatile	Relative to end of Centrifuge-1 in P-9
	SIP-1	Default settings	Relative to end of Dry-1
P-11/ NM-101	Nano-Mill-1	Default Settings	Relative to end of Dry-1 in P-10
	SIP-1	Default Settings	Relative to end of Nano-Mill-1
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P-11
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3
	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1

Table 24 Continued 3

P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28
P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

Table 25 Model 4 Reusable Info

Label	Operations	Changes to Default	Scheduling Start Times
P-3/V-101*	Charge-1	V = 2000 L	Relative to Beginning of Batch
	Agitate-1	Default Settings	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default Settings	Relative to end of Transfer-Out-1
P-5/DE-101	Filter-1	Default Settings	Relative to end of Transfer-Out-1
P-4/V-102*	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Transfer-Out-1	Set Volume = 20 mL	Relative to end of Transfer-In-1
	Store-1	Set by Master-Slave Relationship Operation Ferment-1 in P-1	Relative to end of Transfer-Out-1
	Transfer-Out-2	Set Volume = 200 mL	Relative to end of Ferment-1 in P-1
	Store-2	Set by Master-Slave Relationship Operation Ferment-1 in P-2	Relative to end of Transfer-Out-2
	Transfer-Out-3	Set Volume = 4 L	Relative to end of Store-2
	Store-3	Set by Master-Slave Relationship Operation Ferment-1 in P-6	Relative to end of Transfer-Out-3
	Transfer-Out-4	Set Volume = 80 L	Relative to end of Ferment-1 in P-7
	Store-4	Set by Master-Slave Relationship Operation Ferment-1 in P-8	Relative to end of Transfer-Out-4
	Transfer-Out-5	Set Volume = 1600L	Relative to end of Store-4
	SIP	Default Settings	Relative to end of Transfer-Out-5
P-1/TRF-101	Transfer-In-1	Default Settings	Relative to Beginning of Batch
	Charge-1	Volume = 5 mL	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Charge-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
P-2/ DBS-101	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P1
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1

Table 25 Continued 1

	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-12/ AF-101	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-2
P-6/ DBS-102	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-2
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-13/AF-102	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-6
P-8/ DBS-104	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-6
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-14/ AF-103	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-8
P-7/ DBS-103	Transfer-In-1	Default Settings	Relative to end of Ferment-1 in P-8
	Transfer-In-2	Default Settings	Relative to end of Transfer-In-1
	Ferment-1	Final Temp = 28° C Time = 8days	Relative to end of Transfer-In-2
	Transfer-Out-1	100% of vessel contents	Relative to end of Ferment-1
	CIP-1	NaOH (2M) Waste Disposal = $50 per kg	
	SIP-1	Default Settings	Relative to end of CIP-1
P-15/AF-104	Filter-1	Default Settings	Set by Master-Slave Relationship Operation Ferment-1 in P-7

Table 25 Continued 2

P-9/DS-101	Centrifuge-1	Solids Removal Solids Concentration = 1000 g/L	Relative to end of Transfer-Out in P-7
	SIP-1	Default Settings	Relative to end of Centrifuge-1
P-16/ ST-101	Sterilize-1	Default Settings	Relative to end of Centrifuge-1 in P-9
P-10/ FDR-101	Dry-1	Final LOD = 0.01% FBS Media, Lactic Acid and Water are volatile	Relative to end of Centrifuge-1 in P-9
	SIP-1	Default settings	Relative to end of Dry-1
P-11/ NM-101	Nano-Mill-1	Default Settings	Relative to end of Dry-1 in P-10
	SIP-1	Default Settings	Relative to end of Nano-Mill-1
P-23/V-106	Charge-1	Mass = 5 kg	Relative to Start of Nano-Mill-1 in P-11
	Charge-3	Mass = 20 kg	Relative to end of Charge-1
	Charge-2	Mass = 5 kg	Relative to end of Charge-3
	Transfer-In-1	Default settings	Relative to end of Charge-2
	Agitate-1	Default settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Agitate-1
	SIP-1	Default settings	Relative to end of Transfer-Out-1
P-24/GRN-101	Granulate-1	Water is volatile, 5% Final LOD	Relative to end of Transfer-Out-1 in P23
	SIP	Default Settings	Relative to end of Granulate-1
P-25/V-107	Transfer-In-1	Default Settings	Relative to end of Granulate-1 in P24
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	100% of vessel contents	Relative to end of Store-1
P-26/TP-101	Tablet-1	Default Settings	Relative to end of Transfer-Out-1 in P25
	CIP-1	NaOH (2M)	
	SIP-1	Default Settings	Relative to end of CIP-1
P-27/DB-101	Transfer-In-1	Default Settings	Relative to end of Tablet-1 in P26
	Store-1	Default Settings	Relative to end of Transfer-In-1
	Transfer-Out-1	Default Settings	Relative to end of Store-1

Table 25 Continued 3

P-29/V-108	Charge-1	Mass = 48 kg	Relative to end of Tablet-1 in P26
	Agitate-1	Default Settings	Relative to end of Charge-1
	Pull-Out	Set by Mast-Slave Relationship Operation: Coat-1 in P28	Relative to start of Coat-1 in P-28
P-28	Transfer-In-1	Default Settings	Relative to end of Transfer-Out-1 in P27
	Coat-1	Default Settings; 4 cycles	Relative to end of Transfer-In
	Transfer-Out-1	100% of vessel contents	Relative to end of Coat-1

BIOGRAPHY OF THE AUTHOR

Caitlin Murphy was born in Bangor, ME on May 21st, 1991. She was raised in Bangor, ME and graduated from Bangor High School in 2009. She attended the University of Maine and graduated with a Bachelor's degree in Chemistry in 2017. She returned to the University of Maine and entered the Biomedical Engineering department in the fall of 2018. After receiving her degree, she will be joining Bristol Myers Squib, a pharmaceutical company, as an Assistant Scientist to begin her career developing vaccines. Caitlin is a candidate for the Master of Science degree in Biomedical Engineering from the University of Maine in May 2021.