WPI

Designing an Algal Co-culture System for Increased Sustainability in Cellular Agriculture

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Abstract

The global demand for meat is expected to double in the next half-century, but current meat production practices pose significant hazards to the environment and human health. The emerging field of cellular agriculture has the potential to solve problems associated with traditional animal agriculture by culturing animal products *in vitro*. Cellular agriculture is potentially more environmentally sustainable, but there are hurdles to overcome in large-scale production. Cell culture media is the nutrient solution in which the cells are grown and is a limiting factor in the cost and environmental impact of large-scale cellular agriculture. There is a need to extend the lifetime of the media by removing metabolic waste products and replenishing the media with nutrients. This would reduce media-associated costs, water usage, and energy usage of the system. This project aimed to create a co-culture system of primary bovine satellite cells (PBSCs) and the microalga, *Chlamydomonas reinhardtii*, to extend the media lifetime and improve the sustainability of large-scale cellular agriculture. The success of this system was assessed by collecting data on dissolved oxygen concentrations, culture pH, and cell proliferation and viability. The data suggest that PBSCs can remain viable in co-culture with *C. reinhardtii* and that the system increases dissolved oxygen and buffers the pH drop normally observed in animal cell culture. In a 200:1 ratio of *C. reinhardtii* to PBSCs grown in hypoxic conditions, the PBSCs were able to undergo one doubling in three days. Based on the pH data, the media lifetime was extended by 85%. This system should be further explored to optimize the media recycling potential of *C. reinhardtii* co-cultures.

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Chapter 1. Introduction

When recognizing human contributions to climate change, animal agriculture cannot be ignored. Factory livestock contributes to 9% of carbon dioxide, 30% of methane, and 65% of nitrous oxide emissions [1]. This does not account for water, land, and energy use, or the safety and ethical concerns of factory farming. There is currently an effort to develop the field of cellular agriculture to improve our meat production practices. This field focuses on growing animal tissue *in vitro* by applying tissue engineering principles. One model predicts this could reduce greenhouse gas (GHG) emissions by 96%, land usage by 99%, water usage by 90%, and energy usage by 40% when compared to traditional beef production [2]. There is potential for this field to revolutionize our food production industry while improving our relationship with the environment. However, one of the most prohibitive factors in scale-up is cell culture media. The cost of media is high and volatile due to fluctuations in the price of fetal bovine serum (FBS), which makes consistent large-scale operations difficult [3]. While efforts are being made to move away from serum-based media with supplementation of growth factors and hormones, the quantities required for scale-up still warrant new cost reduction strategies.

Media needs to be replaced over time because animal cells take up the nutrients and produce toxic byproducts like lactic acid and ammonium [4]. In our environment, we consume nutrients and excrete waste, which other organisms use and eventually convert back into compounds we can use. The team aimed to capture this symbiotic relationship in the lab to recycle the waste in spent media. The goal is that co-culturing complementary cell types in a micro-ecosystem will increase the lifetime of cell culture media. This would decrease the frequency of media replacement, thereby reducing media-associated costs. Co-cultures of microalgae and mammalian cells can reduce carbon dioxide and ammonium concentrations while increasing oxygen concentration [5]. Algae culture can also increase the proliferation of mouse myoblasts in glucose and amino-acid deficient media [6]. These studies show that co-culture micro-ecosystems can improve cell growth conditions. The team decided to design a co-culture system for primary bovine satellite cells (PBSCs), which are used to make cultured beef.

After background research, and with relevant stakeholders in mind, the team determined objectives and constraints to guide the design process. To meet the societal need, the system must be scalable, allow proliferation and harvesting of PBSCs, and reduce the cost associated with cell culture media. For the cell culture system to work, the PBSCs must remain viable and retain their stem-phenotype, and the co-culture cell must remain viable and metabolically functional. The design was split into two aspects. The first is the co-culture system, which is largely defined by the specific cell species used to co-culture with PBSCs. After comparing the growth conditions and metabolic capabilities of multiple cell species, the microalga *Chlamydomonas reinhardtii* was chosen to be co-cultured with PBSCs. The second aspect of the design is scale-up, which is concerned with bioreactor designs that can incorporate the co-culture system for large-scale cultured meat production. The team determined design needs for the bioreactor and compared multiple conceptual designs to finally choose a hollow fiber bioreactor design that keeps the PBSC and *C. reinhardtii* cultures separate with a semi-permeable membrane.

The team started by testing the co-culture system to confirm that it fulfills the design objectives and constraints. The PBSCs and *C. reinhardtii* were first grown in monoculture to establish baseline growth conditions. Later experiments were focused on exploring the co-culture's effect on PBSC proliferation and viability, the dissolved oxygen (DO) of the media, and the culture pH. The results indicate that the co-culture can promote PBSC proliferation and increase viability in hypoxic conditions. *C. reinhardtii* can remain viable in co-culture conditions, and it increases the DO while slowing the drop in pH. These data indicate that the co-culture can increase media lifetime by 85%, thereby reducing the prohibitive media cost for scale-up. A theoretical model was created for the hollow-fiber design, although further work is necessary to prototype and validate the design. The team concluded from the results that *C. reinhardtii* co-cultures should continue to be explored as a path to reducing media costs for large-scale cellular agriculture.

Chapter 2. Literature Review

2.1 Current Animal Agriculture

Global meat production has increased more than fivefold since 1950, and factory farming is the fastest growing method of animal production worldwide [7]. This practice continues to grow around the world as a cheap method of producing meat for consumption. However, many of the practices associated with intensive animal farming have been criticized by public health professionals and animal welfare advocates [8]. Pork, chicken, and eggs are viewed as important and inexpensive contributors to dietary protein for the expanding urban populations [9]. To help dismantle factory farms, humans need to either change their dietary habits or look for alternative sources of protein.

2.1.1 Environmental Impact

Our current farming practices require large amounts of water, energy, and land use, as well as GHG emissions, making factory farming a grave environmental concern [1]. On average, livestock contributes to 9% of total carbon dioxide emissions, 39% of methane emissions, and 65% of nitrous oxide emissions [1]. Although seemingly minor in comparison, agricultural practices account for 7.4 percent of all GHG emissions, as shown below in Fig 2.1 [10]. This is still however an egregious number of emissions from agriculture, and all aspects need to be lowered to significantly decrease the impact of GHG emissions.

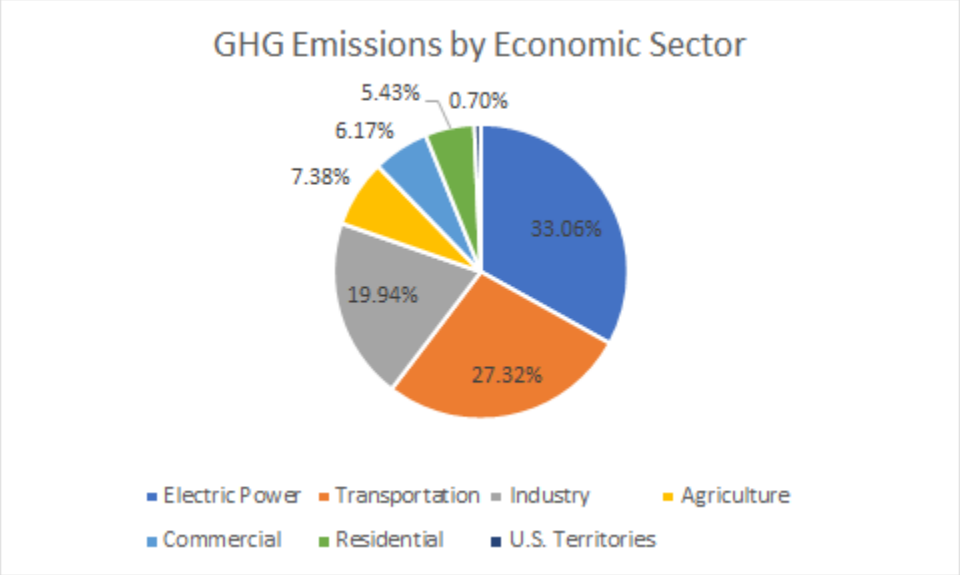

Figure 2.1: Greenhouse Gas Emissions by Economic Sector [10]

Humans currently use approximately 50% of total habitable land for agriculture [11]. This does not include deserts, the arctic, or other land that is not inhabited by humans on a large scale. Also, as shown in Fig 2.2 below, 5.9% of energy in the United States is used for agriculture [12].

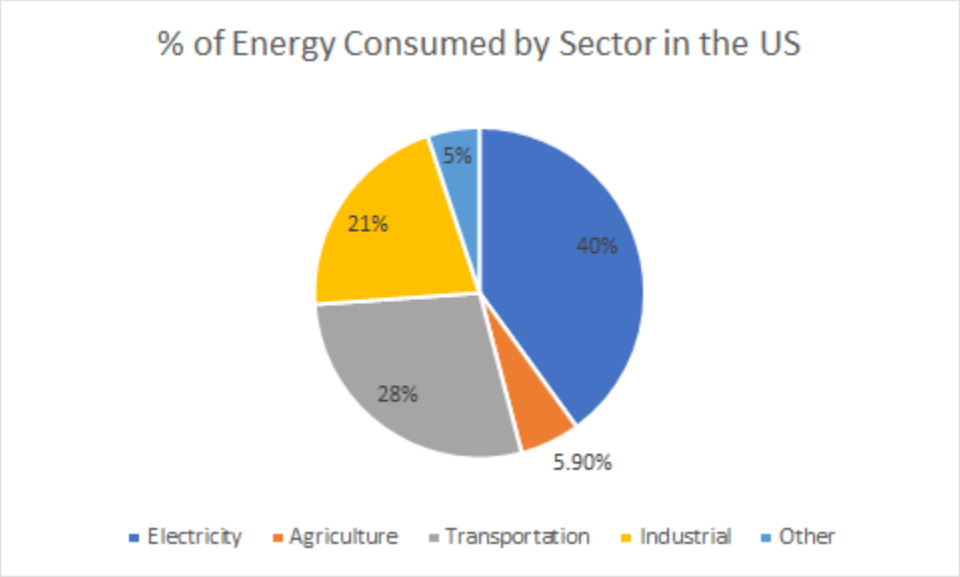

Figure 2.2: Energy Consumed by Economic Sector in the US [12]

Furthermore, Fig. 2.3 illustrates that 75% of the total amount of water used by humans is for agriculture [13]. To lessen the crisis of our shrinking water supply, industries must research and apply creative solutions to decrease their impact on an increasingly scarce resource.

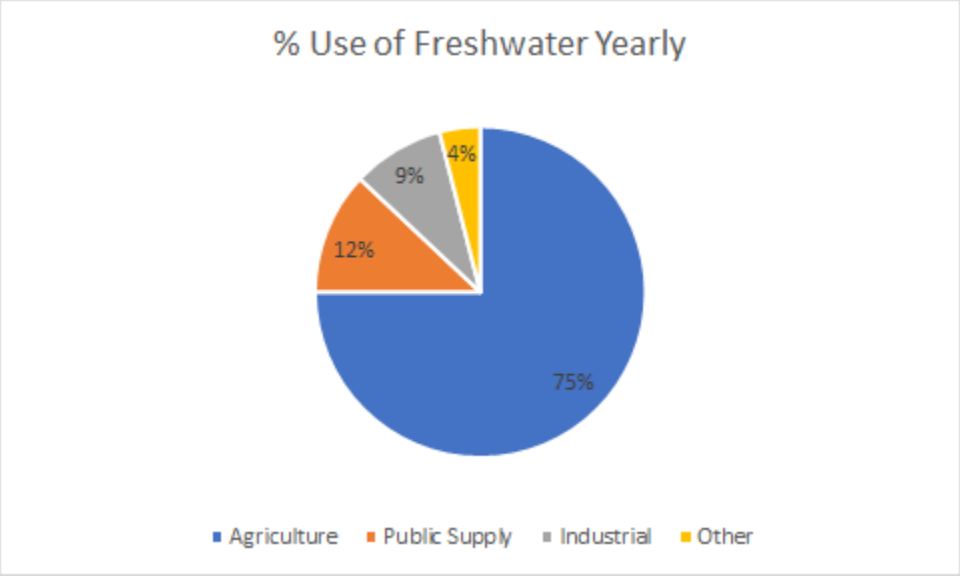

Figure 2.3: Use of Freshwater in the US [14]

2.1.2 Health and Safety Concerns

The risk of disease is embedded within modern factory farming [15]. Animals in factory farms are treated with enriched feeds and veterinary medicines that stimulate growth but also weaken their immune systems. These animals are also almost genetically identical, and with thousands of them housed close together, the environment is a hotspot for disease incubation [15]. As seen in Fig. 2.4, the majority (60.3%) of emerging infectious disease events are caused by zoonotic pathogens, which are defined as those which have a non-human animal source [16].

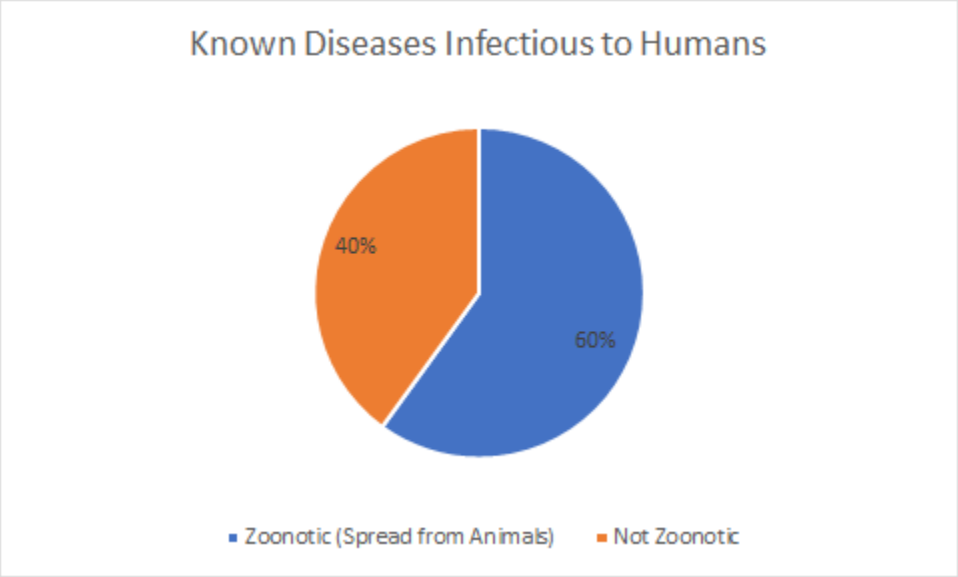

Figure 2.4: Comparison of the Origin of Diseases Infectious to Humans [16]

2.1.3 Ethical Concerns

There are many ethical concerns with factory farming, from the overcrowding and confinement of animals in small spaces to the removal of beaks or tails from livestock. Veal calves are kept in total darkness for most of their lives and fed a diet of iron-deficient milk substitute to make their flesh palatably pale and tender, to a point just above starvation [17]. All these factors cause a great deal of stress to the animals [17].

Some laws have been passed to prevent inhumane treatment. California passed the Prevention of Animal Cruelty Act in 2008. This law requires that "calves raised for veal, egg-laying hens and pregnant pigs be confined only in ways that allow these animals to lie down, stand up, fully extend their limbs and turn around freely" [8]. There is still significant legal headway to be made to ensure all states and countries are treating livestock humanely.

2.2 Cellular Agriculture

Cellular Agriculture is the field that attempts to tackle the issues caused by our current animal agriculture systems. Recent developments in biology and biomedical engineering have led to the ability to grow human tissue *in vitro*, which created the field of tissue engineering [18]. These developments include the ability to isolate stem cells and culture them *ex vivo* [1]. One recent development in the last 15 years is the ability to grow skeletal muscle stem cells, or satellite cells, into full artificial muscle [1]. This new ability paved the way for exploring tissue engineering in the world of animal agriculture [19]. If it is possible to grow human muscle in a lab, it should also be possible to grow animal muscle. This was shown in 2013 when the first cultured meat prototype was produced by Mark Post's lab at Maastricht University [19]. The process of growing cultured meat starts by obtaining animal muscle tissue [18]. The tissue is then broken down, and the satellite cells are isolated for proliferation [18] After enough satellite cells have proliferated with traditional culture techniques, they are differentiated into mature muscle cells [18]. This then leads to the final meat product, which in Mark Post's case, was the first cultured beef burger [20]. Since this demonstration of the potential of cultured meat, this

research has spread to more labs around the world, and some small businesses have begun to develop their own product prototypes [20].

2.2.1 Solves Contemporary Issues

The appeal of cultured meat is that it has the potential to solve many issues which are caused by traditional meat production methods. Some of the most alluring qualities of cultured meat are that it reduces land, energy, and water used in animal agriculture, and overall GHG emissions.

Current farming practices contribute a large portion of the GHG emissions into the earth's atmosphere. One analysis found that cultured meat has the potential to significantly reduce GHG emissions from meat production [2]. This analysis also found that land usage could be reduced by 99% when compared to beef production [2]. There would also be a 90% reduction in water usage and a 40% reduction in energy usage when compared to beef production [2]. The only category that cultured meat did not improve is the energy consumption when compared to poultry farming [2]. Below, in Fig. 2.5, one can see the percentage reduction of each resource if animal agriculture was replaced by cellular agriculture.

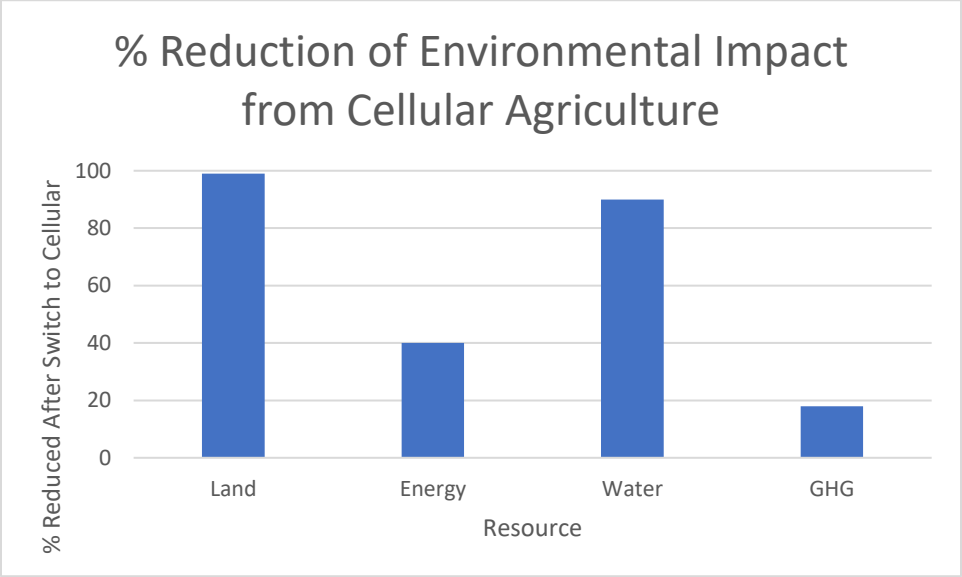

Figure 2.5: Potential Reduction of Environmental Impact of Meat Production by Cellular Agriculture [2]

2.2.2 Cost Comparison

Traditional methods of factory farming have been engineered and modified over the years to make the final product as cheap as possible [21]. The cost comparison between the different meat products, as seen in Fig 2.6, illustrates the massive cost difference between traditional meat culture with cellular agriculture. Traditional meat cost fluctuates depending on where you live, but the average cost is $6.12 per pound [22]. Plant-based meat is slightly more expensive, at $10.30 per pound [23]. Comparatively, cell agriculture meat is very expensive and costs around $100 per pound, which currently cannot compete in the general meat market against farmed products [23]. However, as this field is researched and improved upon, the cost of cellular agriculture meat will only go down [20].

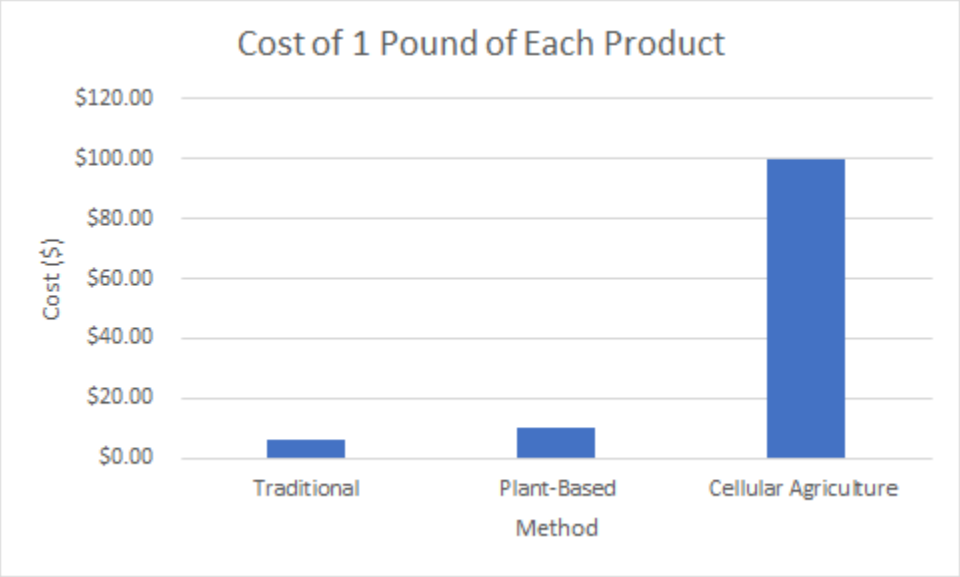

Figure 2.6: Current Cost to Produce One Pound of Each Meat Product [22-23]

2.2.3 Scale-Up Issues

While cultured meat has the potential to improve upon traditional meat in many aspects, this requires the creation of large-scale systems to generate massive quantities to sustain the meat demand. The scale necessary to compete with traditional meat production is said to be the largest ever system for tissue engineering [20]. Some of the biggest limitations that researchers face for scale-up are related to the cells, suitable scaffolds, cell culture media, and the large-scale bioprocess [15].

For large-scale production of cultured meat products, many limitations are specific to the needs of the cells which are part of the final meat product. One limitation is how long cells can be cultured, as it has been shown that human myoblasts can double up to 45 times for *in vitro* culture [24]. This is limited because there is difficulty preventing satellite cells from differentiating [1]. However, there is some evidence that aged populations of satellite cells have similar regenerative abilities when compared to a young standard [25]. The main two stages for consideration in cell culture are proliferation and differentiation, and there has been much focus in delaying the latter to lengthen the former [1].

Another goal is to control the muscle cell niche to be more like that of stem cells; this may allow the cells to extend proliferative abilities [26]. Besides finding ways to improve and control the proliferation and differentiation of satellite cells, there is a focus on the other cell types that make up meat [18]. Skeletal muscle is not just one type of tissue; it contains muscle, fat, and connective tissues which contribute to the texture, flavor, and nutritional make-up of meat [27]. Taste is an important factor for cultured meat, and researchers are beginning to understand the combination of peptides responsible for these flavors [28]. However, the lipids in meat cannot be ignored when replicating the taste of traditional meat [29]. Overall, the hurdles to overcome for scale-up to commercial products are controlling proliferation, differentiation, and the mixture of cell types.

Scaffolds are an integral part of tissue engineering, as they guide cells in 3D culture and can help promote the growth of functional tissue [30]. This is especially important for muscle cells since they form tissues with specific orientations [30]. Therefore, it is necessary to form

scaffolds that help to replicate the natural environment of the target cells [1]. One necessary complication to overcome is nutrient transport, which is often addressed by increasing scaffold porosity [1]. Another way to achieve this is using decellularized plant scaffolds, which serve as an effective scaffold for cardiomyocytes [31]. The benefit of these scaffolds is that they take advantage of the natural vasculature of plants for nutrient transport [31]. The fabrication of these scaffolds is relatively simple when compared to the synthetic formation of such a vascular network [31]. Since they are made of natural polymers, these scaffolds are edible [31]. The idea of an edible scaffold is incredibly important for cultured meat, as the product is intended for consumption [32]. The scaffold does not have to be edible, but this would mean that cells must be removed before further processing [32]. Another benefit of having an edible scaffold is that it may contribute positively to the texture of the cultured meat product [15]. The type of scaffold used will influence the success of large-scale cultured meat production.

Growing cells require cell culture media to supply nutrients and other necessary biomolecules [3]. Currently, the gold-standard medium for growing mammalian cells uses FBS to supply necessary growth factors and growth inhibitors [33]. One concern with this method for cultured meat is that the use of FBS conflicts with the advantage of "slaughter-free" meat production [3]. Serum-based media are also flawed because the serum composition varies for each batch, which makes quality control more difficult [3]. Serum-based media is also prohibitive for scale-up because of its cost [32]. In recent years, the price of FBS in the United States has gone up by 300% [34]. These kinds of volatile fluctuations in FBS cost are not uncommon and are not conducive to sustainable large-scale operations [34]. Another concern for cell culture media is the production of waste products. There are some concerns that, like other large-scale operations, waste products may be released into the water supply [35]. Currently, serum-based media are more effective than completely synthetic media, but the cost and non-standard nature of FBS are prohibitive for scale-up [1]. However, there is some preliminary evidence that recycling nutrients might improve the capabilities of synthetic media [2].

2.2.4 Bioreactors

A universal definition of a bioreactor does not exist [36]. There are, however, two main types of bioreactors: chemostat and turbidostat. A chemostat bioreactor includes an ongoing feed of fresh nutrient medium, while also discharging an equal mass of used medium, including biomass. Conversely, a turbidostat bioreactor keeps the biomass constant [37]. Bioreactors operate in batch, bed-batch, and continuous processes [38]. Depending on the type, such as a fixed wall or rotating wall, the bioreactor can be set up for continuous or semi-continuous harvesting.

Bioreactors have many applications in the bioprocessing field, including cell culture and tissue engineering. This ranges from bone, ligaments, blood vessels, and even heart valve tissue. Bioreactors have already improved the processing and the results of skin and cartilage regeneration, the two main laboratory-grown products that are currently commercially available [36].

Some important considerations for bioreactors are how fast the effluent flow rate is as this can help the system in saving water with a recycle stream if the effluent is fast enough [15]. Another consideration when designing a bioreactor is the equipment involved to keep the cells alive and keep operating conditions running smoothly. This includes media storage tanks, heat exchangers, monitoring and control systems, and a means of maintaining isothermal conditions within the bioreactor [15]. Each bioreactor must be designed specifically for each tissue because

if the signals are inappropriate or absent, cells cannot proliferate or form organized tissues, and instead they become disorganized which leads to cell death [39].

2.3 Animal Cell Culture

Cell culture is the process of growing cells *in vitro* for scientific purposes. The process for cell culturing includes isolation of the cell of interest to culture, growing the cells in media, and passaging the cells as they grow to confluence, or fill an entire container [40]. The skeletal muscle niche has a complex tissue composition with various cell and tissue types that contribute to its quality [41]. Recreating authentic muscle tissue in culture requires many efforts to replicate the structures, texture, and flavors present in traditional meat. Many specifications of culturing conditions must be considered for successful tissue culture.

2.3.1 Culture Requirements

Before considering the chemical components of the media for cell culture, the macroscopic environment for cell growth must be suitable for cell growth by mimicking physiological conditions. Specifically, pH, temperature, and osmotic pressure are conditions that should be monitored and kept consistent. The suitable pH for mammalian cell growth is 7.2-7.4. CO_2 can have a large effect on the pH of a system because of the formation of carbonic acid. A $NaHCO_3$-CO_2 buffer system is often utilized to prevent pH from fluctuating [42]. The temperature of the system should be kept at about 37°C which is consistent with mammalian internal temperature. Oxygen must be kept at high levels to support growth and metabolism as well.

In addition to these essential components for cell growth, considerations should be made to prevent unwanted contamination in the environment. The growth environment must be sterile to prevent the growth of unwanted microorganisms such as bacteria [43]. Sterile techniques must be carefully followed to prevent the incorporation of plasticizers from plastic instruments, trace elements from water, or microorganisms [44]. To prevent contamination and thus product spoiling, antibiotics, and antimycotics are often included in cell culture media [42]. Specifically, penicillin and streptomycin may be utilized in media. It should be noted that these are not essential to growth, and depending on the purpose of the product, antibiotic use may not be preferred by potential consumers since antibiotic-fed farm animals have been a larger concern in the past decade [19].

Satellite cells also need a physical scaffold or structure on which to develop in culture to generate a 3D structure similar to muscle tissue. The scaffold must allow for nutrient flow, proper anchorage, and generate a viable product [41]. A tissue cell culture environment may be simulated with biomaterials such as hydrogels or macroporous structures, which may even be edible materials such as decellularized plants [41].

The necessary nutrients required for cell culture include sugars, inorganic salts, pH buffers, amino acids as a nitrogen source, vitamins, fats, nucleic acid precursors, growth factors, hormones, antibiotics, and O_2 [40]. Each cell type has a unique set of nutrients in its ideal media which must be determined experimentally. Often found in cell culture media, which is artificial, or not derived directly from animal tissue, is serum. Serum provides many essential nutrients such as salts, various growth factors, and essential proteins for cell growth, however, there are serum-free media available for cell culture [43].

Growth factors present in media designate conditions either of the two main growth pathways of satellite cells. The two major pathways for cells when in culture are proliferation and differentiation. For proliferating cells, key growth factors must be present at different cell cycle growth determining steps. Fibroblast growth factor (FGF), specifically FGF2, is an essential growth factor involved in signaling pathways to initiate continuous satellite cell proliferation [45]. *In vivo*, FGF is released when skeletal muscle cells are injured to induce satellite cell proliferation for tissue regeneration [46]. FGF also blocks the differentiation of satellite cells, thus reinforcing proliferation pathways [47]. In a medium dedicated to satellite cell proliferation, there must be a source of growth factors which is typically serum [48]. Serum-rich media support the proliferation of cells, whereas serum-poor media are needed for cell differentiation.

Albumin is an essential molecule to preserve the integrity of the grown satellite cells. Bovine serum albumin (BSA) is a common component of media for cell culture. BSA in serum is typically 60% of the total protein in serum at a concentration of about 50 mg/mL [49]. There are many positive effects associated with albumin. Albumin plays a role in reducing oxidative stress, acting as an antioxidant to aid in circulation *in vivo*, which is also essential *in vitro* [50]. Albumin also has been suggested to play a role in preventing apoptosis by modifying forces in the extracellular environment of large bioreactors [51]. With the ability to bind a variety of ligands of vastly different chemical makeup, including fatty acids, metal ions, and amino acids, albumin can influence many cellular processes including waste removal [52].

Amino acids must be in high concentrations in tissue culture media. Amino acids are the nitrogen source for satellite cells and are essential for tissue growth and protein synthesis. Specifically, glutamine is essential at a higher concentration than other amino acids in mammalian cell culture [42].

Other notable nutrients include salts, hormones, and micronutrients. One salt of note is sodium bicarbonate which regulates pH by buffering CO_2 concentrations. Insulin may also be added to media for its hormone activity in regulating and promoting the use of glucose and amino acids [42]. Various inorganic salts, amino acids, vitamins, and other compounds are common in formulations of media such as DMEM basal medium [40].

2.3.2 Limiting Factors

The health of animal cell cultures can decline due to the accumulation of toxic metabolic byproducts. The primary by-products of concern are ammonium and lactic acid, which can limit cell function and proliferation [53-54]. Ammonium is the main nitrogenous waste product of mammalian cells and is secreted as a waste product of protein synthesis. Lactic acid is a carbon-based compound produced by anaerobic respiration. During aerobic respiration, the carbon byproduct is CO_2. When maintaining cell cultures, these waste products are removed by replenishing the culture with fresh cell culture media.

2.4 Co-cultures

2.4.1 Benefits

Co-culture of animal cells with other species is useful in many different contexts. Co-culture systems can be used to help better mimic *in vivo* tissue models by creating a more accurate cellular environment. Other than studying the natural interactions between the cell

cultures, researchers can also use it to better understand ways to improve the cultivation success of cell lines [55-57]. Researchers have been using co-culture systems to help better understand the microenvironment in cancer research [58]. It is also widely used by scientists to help determine the potential cytotoxicity of drug compounds due to a more accurate *in vivo* environment [56]. Furthermore, in the field of synthetic biology, the study of various co-culture systems can also help create new synthetic interactions between different cell populations [55].

In the realm of synthetic biology, researchers have been studying different ways to employ a co-culture of different organisms to achieve various metabolic end-products [59-60]. Before this discovery, researchers have been using a single type of engineered microbe to help aid in the conversion of substrate to the desired product. However, research has shown that using a combination of organisms in a "divide and conquer" technique can improve the efficiency of the biosynthesis of desired compounds [59]. In addition to a "divide and conquer" technique, studies have also revealed that one can manipulate and modulate the production of compounds through co-cultures [60].

2.4.2 Carbon Cycle

Carbon must be present in usable sources for animal cell metabolism. The most common carbon sources in media are sugars, specifically in monosaccharide form, most commonly as glucose for aerobic respiration [42]. In commonly used media for optimal mammalian cell culture, DMEM, glucose is present at 4500mg/L concentration [61]. Carbon sugar sources are transformed into metabolic chemical compounds in the cells that generate chemical energy in the form of carrier molecules such as ATP, NADH, and $FADH_2$. Energy from these carrier molecules is used in the cell for various metabolic processes including protein synthesis, DNA synthesis, waste removal, vesicle trafficking, among others. During aerobic respiration, which requires O_2 as a reactant, the metabolic byproduct is CO_2, as seen in Fig. 2.7 [62].

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 38ATP$$

Figure 2.7: Overall Equation of Aerobic Respiration

Carbon dioxide in the atmosphere is recycled via photosynthesis by plants and other photosynthetic organisms by using water as a hydrogen source to ultimately produce sugars [63]. This process is shown below in Fig. 2.8.

$$6CO_2 + 6H_2O + \text{Light Energy} \rightarrow C_6H_{12}O_6 + 6O_2$$

Figure 2.8: Overall Equation of Photosynthesis

If oxygen is not present, the animal cells undergo lactic acid fermentation, as seen in Fig. 2.9 [62]. The by-product, lactic acid, is written as $C_3H_6O_3$.

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 + 2ATP$$

Figure 2.9: Overall Equation of Lactic Acid Fermentation

2.4.3 Nitrogen Cycle

The nitrogen cycle is a biogeochemical process where inert nitrogen found in the atmosphere is transformed into more usable forms for various organisms. The nitrogen cycle can be characterized as five interconnected stages: nitrogen fixation, nitrification, assimilation, ammonification, and denitrification. Nitrogen fixation consists of converting inert nitrogen, found in the atmosphere, into a more usable form, ammonia. Nitrification is when ammonia is converted into nitrite and subsequently into nitrates. Assimilation is the process where plants uptake the various nitrogen compounds used in the formation of amino acids and proteins. Assimilation is the process that allows nitrogen to enter the food web. Ammonification is when various bacteria and fungi convert organic matter containing nitrogen back into ammonium when an organism dies. Denitrification is the process where nitrogen is released back into the atmosphere by converting nitrates into nitrogen gas [64].

Nitrogen metabolism is an aspect of the nitrogen cycle that plays an important role in the survival of many living organisms [65]. Nitrogen metabolism is the process in which organisms uptake and recycle ammonia, or its charged form, ammonium, and convert it to various compounds including proteins, nucleic acids, hormones, neurotransmitters, and nucleoside triphosphates [65-66]. Various studies have suggested that the manipulation of this mechanism in algae and cyanobacteria can be used to recycle built-up ammonium in animal cell cultures [67-69].

2.4.4 Potential Cell Types

2.4.4.1 Plant Cells

Plant cells are a potential cell type to consider for their low cost, easily replicated growing conditions, and photosynthetic capabilities. Plant cells for culture are cheap and easy to isolate and general methods for harvesting metabolically active and photosynthetic cells are well established [70]. Developing a plant cell culture includes isolation of tissue from the explant, culturing of the callus, and generation of a cell culture suspension from the callus in media [71]. The growth condition for plant cell culture generally requires a temperature of 24-25°C with photoperiodic light [71]. The short life cycle and easy manipulation of their small genome make plant cells ideal candidates for research purposes [72]. Additionally, due to plant cells being photosynthetic, they require few nutrients besides oxygen, water, and ample light to thrive. An additional benefit to the photosynthetic abilities of plants in co-culture is the uptake of CO_2 generated by cellular respiration.

Of the many possible plant species to use, *Arabidopsis thaliana* is a well-characterized species that can grow successfully in suspension culture [73]. Studies of *A. thaliana* demonstrate the simple techniques to generate large cell suspensions from callus cultures [74]. Other cell types to consider for a co-culture environment would be those with high capacity for cell growth, high viability with high cell density, and efficient metabolism for utilizing waste products of animal cell metabolism.

2.4.4.2 Microalgae

The application of algae to help remove waste has been studied and practiced for years. For example, a mixed culture of microalgae and bacteria can remove ammonium and phosphate from domestic sewage and convert them into various valuable compounds such as algal metabolites and biogas [68]. Research has also suggested the potential benefits of using algae to

recycle waste metabolites produced in mammalian cell culture [5]. The microalga, *Chlorococcum littorale*, can increase the proliferation of both C2C12 (mouse myoblast) and rat cardiac cells through co-culture [5]. Co-culture of *C. littorale* with mammalian muscle cells can decrease glucose consumption and lactic acid production, increase O_2 concentrations by metabolizing CO_2, and significantly reduce the ammonium buildup in media [5]. This co-culture also reduced the cardiomyocyte production of creatinine kinase, which is an enzyme that indicates muscle cell damage [5, 75]. This indicates that co-cultures of microalgae can increase the health of muscle cells. The microalga *Chlamydomonas reinhardtii* is also able to uptake ammonium as a nitrogen source [76-78]. *C. reinhardtii* is a model organism for algae [78], and it has been used in co-culture with fibroblasts to reduce hypoxic distress in low-oxygen conditions [79]. *Chlorella vulgaris* is another microalga species that can uptake ammonium from cell culture media [80-81]. Both *C. reinhardtii* and *C. vulgaris* are edible and contain essential amino acids, fatty acids, and minerals [82].

2.4.4.3 Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that are thought to be one of the earliest organisms to have lived on this planet [67]. Aside from being known for their photosynthetic abilities, studies have shown that they can also be used to treat distillery wastewater [67, 83]. Their ability to fix nitrogen and their photoautotrophic nature make them cheap to maintain [67]. Their ability to produce reactive oxygen species serves to break down recalcitrant contaminants that are usually non-biodegradable [67]. In addition, cyanobacteria are also known for uptaking metal ions and phosphates [67]. There has not been much research on cyanobacteria and animal cell co-cultures because certain metabolites that cyanobacteria secrete are toxic to many organisms [84]. However, research has shown that manipulating culture conditions of cyanobacteria, like temperature, can reduce toxin secretion [85].

Additional research has suggested that cyanobacteria hydrolysate can also be used as a nutrient supplement for muscle cell growth [2]. Culturing meat using cyanobacteria hydrolysate, when compared with traditional beef production, resulted in a 45% reduction in energy use, 96% reduction in GHG emissions, 99% lower land usage, and 96% reduction in water usage [2]. This demonstrates that cyanobacteria can potentially produce nutritional components for muscle cells. However, minimal research has been done on whether cyanobacteria can secrete these metabolites for muscle cell growth.

2.4.5 Genetic Engineering Possibilities

The usage of genetic engineering to help increase the production of various compounds is a relatively new field of biology that researchers have been exploring. Various transgenic bacterial species are used to synthesize compounds such as insulin, biofuels, and enzymes [86]. However, genetically engineered bacteria can harbor harmful side effects and be a potential health hazard [87]. Therefore, the usage of genetically engineered bacteria has been mainly limited to pure production purposes. The potential use of genetically engineered plants or algae to produce desired metabolites has also been an area of interest for many. Genetically engineered plants and algae can produce many more complex proteins, due to their increased cellular complexity compared with bacteria. Many bacteria lack the proper post-translational modifiers to

process and modify more complex proteins, which makes plants and algae more promising candidates for producing complex proteins [88].

Chapter 3. Project Strategy

3.1 Initial Client Statement

Our team aimed to alleviate some of the limitations of large-scale cultured meat production. The initial background research showed that the cost of media in large-scale production was prohibitive and raised environmental concerns. With this research, and input from scientists in the field of cellular agriculture, this initial client statement was developed:

Recycle the spent cell culture media to be reused for future cell culture.

3.2 Technical Design Requirement

The team found that recycling the media required the design of a novel system. There has been minimal research on the feasibility of a media recycling system using symbiotic co-cultures. However, no significant work has been made on the development of a prototype or proof-of-concept. The team determined multiple design objectives that would satisfy the need for this system. Various cell culture constraints were determined to ensure that the co-culture system is feasible. These objectives and constraints informed the design process and experimental setup.

3.2.1 Objectives

The team determined objectives that, if achieved, would fulfill the need for this co-culture system. To meet the need, the system must be scalable, allow proliferation and harvesting of PBSCs, and reduce the cost associated with cell culture media.

3.2.1.1 Scalable System

Scalability is necessary for this system to improve the large-scale production of cultured meat so it can compete with traditional meat production. To create a scalable system, the cells must be grown in a bioreactor, which can range from small-scale to industrial-sized. There must be a scaffold for the PBSCs to grow on since they are adherent cells. The system must transport the spent media to be recycled and transport the replenished media back to the PBSC culture.

3.2.1.2 Proliferate PBSCs

Proliferation is the step in cultured meat production when a large mass of cells is produced, which will be used to form muscle tissue. The PBSCs must continue to proliferate in the system so a large biomass can be produced. The media must contain components that encourage PBSC proliferation, like FGF2.

3.2.1.3 Harvest PBSCs

After proliferation, the cells must differentiate into more specialized tissues to form a meat product. This system must allow easy harvesting of PBSCs when they are ready to move to the differentiation stage. This objective may be accomplished by physically separating the PBSC culture from the symbiotic recycling culture.

3.2.1.4 Reduce Media Cost

The system must reduce media-associated costs. This can be achieved by increasing the lifetime of the cell culture media by 100%. This will reduce the frequency of media

replacements, thus reducing the cost associated with media consumption. The media lifetime can be extended by removing metabolic waste products like ammonium, lactic acid, and carbon dioxide. The system should also add oxygen back into the media.

3.2.2 Constraints

The team determined cell culture constraints that would be necessary to adhere to for the system to be feasible. For the system to work, the PBSCs must remain viable and retain their stem-phenotype, and the co-culture cell must remain viable and metabolically functional.

3.2.2.1 PBSC Viability

The PBSCs must remain viable in the co-culture system. The co-culture cell and the co-culture conditions must not significantly hinder the growth of PBSCs. The PBSCs must be viable after proliferation to differentiate into muscle tissue.

3.2.2.2 Maintenance of PBSC Stem Phenotype

PBSCs can proliferate well because they are stem cells. After differentiation, the proliferative ability of the cells diminishes. To produce a large cell mass, the co-culture system must encourage PBSCs to maintain their stem-phenotype.

3.2.2.3 Co-culture Cell Viability

For the co-culture to function, the co-culture cell must remain viable throughout the proliferation process. This means that the co-culture cell must be able to survive in similar culture conditions to the PBSCs.

3.2.2.4 Co-culture Cell Functionality

Along with remaining viable, the co-culture cell must maintain its ability to metabolize animal cell waste products. The co-culture conditions must not inhibit the uptake of waste products or the production of vital nutrients.

3.3 Standard Design Requirements

For the design of this project, there are standards of regulation that must be followed. Since the goal of this bioreactor system includes food production, both the FDA and USDA standards of meat production must be followed as it relates to the quality and safety of meat production for consumption. There are also design regulations for the bioreactor set forth by Good Manufacturing Practices and International Organization for Standards. Both regulations cover safety, cleanliness, and general good operations for this bioreactor to avoid accidents or contamination.

3.3.1 Bioreactor Standards

For the bioreactor itself, many standards must be complied with to consider it valid. The bioreactor must be sterile and easy to be cleaned, along with safe to work with for employees. Through the design process, the following regulations must be met:

- ISO 22000 - Food safety management systems - Requirements for any organization in the food chain. [89]

- ISO 11737-2 - Sterilization of health care products — Microbiological methods — Part 2: Tests of sterility performed in the definition, validation, and maintenance of a sterilization process [90]
- ISO 31000 - Risk management [91]

ISO 22000 shows companies what they need to do to demonstrate how they can control food safety hazards. Mostly just a guideline on how to safely handle and store food. ISO 11737-2 is a guide to properly sanitize equipment. ISO 31000 lays a guideline for organizations facing any risks, no matter the size of the organization. It is just a general approach to solving some common risks or problems.

In addition to the ISO regulations, there are Current Good Manufacturing Practices guidelines set by the FDA to set the standards for pharmaceutical products. Although our bioreactor isn't strictly for pharmaceutical use, this is still a product obtained from cell culture and falls under these guidelines. The main CGMP standard we will follow is below:

- CGMP Part 210 -- Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs [92]

This regulation is to ensure the product meets quality design requirements and the whole assembly process is followed safely. Failure to comply with these regulations, "shall render such drug to be adulterated under section 501(a)(2)(B) of the act and such drug, as well as the person who is responsible for the failure to comply, shall be subject to regulatory action" [92].

3.3.2 Standards for Meat Production

For the meat production standards, the group will follow the regulations set by the USDA. The role of the USDA is to regulate packaging, handling, and labeling. Many of the current USDA meat standards relate to livestock so we can disregard a good amount as they include living conditions or ways to correctly handle the animals. Several of the relevant meat production standards are shown here:

- 315 Pathogen Intervention – The harvest process shall include at least two pathogen intervention steps. One of the intervention steps shall be a critical control point (CCP) in the establishment's FSIS recognized harvest process Hazard Analysis Critical Control Point (HACCP) plan. The CCP intervention(s) shall be scientifically validated to achieve a three-log reduction of enteric pathogens [93]
- 318.2 Handling - All boneless beef shall be maintained in excellent condition. [93]
- 318.7 Microbial Testing – All lots of fresh chilled boneless beef shall be tested for all indicator microorganisms [93]
- 331 Fat Percentage - The limits for percent fat in a boneless beef product should be within 12.0-18.0% [93]

Standard 315 covers the intervention of pathogens, which is the methods the production company uses to prevent diseases in their meat products. Of course, the best methods are preventative methods, but this is up to the company on how to handle this. Standard 318.2 is just to make sure the boneless beef is stored correctly. Standard 318.7 says to test all boneless beef

for microorganisms, which is to make sure correct storage guides are followed and protect consumers from disease. The final standard, 331, covers the fat percentage of boneless beef products, which is relevant to cellular agriculture.

It should be noted that the FDA does not regulate meat production for most farm animals. It does, however, require all food manufacturers except seafood and juice processors to create a Food Safety Plan. This plan has the owner evaluate the hazards that could affect food manufactured, processed, packed, or held by such a facility. It also wants them to identify and implement preventive controls to significantly minimize or prevent the occurrence of such hazards and provide assurances that such food is not adulterated or mislabeled [94].

- 21 U.S. Code § 350g - Hazard analysis and risk-based preventive controls [94]

3.4 Revised Client Statement

Addressing the prohibitive cost of media in large-scale cultured meat production is necessary to compete with the traditional meat market. The requirements previously explained must be met to address this limitation. With these requirements in mind, the team formed the revised client statement:

> *Develop a scalable system to recycle spent media so it can be used again for PBSC culture. The system must keep PBSCs viable and promote proliferation. The PBSCs should be harvestable from the system for relocation to the differentiation stage. The system must reduce media-associated costs by extending the media lifetime by 100%. This can be achieved by removing metabolic waste products and replenishing vital nutrients. The cells responsible for media recycling must remain viable and functional in the co-culture system.*

3.5 Project Approach

The project began in August of 2020, at the beginning of A term. The team began with background research on the need for a co-culture media recycling system. The team also brainstormed conceptual designs for a bioreactor to house the co-culture. By the end of A term (late October), the team chose to use *C. reinhardtii* as the co-culture cell and began to learn techniques for growing PBSCs. The team spent B term (late October through early December) starting preliminary experiments and learning proper techniques for culturing PBSCs and *C. reinhardtii*. The preliminary experiments explored the growth of PBSCs and *C. reinhardtii* in various media conditions and temperatures. The team also began experiments to assess the ability of *C. reinhardtii* to uptake waste metabolites and extend the media lifetime. Over winter break, the team began co-culturing PBSCs with *C. reinhardtii*. The team spent C term (late January through mid-March) exploring the effects of the co-culture on dissolved oxygen and pH. The effects of growing the co-culture with a semi-permeable membrane were also explored. In D term (mid-March through early May), the team finished all validation experiments. The data was analyzed, and the report was finalized. The team participated in Project Presentation Day with a recorded presentation and a live Q&A session.

Chapter 4. Design Process

This chapter will outline the needs analysis for the two main aspects of the system, a description of various conceptual designs, and the selection of a final design using a custom value analysis matrix.

4.1 Needs Analysis

The needs were addressed for two aspects of this design. The first is the co-culture system, which is largely defined by the specific cell species used to co-culture with PBSCs. The second is the scale-up, which is concerned with bioreactor designs that can incorporate the co-culture system for large-scale cultured meat production.

4.1.1 Co-culture System

The team brainstormed needs for the co-culture system, and each need was assigned a weight based on relative importance in Table 4.1. The weight ranged from 1 to 5, with 1 being not very important and 5 being very important.

Table 4.1: Needs and Relative Weights for the Co-Culture System

Need	Weight
Metabolize CO_2	5
Uptake Ammonium	5
Uptake Lactic Acid	5
Overlapping Temp. Requirement	4
Overlapping pH Requirement	4
Well Documented Species	3
Transfectability	2

The most important needs are metabolizing CO_2 into O_2, uptaking ammonium, and uptaking lactic acid. These are the primary metabolic waste products in animal cell culture. Removing them from the media is the first step in media recycling.

The next most important needs are having an overlap in temperature and pH requirements for the two cell types. Normal culture conditions for PBSCs are 37°C and a pH of 7.2. PBSCs can be grown at a lower temperature, but their metabolic rate will be slowed. The cell type used in co-culture should be able to survive well in similar conditions to the PBSCs.

The next need in order of importance is having a cell type for co-culture that is well documented. It is advantageous to work with a species that is well studied, so more time can be spent on experimental design instead of learning to culture the cells.

The least important need is transfectability. The team discussed the possibility of creating a transgenic cell that produces growth factors that promote PBSC proliferation. This may become a more important objective following the success of toxic byproduct removal.

4.1.2 Scale-Up

For scale-up of the co-culture system, the team brainstormed multiple needs and gave them relative weights, as seen in Table 4.2.

Table 4.2: Needs and Relative Weights for Scale-Up

Need	Weight
PBSC Harvestable	5
Scalability	5
Keep Co-culture Separate	4
Suitable Scaffolding	4
Variable Temperature Control	3
Durability/Ease of Maintenance	3

The harvestability of primary PBSCs was deemed to be the most important because this is the whole idea behind culturing the cells in a bioreactor. If the cells cannot be harvested, then there is no point in scaling up the process. Scalability of the design is also very important, as that pertains to taking the small-scale model and recreating an industry-scale version for marketing and sale.

Next separating the co-culture and suitable scaffolding for the cells were decided to be given a weight of four. Although these were not the most important factors in the scale-up of our design, they are very integral and important to the success of the scale-up. Separating the co-culture is important in our design to avoid contamination between the algae and PBSCs. Suitable scaffolding for the cells pertains to the holding system for the cell interactions, which is important to ensure the proliferation of the PBSCs.

Finally, ease of maintenance and variable temperature control were both given a weight of three. Ease of maintenance is how easy the equipment can be cleaned after use or how often material needs to be changed to avoid breaking or damage. Variable temperature control is keeping the cells in viable living conditions.

4.2 Conceptual Designs

Multiple designs were brainstormed as solutions to the media-use issue in large-scale cellular agriculture. For the basic co-culture system, multiple cell species were explored to find a suitable co-culture cell. For scale-up, multiple conceptual designs were created for bioreactor systems.

4.2.1 Co-Culture System

The main component of the co-culture system is the species of co-culture cell that is being co-cultured with PBSCs. Inspiration was drawn from the carbon and nitrogen cycles in our ecosystem, so multiple photosynthetic cell species were explored.

4.2.1.1 Chlamydomonas reinhardtii

The unicellular microalga *Chlamydomonas reinhardtii* was explored as an option for the co-culture cell in the co-culture system. *C. reinhardtii* is a model species for algae, so its culture techniques and conditions are well documented [95]. The microalga is photosynthetic, motile, and phototropic. *C. reinhardtii* can uptake ammonium, which is a necessary step in media recycling [95-97]. The optimal temperature for culturing the microalga is 28°C, but it has been grown from 18°C to 37°C [98]. *C. reinhardtii* is often grown in TAP media, which can have a pH of 6.9-8.8 [99]. Transfection of *C. reinhardtii* has been successful by electroporation [100] and CRISPR [101].

4.2.1.2 Arthrospira platensis

Arthrospira platensis is a cyanobacterium that was explored as a cell species for the co-culture system. *A. platensis* is an edible and photosynthetic bacterium that is sold as a dietary supplement, more commonly referred to as spirulina [102]. The cyanobacterium can uptake ammonium in culture [103]. The optimal temperature for growth is 29°C, and it has been grown at a range of 23°C to 34°C [104]. *A. platensis* is grown at an optimal pH of 9.5 [104]. Transfection of *A. platensis* has been successful by electroporation [105] and using agrobacteria [102].

4.2.1.3 Arabidopsis thaliana

Arabidopsis thaliana is a model organism for plants that was explored as a source of cells for the co-culture system. This is the only multicellular organism proposed, but it is well-characterized, and its cells can be isolated and cultured. There is some evidence of *A. thaliana* uptaking ammonium, but this process is downregulated as ammonium is taken into the roots and then the cytoplasm [106]. The optimal culture temperature for *A. thaliana* cells is 24°C, and the culture pH is 5.5 [107]. Transformation of *A. platensis* has been demonstrated at the organismal and cellular level using agrobacteria [108] and tymovirus [109] transfection.

4.2.2 Scale-Up

To be able to scale up the design the group must develop a system to contain the cells that will allow the nutrient exchange but keep them separate to avoid contamination. Several concepts of this design were created and are shown below.

4.2.2.1 Simple Pump

The first design the group considered was keeping both cells separate with two vats connected with pumps, pictured below in Fig. 4.1. This design, at its core, is likely the simplest and easiest to design and build due to its simplicity. The main drawback to this design is its maintenance with many parts that require cleaning such as the membranes and pumps.

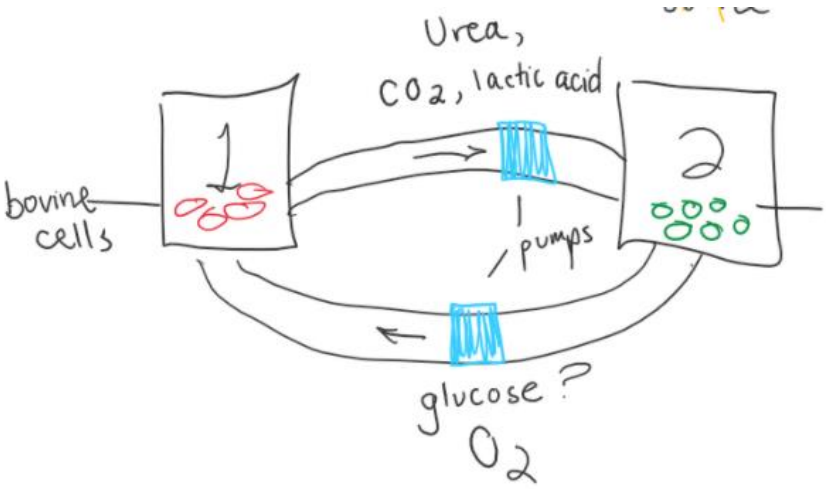

Figure 4.1: Simple Pump Rough Sketch

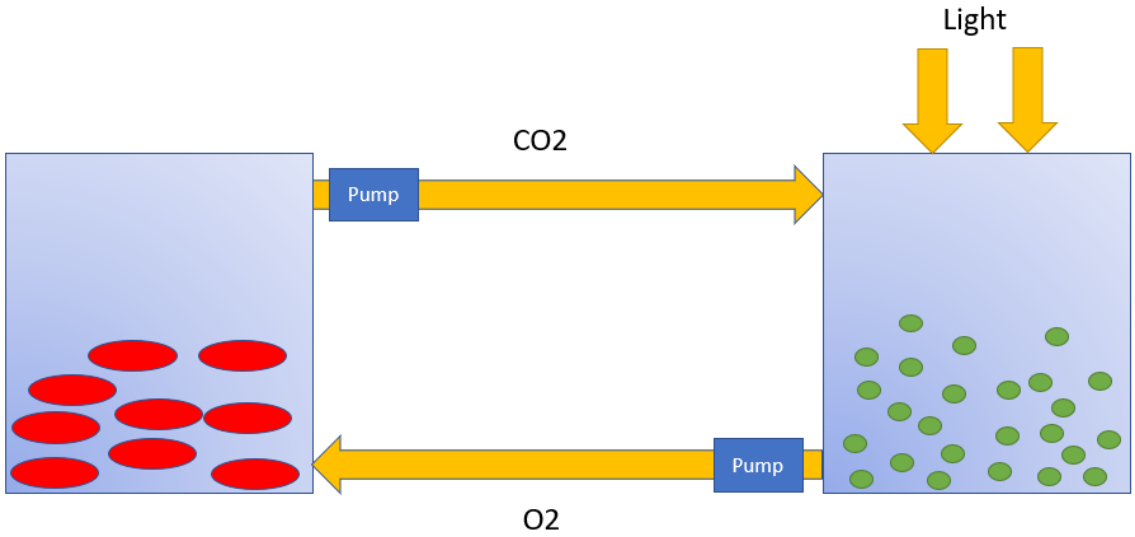

Figure 4.2: Finalized Simple Pump Design

4.2.2.2 Hourglass

The second design considered was an hourglass design that replaces the two pumps with a gravity-fed transfer system. A semipermeable membrane separates the two containers in the center, which allows the byproducts to pass through. The hourglass will also be on a motorized spinning wheel, to avoid the buildup of waste on one side and allow even transfer. This design is a little out of the box and would hopefully be little maintenance, but the biggest issue is how the PBSCs would be harvested.

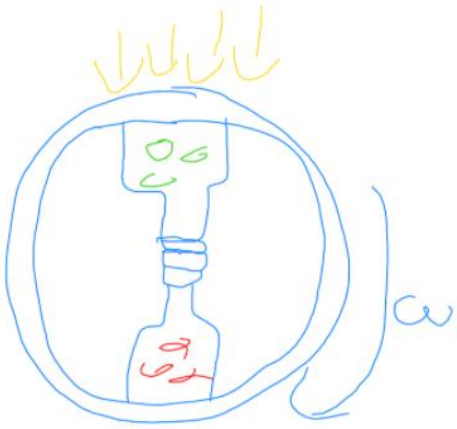

Figure 4.3: Sketch of the Hourglass Design

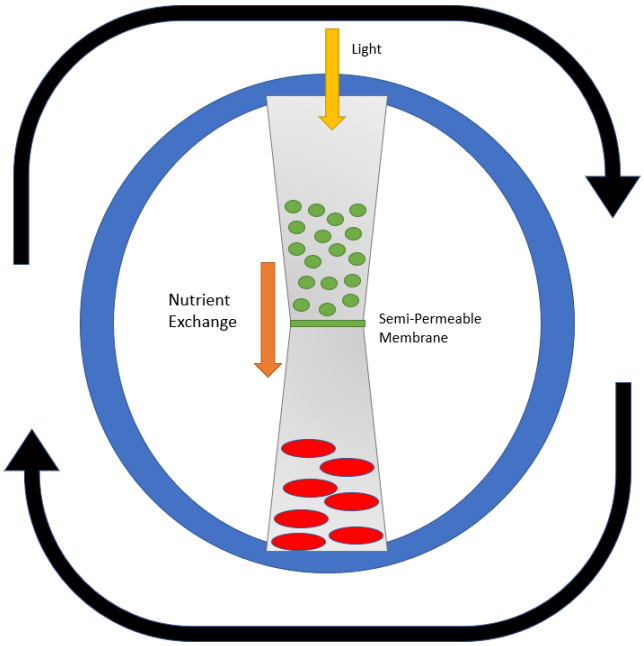

Figure 4.4: Final Design of Hourglass

4.2.2.3 Hollow Fiber

Hollow Fiber bioreactors are already widely used in cell culture making them a prime option for this project. The idea is to pump the algae cells in DMEM around the bioreactor, where the algae cells enter a cartridge. This cartridge allows the flow of algae to separate into many different capillary tubes, with PBSCs on the outside of these tubes. A semipermeable membrane separates the two cells which will keep them from contaminating each other but allow the exchange of the CO_2 and O_2. This design concept is well established and has been studied extensively.

4.2.2.4 Teabag

This design is unique in that the cells are grown in the same vat and mostly stay there together. The algae cells would sit inside a semipermeable membrane packet, like a teabag, and would allow the nutrient exchange into the PBSCs surrounding the packets. This idea is like how tea diffuses from leaves into water to make tea, which inspired the name.

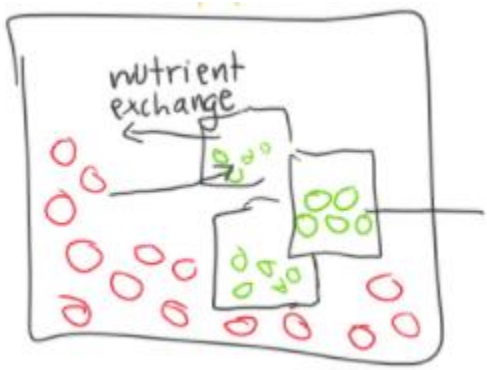

Figure 4.5: Sketch of the Teabag Design

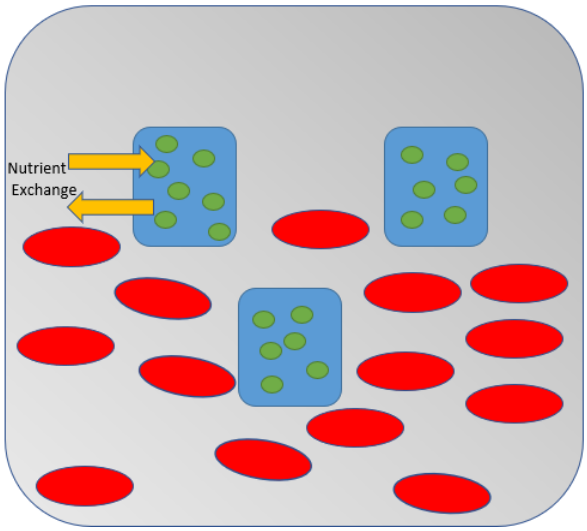

Figure 4.6: Finalized Image of Teabag Design

4.3 Final Design Selection

To choose a final design, a custom value analysis matrix was used to compare each conceptual design. This considered the needs and weights stated in section 4.1 and how well each design satisfied the needs.

4.3.1 Co-culture System

To choose a cell species for co-culture with PBSCs, a value analysis matrix was used to compare the proposed co-culture cells. Each species was rated on a scale from 1 to 5 based on how well it satisfied each objective. The satisfaction score was multiplied by the weight, and

each of these values was added together for a total score. The highest score indicates the design that best fulfilled the needs, taking relative importance into account.

Table 4.3: Value Analysis Matrix for Evaluating the Co-Culture System

		Chlamydomonas reinhardtii		*Arthrospira platensis*		*Arabidopsis thaliana*	
Need	Weight	Satisfaction	Total	Satisfaction	Total	Satisfaction	Total
Metabolize CO_2	5	5	25	5	25	5	25
Uptake Ammonium	5	4	20	4	20	3	15
Uptake Lactic Acid	5	0	0	0	0	0	0
Overlap of Temp. Requirement	4	3	12	3	12	2	8
Overlap of pH Requirement	4	5	20	3	12	2	8
Well Documented	3	5	15	4	12	5	15
Transfectable	2	4	8	4	8	4	8
Total			**100**		**89**		**79**

Based on the results of the value analysis matrix, *Chlamydomonas reinhardtii* best fulfills the design needs for the co-culture system. *Arthrospira platensis* was promising, but the high pH requirement was its most detrimental factor. *Arabidopsis thaliana* also had issues with pH, as well as temperature, and its ability to uptake ammonium is not very promising. None of the proposed cell species could uptake lactic acid, which suggests that a second cell species may be necessary for further development of the co-culture system.

4.3.2 Scale-Up

Shown in Table 4.4 is an analysis of the group's several scale-up designs. Based on the scale-up objectives laid out above in Table 4.2, each design was scaled on how much it satisfies each objective and given a score according to the importance of that objective.

Table 4.4: Value Analysis Matrix for Evaluating the Scale-Up Design

		Simple Pump		Hourglass		Hollow Fiber		Teabag	
Need	Weigh t	Satisfaction	Total	Satisfaction	Tota l	Satisfaction	Total	Satisfaction	Total
PBSC Harvestable	5	4	20	4	20	5	25	4	20
Durability	3	3	9	3	9	5	15	4	12
Co-culture Separate	4	5	20	4	16	5	20	4	16
Temp Control	3	5	15	2	6	3	9	2	6
Scaffold	4	4	16	3	12	5	20	4	16
Scalability	5	5	25	2	10	5	25	4	20
Total			**105**		**73**		**114**		**90**

Based on the totals given in Table 4.4, the hollow fiber bioreactor design satisfies the objectives the most. The simple pump design is very close to the hollow fiber design, due to its simplicity and ease of scalability, but the hollow fiber is a more solid and widely used design.

Chapter 5. Final Design Verification

Section 5.1 Standard Laboratory Procedures

5.1.1 PBSC Feeding

PBSCs were regularly maintained according to the volume of the culture flask. For T-25 flasks of PBSCs, after aspirating media, cells were fed with 5 mL of DMEM + 10% FBS, 1% penicillin/streptomycin, and growth factors. For T-75 flasks of PBSCs, after aspirating media, cells were fed with 11 mL of DMEM + 10% FBS, 1% penicillin/streptomycin, and growth factors. Cells were fed one day after passaging, and every 2-3 days beyond the initial feeding until the next passage. See Appendix A.

5.1.2 PBSC Passaging

The purpose of PBSC passaging is to either increase the cell population for experimentation or to prevent PBSC contact inhibition. PBSCs were passaged once the cells reached around 70% confluency. The PBSC passaging protocol is detailed in Appendix A.

5.1.3 PBSC Thawing

Frozen PBSCs were removed from liquid nitrogen and immediately thawed according to the detailed protocol in Appendix A. The cells were taken from the same cow sample for each thaw, except for the final reader experiment, results from these cells are shown in Section 5.3.3.

5.1.4 *C. reinhardtii* Maintenance

C. reinhardtii maintenance was carried out to ensure a healthy population of microalgae for experimentation. The *C. reinhardtii* culture was maintained on a shaker in an incubator at 27℃ with a 12-hour light cycle. The health of the algae was examined by the coloration of each flask, with deep green indicating a healthy culture with minimal death and high proliferation, and a bright yellow indicating more cell death. See Appendix B for more information on algae culture maintenance.

5.1.5 Transwell Experiments

The Transwell experiments conducted to verify the feasibility of algae and PBSC co-culture utilized 6-well plates with Transwell inserts (VWR, Corning). The protocols used for these experiments are included in Appendix D.

5.1.6 Oxygen Evolution with Hill Reaction

The hill reaction is an oxidation reaction that quantifies the amount of a colorimetric indicator, DCPIP, which is oxidized by a sample. The goal of the hill reaction for our purposes was to quantify exactly how much oxygen is evolved by the algae in different media conditions and at different concentrations. This would determine the concentration of algae needed to fulfill the oxygen requirements of the PBSCs, without oversaturating PBSC cultures with oxygen. Multiple different protocols were developed, however there were no conclusive results from the concentrations of algae per volume studied. The hill reaction results were no longer deemed necessary when new equipment was obtained by the lab which provided direct readings of

dissolved oxygen and pH in any cell culture flask. The protocol which achieved inconclusive results is included in Appendix C.

5.1.7 Staining

Hoechst stains were conducted on PBSCs to help with cell counting and imaging upon conclusion of co-culture experiments with algae to help define the two cell types from each other. The Hoechst staining protocol is detailed in Appendix E.

A live/dead stain was used to identify the viability of PBSCs after co-culture with algae cells for three days. Live/dead stains were conducted on PBSCs for cell imaging of viable cells using Calcein AM and Ethidium homodimer-1 (Invitrogen) according to protocols available from the product manufacturer. See Appendix F for protocol details.

5.1.8 ID Reader Set-up

Readers and biosensors were supplied from Scientific Bioprocessing (SBI). The two readers we used for data collection required setup and calibration per the manufacturer instructions which are present in Appendix G. Biosensors for both dissolved oxygen (DO) and pH were placed in experimental flasks according to manufacturer instructions (Appendix G).

Section 5.2 Determining Characteristics of Monoculture

5.2.1 PBSC and *C. reinhardtii* viability and growth in various media compositions

Before growing the two cell types of interest together and assessing their growth in a co-culture environment, it was important to identify whether each cell type could survive in conditions that met the needs of both cell types. In other words, we needed to determine the ideal culture conditions which would proliferate PBSCs without differentiation, and in which algae cells could also survive and undergo photosynthesis.

Initial experiments were conducted to compare the growth of PBSCs in DMEM, the ideal growth medium of PBSCs, and in TAP, the ideal growth medium of *C. reinhardtii*. Passage 4 PBSCs were plated at 10,000 cells per well in a 6-well plate and various ratios of DMEM and TAP were used as the media. The cells were grown at 37℃, with 20% O_2, and 5% CO_2. Unfortunately, all trials were contaminated due to an issue with serum contamination and results were inconclusive although they suggested that PBSCs could not survive in higher ratios of TAP, since no nutritional needs were met by the contents of TAP medium for PBSC growth. It was clear that DMEM should be the growth medium if our objective were to proliferate PBSCs.

Similar to the goal of the previous experiment, the next experiment aimed to test the viability of *C. reinhardtii* in DMEM compared to TAP. The algae were plated in a 6-well plate at 10,000,000 cells per 3 mL well at 30℃, with 5% O_2 and 5% CO_2 in a 12-hour on/12-hour off light cycle. Two of the wells had DMEM media and the other two wells had TAP media. The initial cell count was recorded, and the final cell count was recorded after four days of culturing. The results showed that the algae population increased significantly in TAP from about 3 million cells/mL to nearly 14 million cells/mL and remained at about the same population size when in DMEM, specifically decreasing by about 33,000 cells/mL (Fig. 5.1).

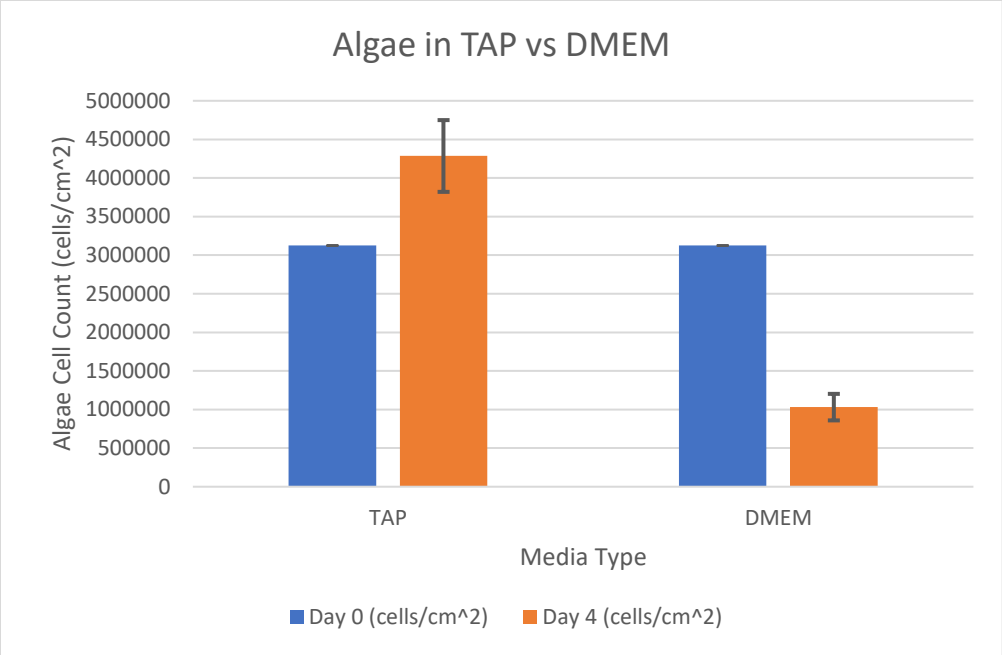

Figure 5.1. Comparison of *C. reinhardtii* growth in TAP media and DMEM media after four days of culture.

As seen in the bar chart of Fig. 5.1, *C. reinhardtii* was unable to exhibit any significant growth in the DMEM media in four days, however, upon examination of the algal cells, it was determined that the algae remained viable despite not being able to proliferate. The viability of the algae was assessed based on the color, movement, and "clumpiness" as dead algae forms clumps in floating media. An image of the two treatments of TAP and DMEM is displayed in Fig. 5.2 below.

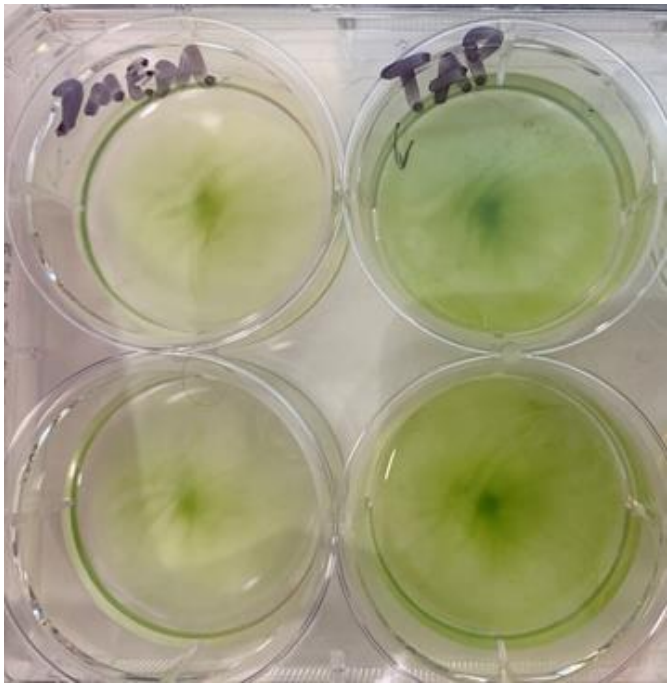

Figure 5.2: Algae media comparison in DMEM and TAP
Image taken after 2 days in at 30°C, with 5% O_2 and 5% CO_2 in a 12-hour on/12-hour off light cycle. The left column shows algae in DMEM, while the right shows algae in TAP.

5.2.2 Experiment with Algae Agitation

Once it was determined that *C. reinhardtii* was able to remain viable in DMEM, our group wanted to investigate whether the algae required constant agitation for successful proliferation. This would determine whether we needed to culture algae separately from PBSCs since PBSCs needed consistent, undisturbed conditions to properly adhere to the plate surface and remain viable. The stock cultures were known to proliferate best in Erlenmeyer flasks on shaker platforms at larger volumes of 50 mL or more. The angled sides of Erlenmeyer flasks ensure equal distribution of nutrients on a shaker platform. In this experiment, *C. reinhardtii* was cultured in a 30℃ incubator in TAP, 5% O_2, 5% CO_2, with 500,000 algae initially plated on a 6-well plate. The experiment was done with 2 replicates with one biological sample. The 6-well plate design was not as conducive to algae cell growth but was conducive to PBSC growth since 6-well plates are tissue-culture treated and provide a consistent flat surface to which cells can adhere. The sides of the 6-well plate are shallow and thus placing the plate on a shaker would potentially shake media out of the plate. Thus, the small magnetic stir bars provided agitation without the risk of losing media from the plate. Half of the 6-well plate contained stir bars, and the other half did not contain stir bars as the control as seen in Fig. 5.3.

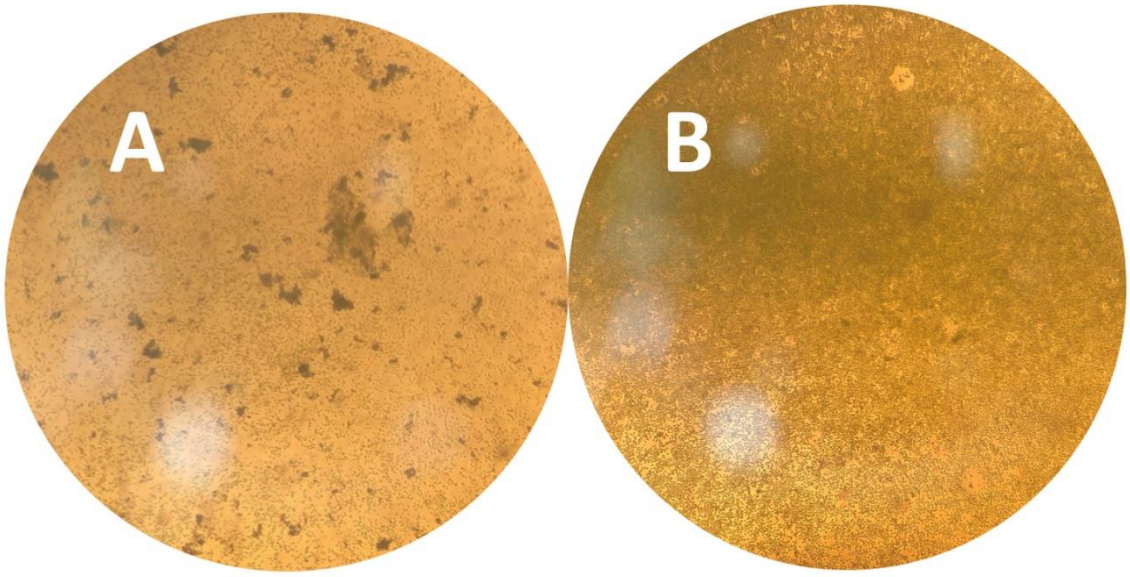

Figure 5.3: (A) *C. reinhardtii* culture after 48 hours with constant stir bar agitation. (B) *C. reinhardtii* culture after 48 hours without any stir bar agitation.

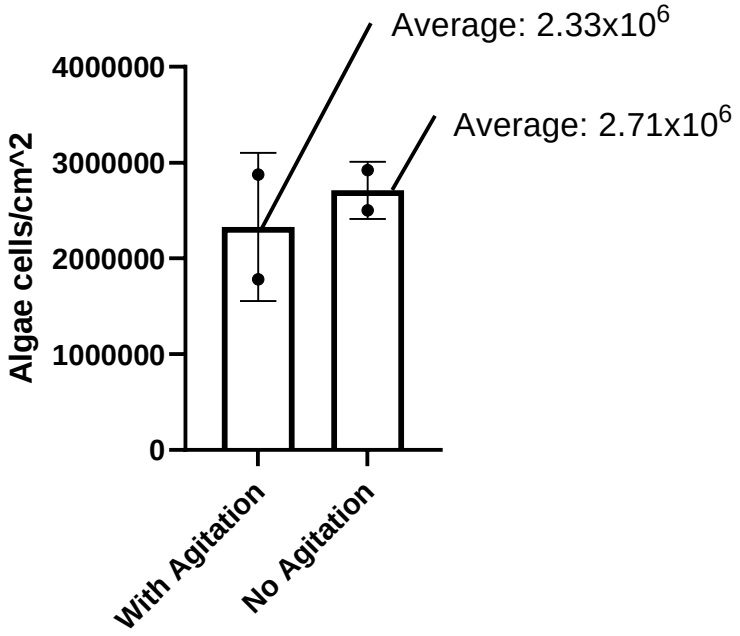

Figure 5.4: Graph of cell counts for algae agitation experiment (n=2)

After determining the ideal media conditions and whether algae required agitation for proper cell growth, we aimed to evaluate if PBSCs could tolerate the light conditions needed for algae survival and photosynthesis. Literature suggested that light conditions would not be specifically detrimental to PBSCs, although regular physiological conditions do not provide the cells with light [110]. Additionally, the optimal temperature of the co-culture would have to be experimentally determined in the next experiments to facilitate both algae and PBSC growth.

Our next tests aimed to synthesize the findings from the separate growth cultures and apply the newfound knowledge about ideal culture conditions to an algae-PBSC co-culture.

Section 5.3 Creating a Co-culture System

5.3.1 First co-culture experiment

In the first co-culture experiment, the PBSCs and *C. reinhardtii* were seeded in a T-75 flask in DMEM with no IGF at 37℃, with 20% O_2 with no growth lights. The aim of this experiment was to investigate whether the cell types could be cultured together without physical separation as well as the temperature requirements for the co-culture system. Literary research has suggested that *C. reinhardtii* will not function normally at 37℃ due to heat stress and that 30℃ is the maximum temperature that still allows *C. reinhardtii* to be metabolically viable. Our group demonstrated the effects of heat stress on algae experimentally. Fig. 5.5 shows the algae with a darker brown color, which is indicative of heat stress or a lack of light [5, 110].

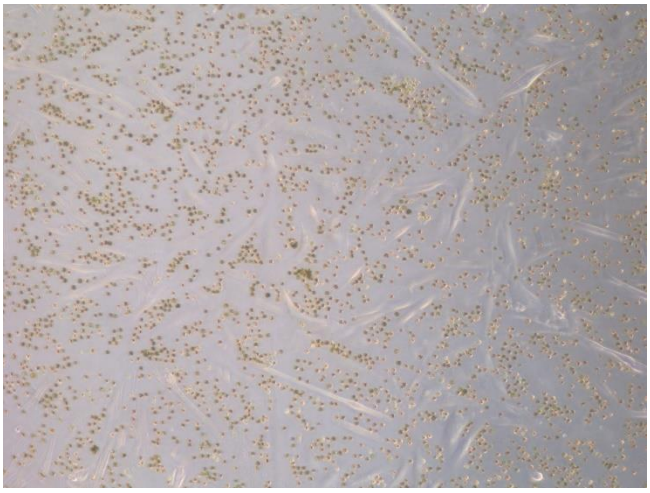

Figure 5.5: Image of the co-culture on Day 5 at 20x magnification

Upon examination of the PBSCs, it was determined that the cells did not appear to exhibit any differentiation or stress responses as a result of direct co-culture with algae cells. This experiment was not held in hypoxic conditions, so it cannot be concluded that algae contributed significantly to the successful proliferation of PBSCs. From this crude example of the effects of co-culture of algae and PBSCs, the group needed to further characterize the effects of algae on PBSCs at a molecular level and determine if there were more ideal conditions that would support the growth of algae in addition to PBSCs. Certainly, moving forward, the growth conditions needed to be more amenable to the needs of algae, specifically with temperature and light conditions.

5.3.2 Transwell Experiments

The goal of the experiment was to compare the growth rates and viability of PBSCs in co-culture, in hypoxic conditions, and with various concentrations of algae. This would determine the ideal concentration of algae needed to fully support the oxygen requirements of PBSCs and adequately recycle CO_2 produced by PBSCs. This would also determine if there were any toxic effects on PBSCs with higher or lower concentrations of algae. From previous experimentation with algae and PBSCs, we determined that a ratio between 1 to 50 PBSCs to algae cells and 1 to 200 algae cells provided significant oxygen to the system. Additionally, it was determined that the PBSCs would grow best on the tissue-treated plate rather than the top well insert of the Transwell. This experiment tested the effect of no algae, a 1:100 ratio of PBSCs to algae, and a 1:200 ratio of PBSCs to algae. The cells were plated in a 6-well Transwell plate with the algae in the top well insert and the PBSCs in the well of the plate at 50,000 PBSCs per well. The total algae plated in the 1:100 ratio wells was 5,000,000 algae cells, and the total algae plated in the 1:200 ratio wells was 10,000,000 algae cells.

After three days, the well inserts containing the algae were removed. It was noted that some algae cells were small enough to fit through the filter of the Transwell insert and entered the culture directly with the PBSCs. This is noted by the green arrows in Fig. 5.6. The lower wells containing the PBSCs only were stained with Hoechst stain to identify individual cells. The samples were fluorescently imaged with a 4X objective, and the images shown in Fig. 5.6A-C are representative of an 8X magnified image. There were 4 images taken from each well to count and identify PBSC concentrations. Representative images are shown in Fig. 5.6. Appendix I

includes the calculation of the PBSC cell count from the images taken on the fluorescent microscope and its quantification can be seen in Table 5.1.

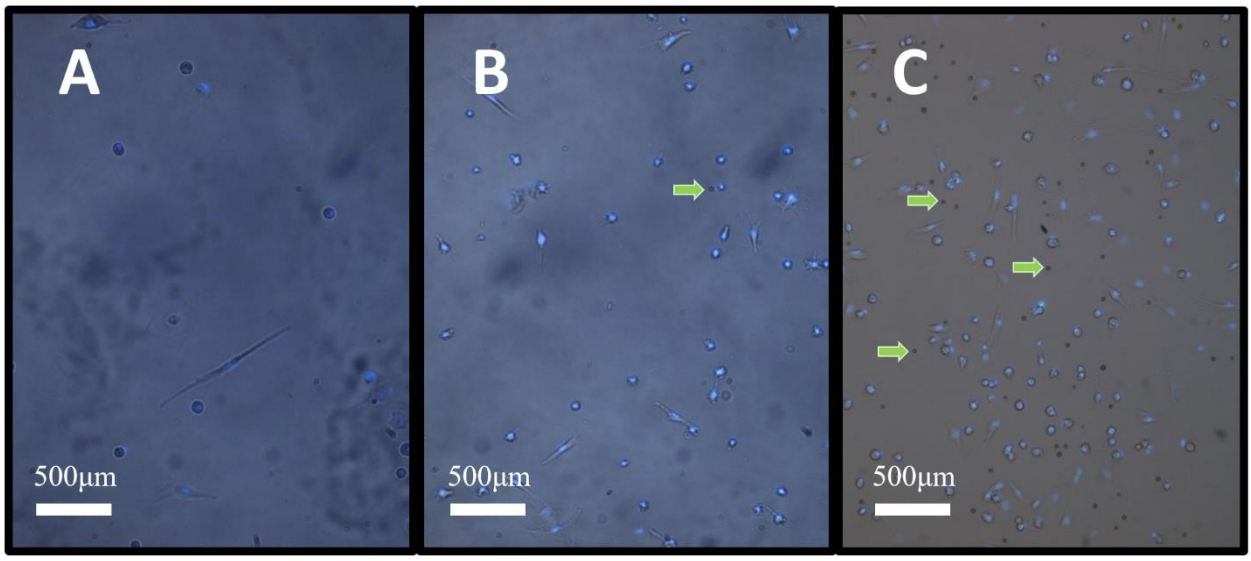

Figure 5.6 A-C: Representative images from the Transwell Hoechst stain (Left to right: no algae, Algae 100:1, Algae 200:1). Blue fluorescence represents the stain of DNA in PBSCs. Green arrows point to examples of algae cells in the images within panels B and C.

Table 5.1: Average number of PBSCs in varying PBSC - *C. reinhardtii* ratio with standard deviation

PBSC:Algae Ratio	Total PBSCs/cm^2	Attached PBSCs/cm^2	Detached PBSCs/cm^2
1:100	61220 ± 32297	24242 ± 12960	36979 ± 20145
1:200	122029 ± 78616	45196 ± 28239	76833 ± 50687
1:0 (control)	31637 ± 18498	12326 ± 9187	19311 ± 10418

Table 5.1 is a quantification of the average number of PBSCs in the fluorescent microscopy pictures for the varying PBSC - *C. reinhardtii* ratios. The data gathered in Table 5.1 was compiled through an experiment with two samples for each ratio with two pictures per well. The data obtained from the pictures illustrated that the 1:200 ratio contained the highest number of PBSCs. In all three ratios, there seemed to be more detached PBSCs than attached PBSCs. However, since no live-dead staining was made, no conclusion can be drawn regarding the viability of the PBSCs in the co-culture system. From Table 1, our group was able to determine the size of each of the images and thus estimate the total number of PBSCs in a well after three days of co-culture as seen in Fig 5.7.

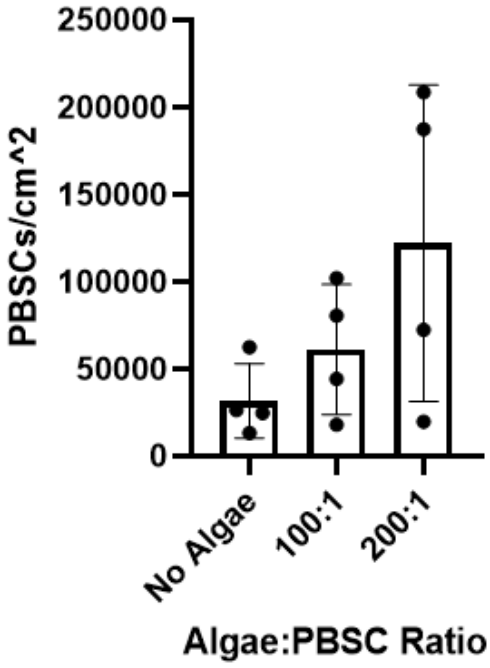

Figure 5.7: Estimated number of PBSCs in a Transwell after three days of co-culture at varying ratios.

The cell counts in Fig. 5.8 are representative of 4 counts taken from different images of different locations in each well plate (See Appendix H for other images). The PBSCs appeared to proliferate at higher rates in the 1:200 PBSC to algae ratio. Fig. 5.8 illustrates the estimated number of PBSCs per well in the three different ratios. Compared to the initial seeding density of 50,000 cells, the hypoxic conditions in DMEM without algae caused cell death since the final cell count after 3 days was only 30,380 cells. In the 1:100 PBSC to algae ratio, the final cell count was 58,787 suggesting a minor increase in proliferation but a lack of significant cell population growth. With the 1:200 PBSC to algae ratio, the cell count was 117,180 cells which are more than double the initial seeding concentration of PBSCs. This suggests that the increase in the concentration of algae cells in relation to PBSCs has a positive impact on the outcome of PBSC viability.

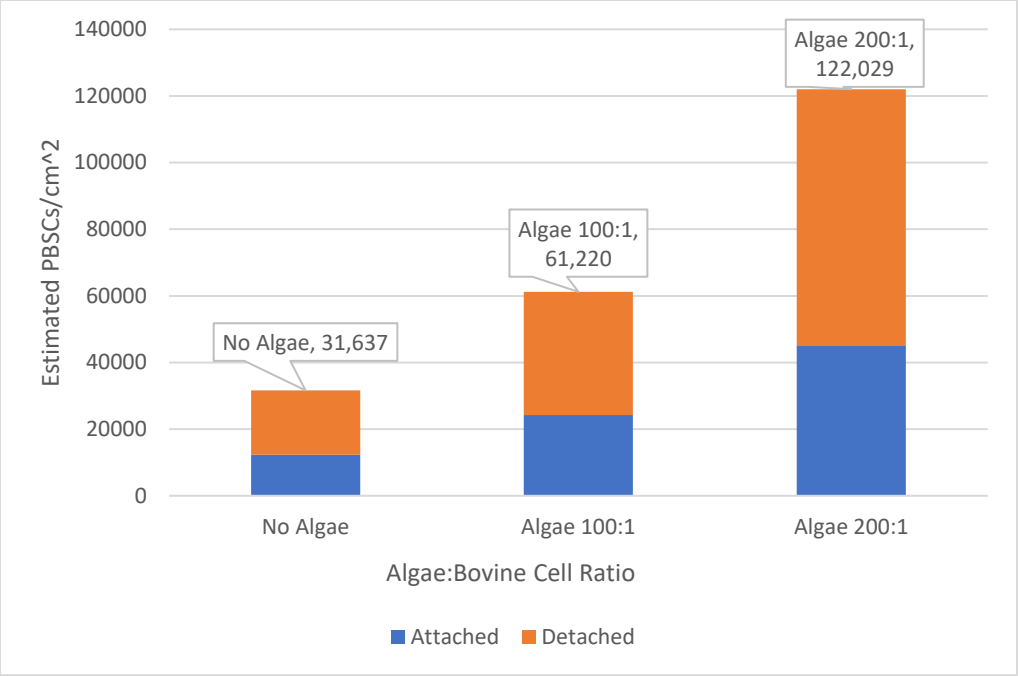

Figure 5.8: Estimated counts for PBSCs with different Algae:PBSC ratio, including attached and detached cell counts (n=4). Total PBSCs counted per condition, including both attached and detached, are labeled at the top of each bar.

5.3.3 Percent Dissolved Oxygen and pH Reader Experiments

In this experiment, we aimed to again determine the viability of the PBSCs in a direct co-culture system by performing live/dead staining. The direct co-culture was conducted in a T-25 flask in the same conditions as the Transwell experiment from 5.3.2, however, this experiment tested the effects of direct interactions between algae and PBSCs indirect co-culture. This would demonstrate the ability of algae cells to grow in direct contact with PBSCs to assess if there were any issues with contact inhibition for either cell type. The viability stain at the end of the three days of co-culture would determine if there were significant impacts of co-culturing PBSCs with algae compared to PBSCs growing without algae in monoculture. Due to the limitation on the number of readers, the sample size of each reader experiment was one. However, the experiment was conducted three times to ensure a detailed analysis.

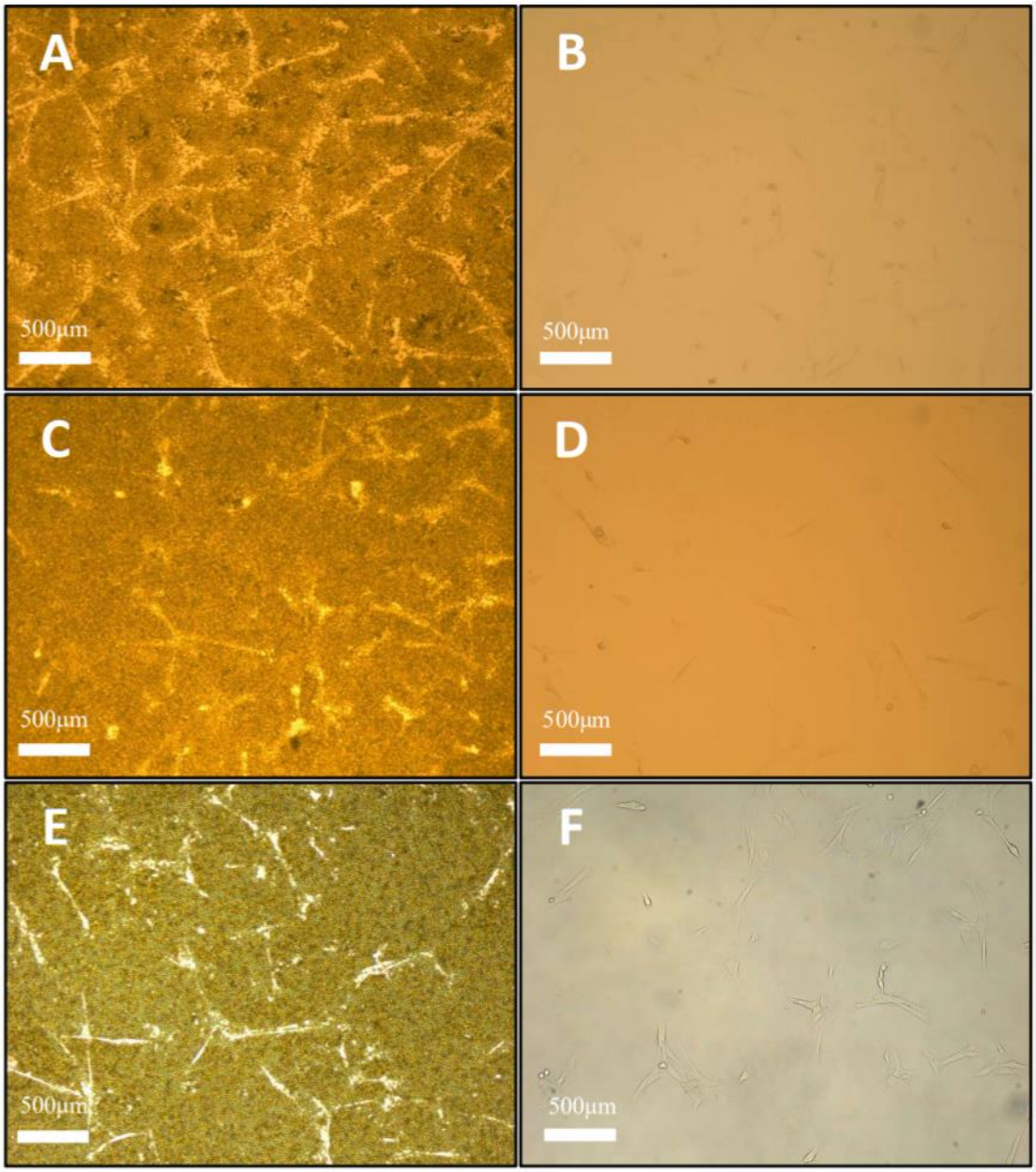

Figure 5.9: All photos are shown at 20x magnification. Co-culture with PBSCs and algae cells at (A) Day 1, (C) Day 2, (E) Day 3, and monoculture at (B) Day 1, (D) Day 2, (F) Day 3.

There was an issue with the live/dead staining of the cells which caused the PBSCs to detach from the culture flask. The cause of this is unknown, however, the stain still showed that cells were viable despite detachment from the flask.

The images in Fig. 5.9 were taken on Day 1-3 of the experiment. The algae cells grew well in direct contact with the PBSCs, and the images of the co-culture show that the PBSCs have proper morphology indicative of proliferation. The live dead staining determined that the viability, regardless of whether cells were detached or attached, was at least 90% in the 200:1 algae ratio (Fig. 5.10).

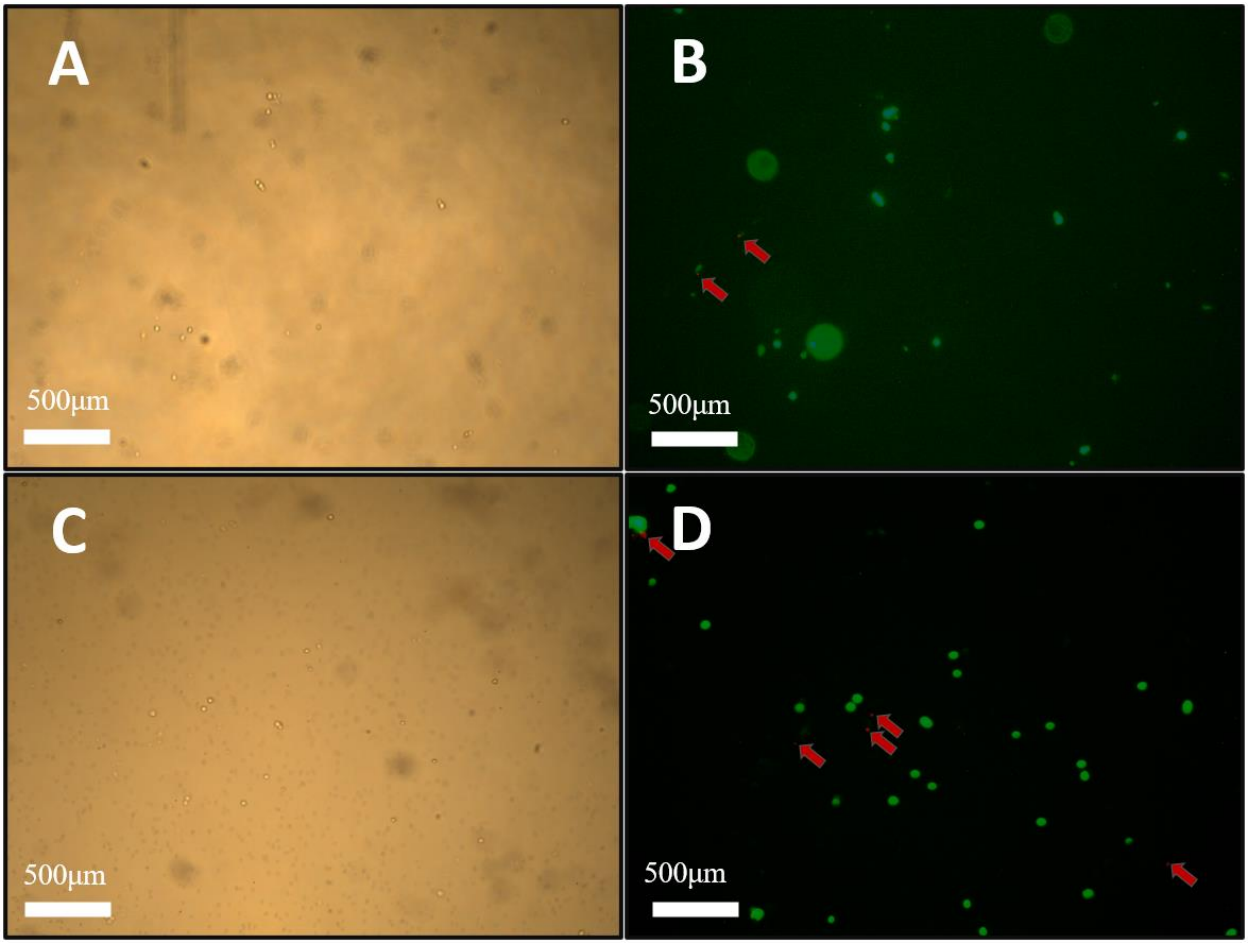

Figure 5.10: Live/Dead stain of monoculture (A, B) and co-culture (C, D) at 8X. Brightfields are shown in A and C of the respective fields of view images in B and D. Panels B and D show the viable cells in green, and dead cells in red. Arrows point to dead cells in red.

To determine the effects of *C. reinhardtii* in a co-culture system with PBSCs, our group has decided to quantify the percentage dissolved oxygen (DO) and the pH over a 10-day period. In this experiment, both the co-culture and the monoculture control were cultured with a 12-hour light cycle at 30°C under hypoxic conditions with a PBSC seeding density of 50,000 cells per well. The ratio of PBSCs to *C. reinhardtii* in the co-culture system was 1:200 as determined in the earlier experiments to be the most optimal ratio. The comparison of the percentage change DO concentration and pH can be seen in Fig. 5.11 and 5.12 respectively.

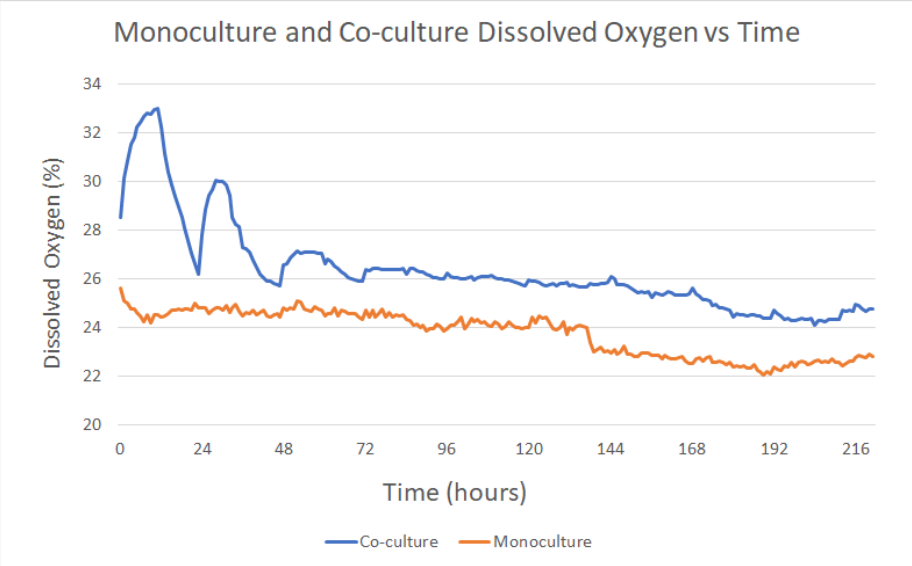

Figure 5.11: Comparison of the % dissolved oxygen in the monoculture and co-culture system over a 10-day period.

As seen in Fig. 5.11, the co-culture exhibited increased DO concentrations when compared with the monoculture's DO throughout the 10-day incubation period. This suggests that the presence of *C. reinhardtii* in the co-culture system has resulted in an increase in the percentage of dissolved oxygen in the system through photosynthesis. It should also be recognized that after three days, the algal cells did not seem to be as productive in generating oxygen for the system. This is an important finding to note when considering the scale-up of the bioreactor on whether the *C. reinhardtii* should be replenished with nutrients or replaced after three days to maximize efficiency.

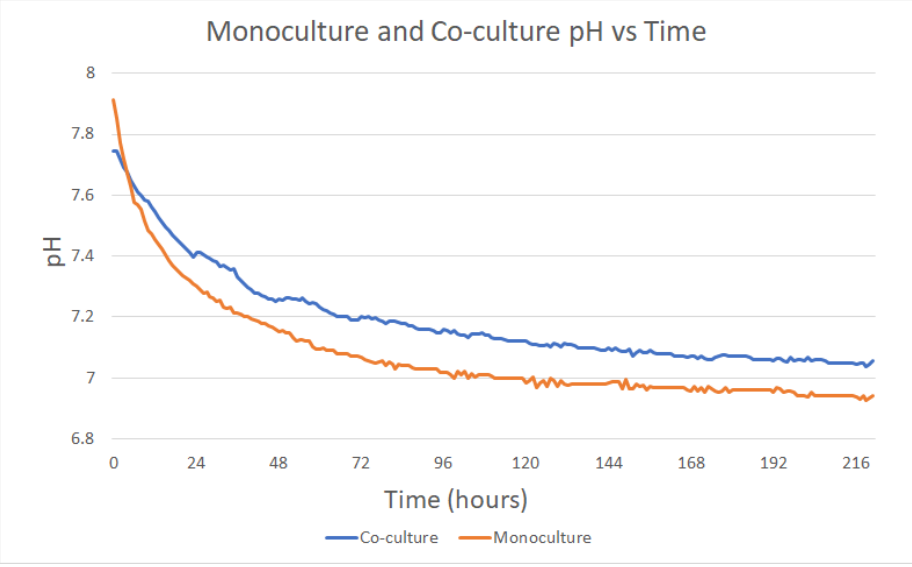

Figure 5.12: Comparison of the pH in the monoculture and co-culture system over a 10-day period.

Fig. 5.12 illustrates a comparison of the pH values of the co-culture with the control over a 10-day period. The data in Fig. 5.12 suggests that the co-culture was able to dampen the decrease in pH over the 10-day culture period. There are multiple possible explanations for this

observed phenomenon. Our team hypothesized that the gradual decrease in pH could be attributed to uptake of CO_2 through photosynthesis, affecting the sodium bicarbonate buffer in the DMEM media. Another possible explanation could be a decrease in lactic acid production due to an increase in aerobic respiration from an increase in the DO concentration.

The data obtained from the two other reader experiments were deemed to be unpresentable due to human and random errors. In the first reader experiment, the graph had multiple asymptote-like spikes because the flasks were removed from the readers periodically for imaging, resulting in a slight misalignment between the sensor and the readers. The results obtained from this experiment can be seen in Appendix J. The data gathered from the other experiment was incomplete due to multiple unexpected shutdowns of the computer. The group decided that the random fragments of the data were not representative of the experimental results and therefore excluded from the analysis.

Chapter 6. Final Design Validation

Section 6.1 Final Design

The final design had two main aspects. The first is the co-culture system, which comprises the cell types, culture medium, temperature, and other culture conditions that will be discussed in further detail. The second is the scale-up, which is the proposed bioreactor design for large-scale production using our co-culture system. Both aspects will be discussed further in the following sections.

6.1.1 Co-culture System

The co-culture system uses the microalga *C. reinhardtii* to uptake metabolic byproducts of PBSC culture and replenish the media with oxygen. The system is closed to gas exchange, so all additional oxygen is supplied by *C. reinhardtii*. The co-culture is seeded at a 200:1 ratio of *C. reinhardtii* to PBSCs in DMEM supplemented with 10% FBS, 1x P/S, and growth factors (see Appendix K for full media making protocol). The cells are grown at 30°C, which slows down the metabolic activity of PBSCs but allows *C. reinhardtii* to function at an ideal temperature. The co-culture is grown with a 12-hour light cycle to allow *C. reinhardtii* to undergo photosynthesis while allowing the PBSCs to grow for 12 hours in their ideal conditions.

6.1.2 Scale-up

The scale-up of the co-culture system will involve the use of a hollow fiber bioreactor. Hollow fiber bioreactors are widely used for cell culture and have lots of different models and size versions, which is perfect for the plan of scale-up of our system. The group did several instances of modeling and calculations for the bioreactor, as discussed below.

6.1.2.1 Hollow Fiber Reactor

For the hollow fiber bioreactor, the PBSCS will grow on the outside of each fiber. There are 100 fibers in this chamber, with about 80% of the surface area covered with PBSCs. The *C. reinhardtii* cells will flow through the bioreactor in the DMEM and will be separated inside the hollow fiber chamber by a semipermeable membrane. This allows the waste metabolites to transfer from the cells while keeping the two cells separate. A depiction of the cells flowing through the lumen is shown below in Fig. 6.1.

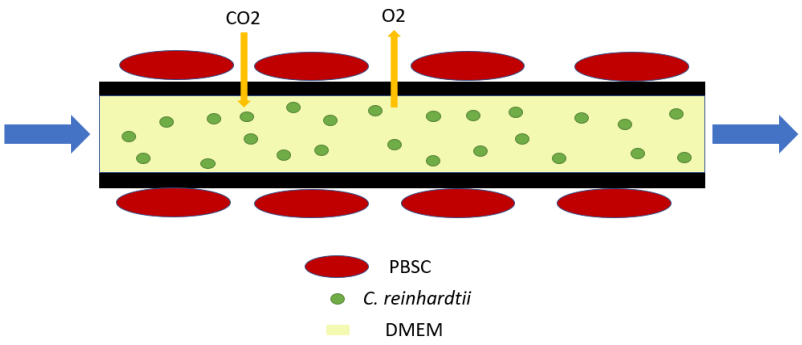

Figure 6.1: Cross-section of the Cells Flowing Through a Hollow Fiber

The process is driven by a pump pushing the DMEM throughout the system. There will also need to be a light source for the algae cells to allow for photosynthesis of the cells to occur. A process flow diagram of the model can be seen below in Fig. 6.2. SBI sensors measuring pH and dissolved oxygen (DO) will also be utilized for monitoring the status of the system. One sensor will be placed just before the media enters the hollow fiber chamber and one at the end of the chamber. This can show how each pass through the cartridge changes the DO and if the system can stay at a steady state of oxygen.

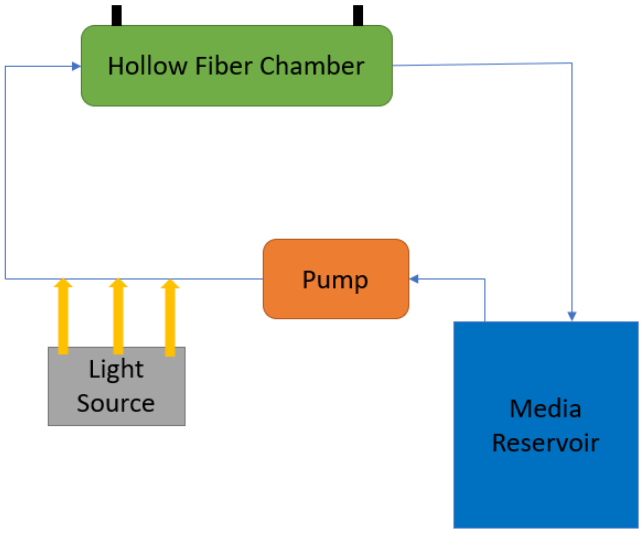

Figure 6.2: Process Flow Diagram of the Bioreactor

6.1.2.2 Hollow Fiber Model

To further model the bioreactor, COMSOL was used to model the diffusion of oxygen inside the lumen. The diffusion rate of oxygen was found to be $2x10^{-5}$ cm^2/s [111]. Although this is the value used for oxygen diffusion in water, there was little research into the diffusion rate of oxygen in DMEM or other media, and the group did not have time to test for it. Using this information, the model was created using a boundary value problem of a constant flow rate with no advection or convection in the system. Below is a cross-section of the diffusion of the oxygen in the lumen of a single hollow fiber. Using this model, the group was also able to determine the inlet concentration of oxygen, which is 0.22 mol/m^3.

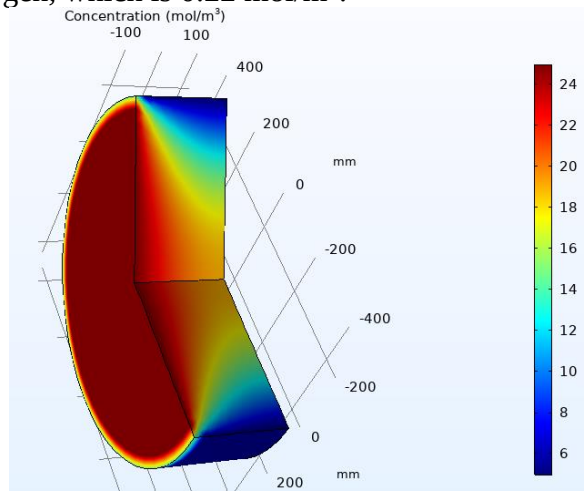

Figure 6.3: Model of Oxygen Diffusion in the Lumen

6.1.2.3 Governing Equations

To begin with the general calculations of the bioreactor, we first needed to make some assumptions. First. the group assumed the flow rate was constant and there is no advection in the system, as stated above in section 6.1.2.2. The flowing media and algae cells are well mixed for this problem, meaning the algae cells are assumed to not settle at the bottom of the media reservoir. The group also assumed that 80% of the surface area of the fibers was covered in PBSCs, with a 200:1 ratio of algae to PBSCs. We are also currently neglecting the replication rate of both cells. The Navier-Stokes equation, along with the continuity equation, were also good governing equations used for finding flow velocity from density and viscosity.
The fibers are each 0.2 meters long and have a diameter of 200 micrometers while the hollow fiber membrane will have an outer diameter of 400 micrometers, also 0.2 meters long, a pore size of 0.1 microns, and be made of polypropylene. Polypropylene is a durable, cost-effective material to use for the semi-permeable membrane and does not contain BPA like some plastics. This means that polypropylene is non-toxic to humans and safe to use in a cell culture system for eventual human consumption.

We calculated the surface area of one fiber to be 252 m^2, which means 201.6 m^2 is covered in PBSCs since we assumed a maximum of 80% of the surface to be covered in PBSCs. The area of a bovine cell is about 500 um^2, which means 403,200 PBSCs are attached to each individual fiber. Since our system uses 100 fibers, this means there are a total of 40,320,000 PBSCs and 8,064,000,000 algae cells within the bioreactor system initially. Using the minimum oxygen concentration in the hollow fiber bioreactor and the concentration of oxygen at the inlet calculated from COMSOL, along with constants in the analytical operating equation, the reduced Peclet number for the fiber was calculated. The Peclet number can then be used to determine the velocity inside the lumen [112].

The inside velocity of the lumen was calculated to be about 1.2 centimeters per second. From the velocity, the group calculated the overall flow rate of the system to be 3.77 um^2/s. Thiscauses the *C. reinhardtii* cells to be subjected to a shear force of 1.536 pascals (Appendix L). Further research will need to be conducted on whether the cells can withstand this shearing force. We will also need to conduct further research into the oxygen consumption rate of PBSCs, to determine whether this system will ever need oxygen pumped into the chamber to help make up for the loss of dissolved oxygen as the *C. reinhardtii* cells die. This likely shouldn't be the case if the media is changed every 4 days, which already doubles the current lifetime of cell culture media, as evidenced from our SBI sensor data.

Section 6.2 Evaluation of Design Criteria

Multiple objectives and constraints were determined in Chapter 3 to guide the design and testing processes. This section will describe how well the final design fulfilled these objectives and constraints.

6.2.1 Objectives

6.2.1.1 Scalable

The proposed hollow fiber bioreactor can be scaled up by increasing the number of fibers and the size of the pumps, tubing, and vessels. The benefit of this design is that the hollow fibers provide a large surface area for PBSC growth with a relatively low required volume of media. These bioreactors can take up relatively little space for large-scale production.

6.2.1.2 Proliferation of PBSCs

PBSCs were able to proliferate in co-culture with *C. reinhardtii*. This was quantitatively shown in Fig 5.7. PBSCS cultured in a 200:1 ratio of *C. reinhardtii* to PBSCs proliferated more than those grown in a 100:1 ratio and those grown without algae.

6.2.1.3 Harvesting PBSCs

In our proposed bioreactor, the PBSCs and *C. reinhardtii* are separated by a semipermeable membrane. PBSCs can be removed from the scaffold surface to be harvested for differentiation.

6.2.1.4 Reduce Media Cost

The main indicator for replacing cell culture media is a drop in pH. This indicates the level of metabolic activity because higher levels of CO_2 and lactic acid will lower the culture pH. As seen in Fig. 5.12, after 48 hours of growth (typical time before changing media), the pH of the monoculture was 7.16. The co-culture grown in the same conditions reached a pH of 7.16 after 88.6 hours of growth. Based solely on pH values, the media lifetime was extended by 85%, which means the media can be replaced less frequently. Further testing is necessary to obtain an accurate value of media lifetime, but the results indicate that the co-culture system helps reduce media costs.

6.2.2 Constraints

6.2.2.1 PBSC viability

PBSCs were able to grow in co-culture with *C. reinhardtii*. As represented in Fig. 5.10, the apparent viability is high, with only a few cells stained as dead. Due to the cells detaching, a quantitative comparison of viability was not obtained. However, Fig. 5.9 shows that there were attached cells in co-culture. Future studies will be done to quantitatively confirm PBSC viability.

6.2.2.2 Maintenance of PBSC Stem-Phenotype

The stem phenotype of the PBSCs was not evaluated due to time constraints during the project. Hoechst staining of co-cultures indicated that some cells had multiple nuclei, which only happens after PBSCs have differentiated. This indicates that not all cells retained the stem phenotype, but more specific testing must be performed to assess the fulfillment of this constraint.

6.2.2.3 Chlamydomonas Viability

C. reinhardtii cultures were successfully cultured and exhibited log-phase growth at 30°C. A *C. reinhardtii* culture was sustained during a 10-day co-culture with PBSCs, indicating that they can remain viable in co-culture. Viability was not quantified, so future studies should use live/dead staining for *C. reinhardtii*. For the hollow fiber bioreactor, the effects of shear stress on *C. reinhardtii* must be explored to confirm that they will remain viable.

6.2.2.4 Chlamydomonas Functionality

The literature suggests that phenol red may inhibit the O_2 production of *C. reinhardtii*, so DMEM without phenol red was used for the co-culture system. In co-culture, *C. reinhardtii*

increased the dissolved oxygen concentration during the 12 hours when lights were on. This indicates that *C. reinhardtii* retained its metabolic activity while in co-culture grown in DMEM.

6.2.3 Evaluation of Standards

The goal of this bioreactor system is to be able to produce large quantities of cultured meat, so it is important to look at the design and manufacturing standards laid out earlier in the paper. Cell cultured meat is a generally new field and not all regulations are in place, but it is important to discuss the bioreactor standards as well as the meat product standards.

6.2.3.1 Bioreactor Standards

The hollow fiber bioreactor design meets ISO 11737-2 because this is a closed system that allows for little contamination risks. The main risk of breaking this regulation comes when changing the media or harvesting the PBSCs, which is easily mitigated with safe and sterile lab practices. The equipment can also be emptied and sterilized in an autoclave. The other design regulations can be followed with property industry safety and handling standards.

6.2.3.2 Meat Production Standards

One important USDA standard for meat production shown earlier is pathogen intervention. Section 315 states there must be at least two pathogen intervention steps. The current design of the hollow fiber bioreactor is mostly self-sufficient and has little human interaction which decreases contamination risks. There could also be a sanitization of the equipment after each use to help mitigate the risk. Gloves and other necessary equipment will also be used in a sterile environment around the apparatus to help with this too.

Another important standard is discussed in section 318.7, which is microbial testing. This mostly pertains to the meat produced by the bioreactor, and there are several ways to ensure no microbes are in the meat product. Microbial contamination changes the color of the meat, so this is one obvious way [113]. Another idea is the use of a pH sensor in the media, which can show if the media is contaminated as well. The FDA does not regulate the beef industry but does have some regulations, such as having a hazard analysis and risk preventative control guide. This is for the meat production factory owner, and not for the small-scale version our group has made. This would, however, be followed in a scaled-up factory version with a plan to mitigate risks and hazards when working with the machinery and the dangers to the public that may come from mismanaging the meat.

Section 6.3 Broader Considerations

6.3.1 Economics

Cellular agriculture must be able to compete with traditional meat production on a large scale. The cost of cell culture media in large-scale production is prohibitive, but this co-culture design can alleviate some economic hurdles. As this system is refined and combined with other cost-cutting measures, cellular agriculture can become economically feasible to meet the rising global demand for animal products.

6.3.2 Environmental Impacts

The environmental impact of meat production is evident and explained further in Chapter 2. Cellular agriculture offers a potentially sustainable alternative, but advancements in technology are necessary to make it truly sustainable. Life cycle analyses are required to ensure that all parts of the process are accounted for. The co-culture system offers a way to reduce water and energy usage by reducing the need to produce, transport, and replace cell culture media.

6.3.3 Societal Influences

While the demand for meat is expected to double in the next half-decade, there is not enough land to meet the necessary supply. Animal agriculture requires wide plots of land, especially with a societal rise in concern for animal wellbeing. While factory farms aim to reduce the need for land, there are numerous ethical and safety concerns that are discussed in Chapter 2. The benefit of cellular agriculture is that meat can be cultivated indoors with minimal environmental requirements. Meat could potentially be produced in 'vertical farms', which use skyscrapers to increase the amount of meat produced per area of land. Specific climate and vegetation are not required, so the locations that meat can be grown will be expanded. This will increase access to calorie-dense food throughout the world while decreasing land usage and transportation costs.

6.3.4 Political Ramifications

There is already pushback from large meat producers against the integration of cultured meat products into the market. Lobbyists will continue to push legislators to outlaw the term 'meat' when describing cellular agriculture products. This will be a difficult hurdle to surmount, so it is necessary to start educating lawmakers and their constituents on the reality of cellular agriculture. The cells used for cultured meat products are genetically identical to the cells that make up traditional meat; they just do not require the death of an animal to produce.

6.3.5 Ethical Concerns

Cellular agriculture has the potential to obsolete many careers in the meat production industry. These ramifications cannot be ignored, especially since a goal of cellular agriculture is to better the conditions of humankind. Cellular agriculture researchers must engage in conversations with farmers, meat producers and distributors, and other workers in the field. It will be important to find ways to integrate cellular agriculture with some current practices to prevent the destruction of livelihoods. When this is not possible, there must be free access to education and retraining so those affected can continue to contribute to society and support themselves financially.

6.3.6 Health and Safety Concerns

Animal-borne illness is a major concern in modern society. Novel diseases like COVID-19 and the swine and avian influenzas are becoming more common due to our animal agriculture practices. The widespread use of antibiotics in factory farms increases the chance of these novel pathogens being drug-resistant. The safety of our society can be increased by shifting away from our reliance on factory farming. Cellular agriculture offers an alternative that would reduce the chance of spreading novel pathogens to the human population.

6.3.7 Manufacturability

The success of this design is only possible if it can be scaled for the mass production of cultured meat. The hollow-fiber bioreactor design can be scaled up by increasing the number of hollow fibers and the size of pumps and vessels. As the large-scale manufacturability of cellular agriculture products increases, cultured meat will become a more competitive option when compared to traditional meat. Due to the potential for reduced land, water, and energy usage, large-scale cellular agriculture can surpass the meat-producing capabilities of traditional animal agriculture.

Chapter 7. Discussion

Section 7.1 Ideal Co-culture Conditions

Various co-culture conditions were investigated throughout the experimental process to determine the most ideal growth conditions for co-culturing PBSCs with *C. reinhardtii*. Through the different experiments, the established conditions for ideal growth of both cell types were to use DMEM (without phenol red), at 30℃, with a 12-hour on/12-hour off light cycle, with no agitation. Although the current co-culture condition allows for PBSC proliferation with minimal cell death while extending the media lifetime by 85%, further experimentation is needed to fully investigate each parameter.

7.1.1 PBSC and *C. reinhardtii* viability in co-culture media

The results of the experiments shown in section 5.2 suggest that the ideal conditions established for the co-culture are not the most ideal for *C. reinhardtii*. The components present in DMEM do not pose any toxicity to *C. reinhardtii*, but DMEM does not provide any nutrients the algae need to proliferate. The inability to proliferate in DMEM is an issue that could be potentially solved by adding certain salts and heavy metals that are present in TAP media. However, the addition of these salts could be detrimental for the PBSCs; therefore, further experimentation will be needed.

The option of using different culture media should also be considered. Our team has initially tried to investigate whether there is an optimal DMEM:TAP ratio that is conducive for both PBSC and *C. reinhardtii* growth. Due to serum contamination and the inherent large amount of variability, the experiment was put on hold. Nevertheless, it should be recognized that although the experiments did not yield any conclusive results, the data did suggest that high ratios of TAP are not ideal for PBSC viability and proliferation. There are varieties of other media that could be used for algae growth, such as plant food, F/2 media, and BG-1; therefore, other algal media should also be explored in the future to properly assess whether a mix of DMEM and algal media could be used to achieve proliferation of both cell types.

An in-depth analysis of the media composition is another avenue of research that should be explored in the future to determine the ideal co-culture media composition. In this experiment, our group's focus was on the carbon and nitrogen cycle. However, there are other media components that need to be considered when trying to extend media lifetime, such as the production of lactic acid, the depletion of growth factors, etc. Our team hopes that an investigation on the composition between the new media, spent media and the media generated from the co-culture system will reveal other critical waste by-products that need to be recycled.

7.1.2 *C. reinhardtii* agitation

The usage of stir bars for the agitation of algae has also been proven to be unnecessary. Data have indicated that the usage of a stir bar has resulted in higher clumping as well as lower cell proliferation compared with no stir bar. The phenomenon observed in the stir bar condition could be potentially explained by the blunt trauma or force experienced by *C. reinhardtii*. The

purpose of shaking the algae culture is to allow for better aeration and allow the cells to receive proper lighting by de-clumping the algae culture. However, the introduction of the stir bar did not achieve the desired results and caused more clumping. The standard procedure for algae agitation is usually achieved through a shaker. The usage of a shaker would negate the possibility of blunt trauma/ force affecting the viability and proliferation of the algal cells. This experimental procedure was not explored during the experimental process due to technical constraints of the oxygen and pH readers, but it remains a possible option to further improve the co-culture conditions.

7.1.3 Possible improvements

It should be noted that there are still many different variables that could be explored to improve the co-culture conditions, such as the lighting conditions. The 12-hour on-off light cycle was a decision made after literary research, but no experiments were conducted to verify whether this was the most optimal condition. The establishment of the 12-hour light cycle was to try and maximize the photosynthetic capability of *C. reinhardtii* without bleaching them with too much light. Further literary research has suggested the usage of strobe LED lights could potentially increase the photosynthetic ability of the algal culture as well as mitigating light attenuation [114]. The experimentation with different lighting conditions is a variable that should be examined to improve the current co-culture specifications.

As aforementioned, the usage of a media analysis could also potentially suggest ways to improve the culture media. By assessing what components are vital or detrimental for the PBSCs and *C. reinhardtii,* a creation of a new media would be theoretically possible. The current system allows for PBSC proliferation with high viability; however, this comes at the cost of reducing *C. reinhardtii* growth. Therefore, the creation of a media that allows for growth of both cell types could potentially reduce the problems of algae maintenance in a large-scale setup.

Section 7.2 PBSC- *C. reinhardtii* Culture in Small Scale

Throughout the design process, the establishment of an ideal ratio is a vital process that will allow the team to determine the best way to scale-up the bioreactors. From the results, we suggest that a 1:200 ratio of PBSCs to *C. reinhardtii* allows for the highest rate of proliferation for the PBSCs. The Hoechst staining of the PBSCs in different algae ratios revealed that the algae, in the 1:200 ratio, was able to produce enough oxygen in the system to allow for PBSC proliferation. After three days, the control only had half of the initial 50,000 cells plated per well. The 1:100 ratio exibited no doubling but no decrease, while the 1:200 ratio exhibited a single doubling in the three-day culture period. In addition to PBSC proliferation, both the PBSCs and *C. reinhardtii* have appeared to survive well in the DMEM conditions with light present with a 12 hour on/off light cycle.

7.2.1 SBI DO and pH Sensors

Our results from section 5.3 suggested that PBSCs were viable, and algae provided enough oxygen to support the growth requirements of PBSCs. The pH was buffered more compared to the control culture, and the dissolved oxygen was higher than the control. The successful increase in viability of PBSCs as the ratio of algae to PBSCs increased suggested that there could be higher ratios explored to determine at which point PBSCs experience toxic

effects. Media analysis would be needed to quantify the impact of the algae directly on the molecular components of DMEM.

The DO increase with algae presence suggested that the algae created oxygen and thus more suitable environments for PBSC growth. The initial increase in the DO of the first 3 days of the 10-day period in the figure describing the pH (Figure 5.12) can likely be explained by the high productivity rate of algae in the first few days of co-culture. This could perhaps be because the PBSCs also became less metabolically active to produce CO_2 as their media became depleted of resources. The DO values also fluctuated significantly between 12-hour light cycles, which suggest that algae have very different oxygen production when they are not under lights. In a larger scale system, the algae productivity as it flows through the hollow fibers of the reactor will have to be considered, since the algae will be moving away from the light source. It should be evaluated in future experiments whether the DO or the pH contributes more to the survival of PBSCs. It was also not determined whether these results were statistically significant compared to the monoculture, however results were consistent among many trials although the trials could not be directly compared.

Additionally, the buffer in pH could be attributed to many different factors. The pH may have decreased due to limited availability of oxygen after algae became less metabolically active after three days. The lack of oxygen would contribute to a decrease in cellular respiration and an increase in fermentation, which would contribute to the production of lactic acid. Certainly, the conditions were not hypoxic, so it could not be confirmed that fermentation was occurring, and lactic acid was present. It is likely that overtime, the algae could not metabolically compensate for all the CO_2 that the PBSCs were producing. Overall, there were limitations with the experimental setup of the sensors and readers which will be discussed more in the next section.

Other questions that remain are whether the algae would be capable of recycling nitrogenous wastes of PBSCs such as ammonium. The team was unable to directly test the presence of ammonium and nitrogenous products over the course of the experiments which evaluated DO and pH. These data would help suggest whether algae could compensate for significant waste production of PBSCs and replenish media.

An additional question is whether growing the algae directly in place with the PBSCs could have contributed to growth inhibition of the PBSCs since algae were taking the physical space of the PBSCs. Alternatively, the algae in direct contact with the PBSCs may have provided for very proximal metabolic conversions of wastes to sources for PBSCs, specifically CO_2 to O_2. The potential inhibition should be accounted for in the hollow fiber model, and the distance from the algae cells will still be very proximal to PBSCs in the model.

7.2.2 Transwell

In the Transwell experiment, the staining protocol did not provide the expected results. The expected viability of 90% may be slightly lower than the actual value. The PBSCs detached from the culture flask in which they were being stained. Perhaps the cells reacted poorly to the live/dead stains, since in previous experiments the cells had not been detached when Hoechst stains were conducted in the same culture conditions. The DMSO in the Calcein AM and Etd-1 stains were at such low concentrations once the stocks were diluted in PBS that it would not have been probable for DMSO causing cell detachment and thus cell death. The cells were not dead however and could have simply detached since they were at room temperature for 30 minutes without any suitable culture medium.

Staining the PBSCs with Ki67 for proliferation was another goal of the project that could not be conducted due to time and COVID-19 related restrictions. Ki67 stains would have informed with precision whether PBSCs were proliferating or differentiating and would have quantitatively proved that we met our original objective of ensuring that PBSCs were proliferating and not differentiating into other bovine muscle cell types. Although these stains were not conducted, qualitative analysis of the cell morphology informed the team that there was minimal differentiation and significant proliferation of the cells as PBSCs.

Section 7.3 Current Limitations of the Experiments

The ID reader software and hardware that the team used to collect DO and pH was new to our team and our advisors. We worked with the manufacturer to improve our knowledge of the sensors, software, and best practices. Initially, the team struggled with accurately placing the small sensors due to issues with the adhesive adhering to the original packaging and not removing properly. This impacted the integrity of the sensor and could have contributed to the accuracy of the sensors to give a proper reading throughout experiments. In the last few experiments, the sensors with technical adhesive issues were replaced with sensors that could be removed from packing more easily. This proved to remove noise from the graphs and provided more consistent results.

The readers had other technical issues due to interference of the LED lights with the reader's mechanism of collecting data. The reader utilizes small LEDs to emit light which interact with the sensors of the culture flask of interest and determine the DO or pH of the culture. The lights interfered with the ability of the LEDs in the reader to accurately acquire proper data points, so data collected during the 12 hour "on" light cycle was slightly skewed. The data could be compared between 12 hour "off" light cycles, and separately compared between 12 hour "off" light cycles.

If the experiments required taking photos of the culture flasks, there was also some uncertainty that the sensors would be misaligned upon being replaced on the readers in the incubator. The team adapted to this uncertainty for the data shared in this report by using separate culture flasks which were held at the same conditions as the flasks on the readers.

Beyond the technical limitations of some of our experimental set-ups, the gas exchange could have not been entirely blocked for the Transwell and ID reader experiments. Parafilm does allow for some gas exchange which over short intervals of time may not have significant impacts however our experiments over 3 to 10 days may have been impacted [115].

Chapter 8. Conclusion

This project culminated in the creation of a proof-of-concept for an algal co-culture system to decrease the cost and environmental impact of large-scale cellular agriculture. The results support the hypothesis that *C. reinhardtii* co-cultures can increase PBSC proliferation in hypoxic conditions. This was assessed with Hoechst staining of co-cultures separated by Transwell inserts. The two species were also co-cultured without separation, and measurements of dissolved oxygen (DO) show that *C. reinhardtii* increased the DO during light cycles. This result indicates that the microalga can remain metabolically functional in co-culture with PBSCs grown in DMEM. The pH readings show that *C. reinhardtii* slowed the pH drop of the co-culture, which supports the hypothesis that the algal co-culture can extend the lifetime of cell culture media. Live/dead staining was used to assess the viability of PBSCs in co-culture, and the results indicate that PBSCS can retain high viability after three days of culture with *C. reinhardtii*. These data indicate that *C reinhardtii* co-cultures should continue to be explored as an avenue for media recycling in large-scale cellular agriculture. A conceptual design of a hollow-fiber bioreactor for scale-up of the co-culture was modeled, although further work is necessary to prototype and validate the design.

Future studies should aim to confirm if the PBSCs retain their stem-phenotype in co-culture since this is necessary to maximize the proliferative potential of the cells. The addition of growth factors from a sustainable and low-cost source is a technological advancement that can help achieve this cell culture constraint. This project only studied co-cultures with a 12-hour light cycle, so the exploration of additional light conditions would generate valuable data for optimizing the co-culture system. The literature suggests that *C. reinhardtii* can uptake ammonium from the media, but time and monetary constraints prevented the team from confirming this experimentally. Measuring ammonium concentrations will be necessary for future studies, as well as characterization of other media components. A complete spent media analysis of various salts, amino acids, small molecules, and proteins will help characterize the full effects of the co-culture system. Lactic acid is a major waste metabolite that is not addressed with *C. reinhardtii* co-cultures. Therefore, the next step in developing a media recycling co-culture system is identifying and testing an additional cell species that can metabolize lactic acid.

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Appendices

Appendix A: Primary Bovine Satellite Cell maintenance protocols

Thawing PBSCs:

Materials:

- DMEM + 10% FBS, 1% P/S, GFs (see making media, Appendix K)
- 2 x 15 mL conical tube
- Water bath at 37℃
- Cryovial of PBSCs
- Aspirating pipettes
- Serological pipettes and pipettor
- Centrifuge

Method:

1. 10 mL of DMEM +10% FBS, 1% P/S, and GFs were measured into a 15 mL conical tube.
2. A frozen cryovial of cells was obtained from a liquid nitrogen tank.
3. Cryovial was placed in a 37℃ water bath just until thawed, with no more frozen liquid.
4. Immediately, using a 1 mL serological pipette, the thawed cells were resuspended and quickly, gently mixed by pipetting up and down.
5. The cell mixture was added dropwise to the 10 mL of DMEM and gently shaken upon the addition of more cells.
6. Once all the thawed cell mixture was added to the DMEM, the 15 mL conical with cells in DMEM was placed in a centrifuge for 5 minutes at 10,000 RPM.
7. After centrifugation, the supernatant was aspirated. The cell pellet was resuspended in 2 mL of media.
8. A count was performed to verify cell viability.

Feeding PBSCs

Materials:

- DMEM + 10% FBS, 1% P/S, GFs (see making media)
- T-75 or T-25 flask with PBSCs
- Water bath at 37 ℃
- Aspirating pipette
- Serological pipettes and pipettor

Method:

9. Warm media in a 37℃ water bath for 15 minutes.
10. Remove T-flask from the incubator.

11. Working in the biosafety cabinet, aspirate media from cells.
12. For a T-25 flask
    a. Add 5 mL of new media
13. For a T-75 flask
    b. Add 11 mL of new media

Passaging Primary Bovine Satellite cells

- Trypsin
- DMEM + 10% FBS, 1% P/S, GFs (see making media)
- 15 mL conical tube
- T-75 or T-25 flask
- Water bath at 37°C
- Serological pipettes and pipettor
- Aspirating pipette
- Centrifuge

Method: (for T-75)

14. Place trypsin and DMEM into a water bath for 15 minutes until warm.
15. Remove flask with cells from the incubator. View the cells under microscope to confirm confluency is about 80% and cells require passaging.
16. Remove trypsin and DMEM from the water bath and move into the biosafety cabinet.
17. Aspirate media from the flask with cells with an aspirating pipette.
18. Measure 5 mL of trypsin and place into the flask with cells.
19. Place the flask in the incubator at 37°C for five minutes.
20. Verify that cells have detached from the flask by viewing cells under a microscope.
21. Add 5 mL of DMEM to flask to deactivate the trypsin.
22. Remove cell suspension from the flask and place it in a 15 mL conical tube.
23. The 15 mL conical with cell suspension was placed in a centrifuge for 5 minutes at 10,000 RPM.
24. After centrifugation, the supernatant was aspirated. The cell pellet was resuspended in 2 mL of media with thorough mixing to ensure full resuspension of pellet.
25. A cell count was performed using a hemocytometer. The following formula was used to calculate cell count:
    c. # of cells counted # of boxes counted 210,000 # of total mL resuspension = # of cells/mL
26. Cells were plated at a density of 750,000 cells/mL.
27. 11 mL of media were added to the newly plated flask.

Appendix B: Algae Cell Culture maintenance:

Feeding C. reinhardtii cells

Materials:

- TAP media
- *Chlamydomonas reinhardtii*
- Grow light (Yoyomax Inc., Model number: BW-C1YQ-8WAE)
- Erlenmeyer flask with metal caps
- Orbital shaker platform
- Serological pipettes and pipettor

Method:

28. Transfer 10 million cells to a sterile erlenmeyer flask
29. Add TAP to cell suspension up to 50 mL in the flask.
30. Place flask on shaking platform in a 12 hour on, 12 hour off light cycle in a 30℃ incubator.
31. Cell suspension should be a bright, deep green. If color becomes yellow-tinted, replenish solution with 10-20 mL of TAP.

Appendix C: Hill Reaction Protocol

Materials:

- DCPIP
- *C. reinhardtii* stock culture flask
- Box (to cover a 96-well plate)
- 6-well plate
- 96-well plate
- Micropipettes and tips

Method:

32. Count algae cells and determine appropriate algae culture volume needed for a 500,000 cells/mL, 5 million cells/mL, and another 5 million cells/mL concentration.
33. Take a control sample without DCPIP of each well in the 6-well-plate and placed into the first column of the 96-well-plate.
34. In a well-lit area, add 10 uL/mL, 20 uL/mL, and 50 uL/mL of DCPIP to each of the respective concentrations. There should be 4 mL of media total per well in the 6 well plate.
35. Start a timer as the DCPIP was added to the 6-well plate. Time 0 samples of 100uL should be taken immediately after the timer is started.
36. Cover the 96-well-plate with the box to prevent further oxidation of DCPIP by algae photosynthesis.
37. Take 100uL samples from every well on the 6-well-plate after 5, 10, 15, 20, 30, 40, and 50 minutes, covering the 96-well-plate between transferring samples and in waiting periods.
38. Read plate at 600 nm wavelength in microplate reader. Record the absorbance values for each sample at each time period to determine oxygen evolution rate.

Appendix D: Transwell Experimental Protocol

Materials:

- *C. reinhardtii* stock culture flask
- Cell culture flask with PBSCs
- 6-well plate with 6 Transwell inserts
- Micropipettes and tips
- Parafilm
- DMEM + 10% FBS, 1% P/S, GFs
- Incubator at 30°C with growth lights.

Method:

39. Perform a cell count for both algae cells and PBSCs.
40. Obtain a 6-well plate with 6 Transwell inserts.
41. Plate PBSCs at 500,000 cells per well in the wells of the 6-well plate in a total volume of 3 mL.
42. Place a Transwell insert in each well of the 6-well plate.
43. Plate algae at desired concentration in the Transwell.
44. Cover Transwell with parafilm to prevent gas exchange.
45. Place the plate in a 30°C incubator with lights on in a 12 hour on/12 hour off light cycle for four days.
46. After four days, remove Transwell inserts with algae and stain PBSCs with Hoescht according to Hoechst staining protocol (Appendix G). Take images of cells.

Appendix E: Hoechst Staining of Primary Bovine Satellite Cells

Materials:

- Hoescht Stain
- Cell culture flask with PBSCs
- PBS
- Micropipettes and tips
- Fluorescence microscope
- Aspirating pipettes
- Incubator at 37 °C
- Box (to cover the flask during the staining protocol)

Method:

47. Remove culture flask from the incubator.
48. Aspirate media from flask in the biosafety cabinet.
49. Wash with PBS by adding enough PBS to coat the entire surface of the flask. Aspirate.
50. Add enough PBS to coat the surface of the culture flask again.
51. Transfer 1 uL of Hoescht dye to the culture flask. Place the flask in a 37℃ incubator for 1-5 minutes, but no longer to prevent background staining.
52. Image the cells under a fluorescent microscope.
    d. Results: Nucleus is stained blue

Appendix F: Live/Dead Staining of Primary Bovine Satellite Cells

Materials:

- Calcein AM solution (Invitrogen)
    - 40 μL each, 4 mM in anhydrous DMSO
- Ethidium homodimer-1 (Etd-1) (Invitrogen)
    - 200 μL each, 2 mM in DMSO/H2O 1:4 (v/v)
- 1 x Phosphate buffered saline (PBS)
- Vortex machine
- Micropipettes and tips
- Aspirating pipettes
- Box (to cover the flask during the staining protocol)

Method: (Adapted from Invitrogen protocol)

Calcein AM - Invitrogen Procedure:

53. Let Calcein AM and EthD-1 vials sit at room temperature until they thaw.
54. Add 20 uL of EthD-1 solution to 10 mL of PBS and vortexing to give a 4 uM EthD-1 solution. Vortex.
55. 5 uL of the 4 mM calcein stock will be added to 10 mL of buffer (PBS)/ethidium solution and vortexed (approximately 2 uM calcein working solution)
56. Aspirate media from flasks.
57. Rinse flasks with PBS two times to remove excess serum.
58. Add enough staining solution to cover the bottom of each flask (2 mL for a T-25 flask). Cover the samples with a box to prevent photobleaching of the fluorophores, and let incubate at room temperature for 30 minutes.
59. Once the incubation period is over, image the cells (Ex/Em 528/617 for Ethidium, 494/517 nm for Calcein).
    e. Results: Viable cells fluoresce green, Dead cells fluoresce red.

Appendix G: ID Reader Set Up

Materials:

- ID reader sensors - DO and pH (Scientific Bio)
- 2 x ID readers (from ID Developer's Kit, Scientific Bio)
- Forceps
- Red calibration disk (enclosed in manufacturer's packaged with ID Developer's Kit, Scientific Bio)
- Access to ID Data Hub Software

Methods:

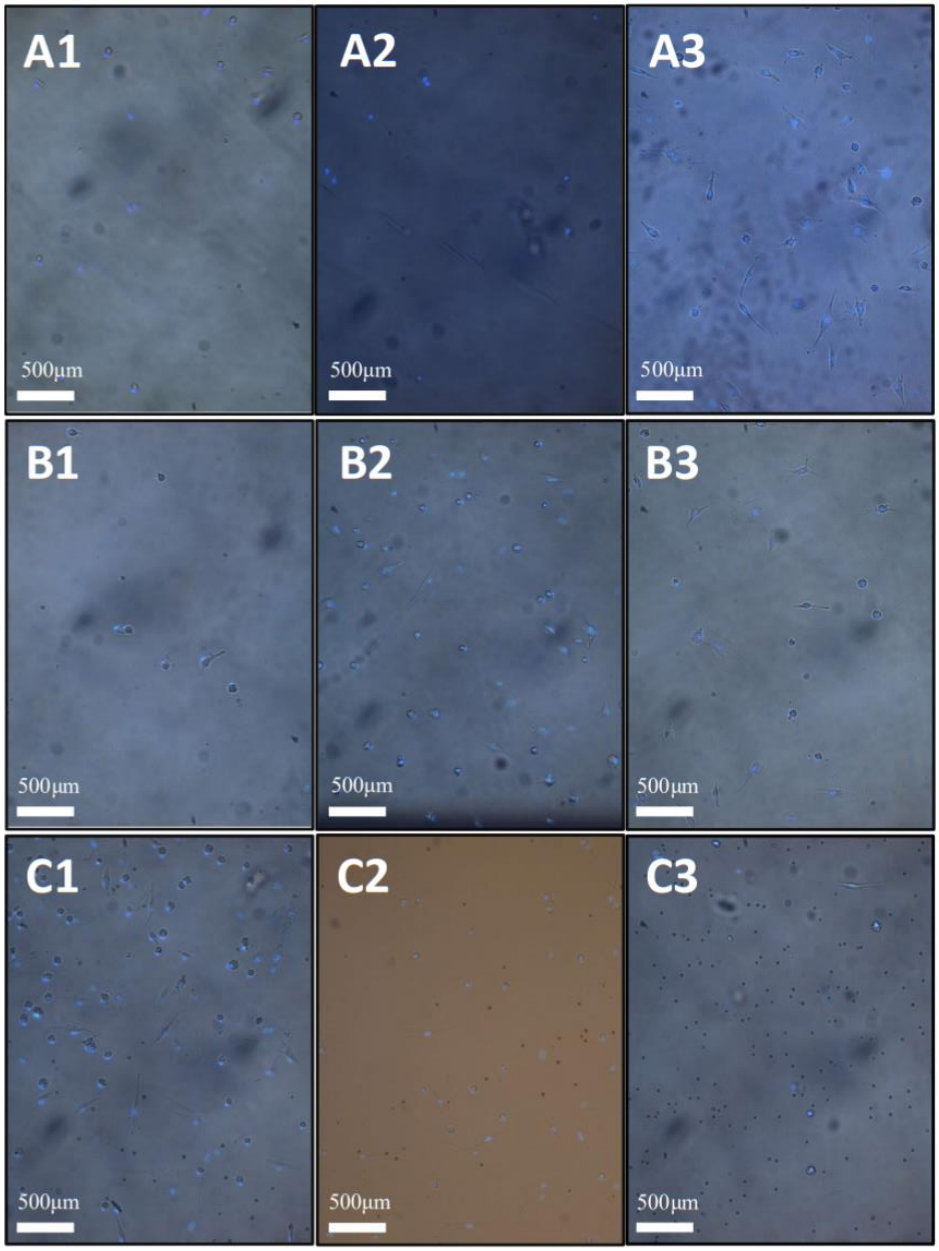
Plating the sensors:

60. Place the readers in the incubator and plug into adapter and computer.
61. Calibrate the reader software using the pH and DO offsets in the sensor packaging. Run the calibration on the ID Data Hub software.
62. Using forceps, place a DO sensor and a pH sensor on the bottom of the experimental flask before adding any test solutions or cells. Sensors should be placed the same distance apart as the LEDs on the ID readers.
63. Place the experimental flasks over the readers in the incubator. Align the sensors with the LEDs of the reader.
64. Set the time between points for data collection. Set the data collection mode to Scan. Set each of the patches for each reader to "Read" for DO and pH.
65. Start collecting data.

Calibration of the reader:

66. Set the software to Scan mode. Read the pH and DO constants and verify that they match the constants which came with the manufacturers details of the sensors.
67. If the pH and DO constants do not match the constants of the sensors, Write the pH and DO sensor constants.
68. Place the reader in the environment for which the data collection will occur.
69. Click the calibration tab. Choose "Measure Offsets and Phase."
70. Follow the instructions on the ID Data Hub interface by placing and removing the red calibration disk when prompted.
71. Repeat for each pH and DO LED on each reader.

Appendix H: Pictures of Hoechst Staining of the Transwell



Images above were used to calculate the cell counts for the Transwell experiments, in addition to the images shown in Fig. 5.6. Values from these images are shown in Fig. 5.7 and Fig. 5.8. Images in Panels A1-3 are from Transwells with no algae, images marked in Panels B1-3 were taken from Transwells with a 100:1 algae to PBSC ratio, images marked in Panels C1-3 were taken from Transwells with a 200:1 algae to PBSC ratio.

Appendix I: Sample calculation for determining cell counts from microscope images

Microscope fields of view were determined to be 0.2702 cm x 0.3603 cm

Updated field area, accounting for 4x objective magnification:

Length of field of view/magnification = 0.2702 / 4 = 0.06755 cm

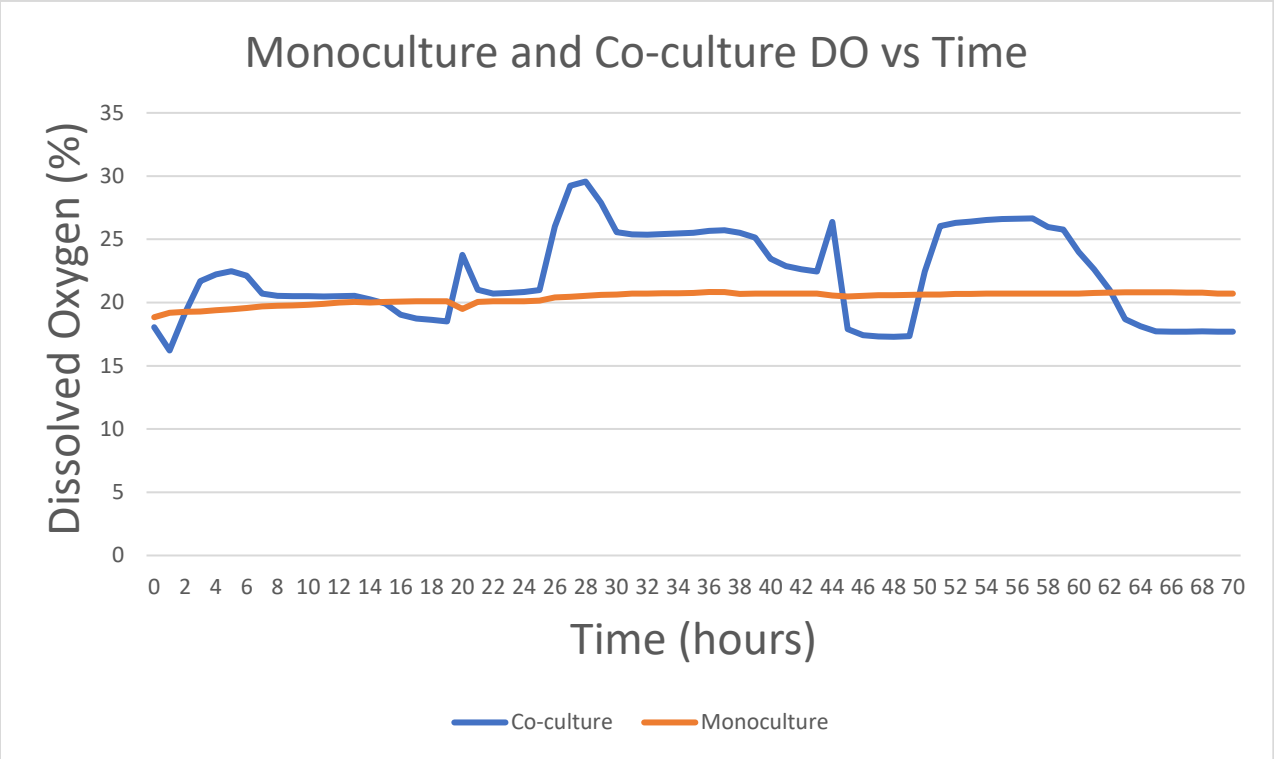
Width of field of view/magnification = 0.03603 / 4 = 0.090075 cm

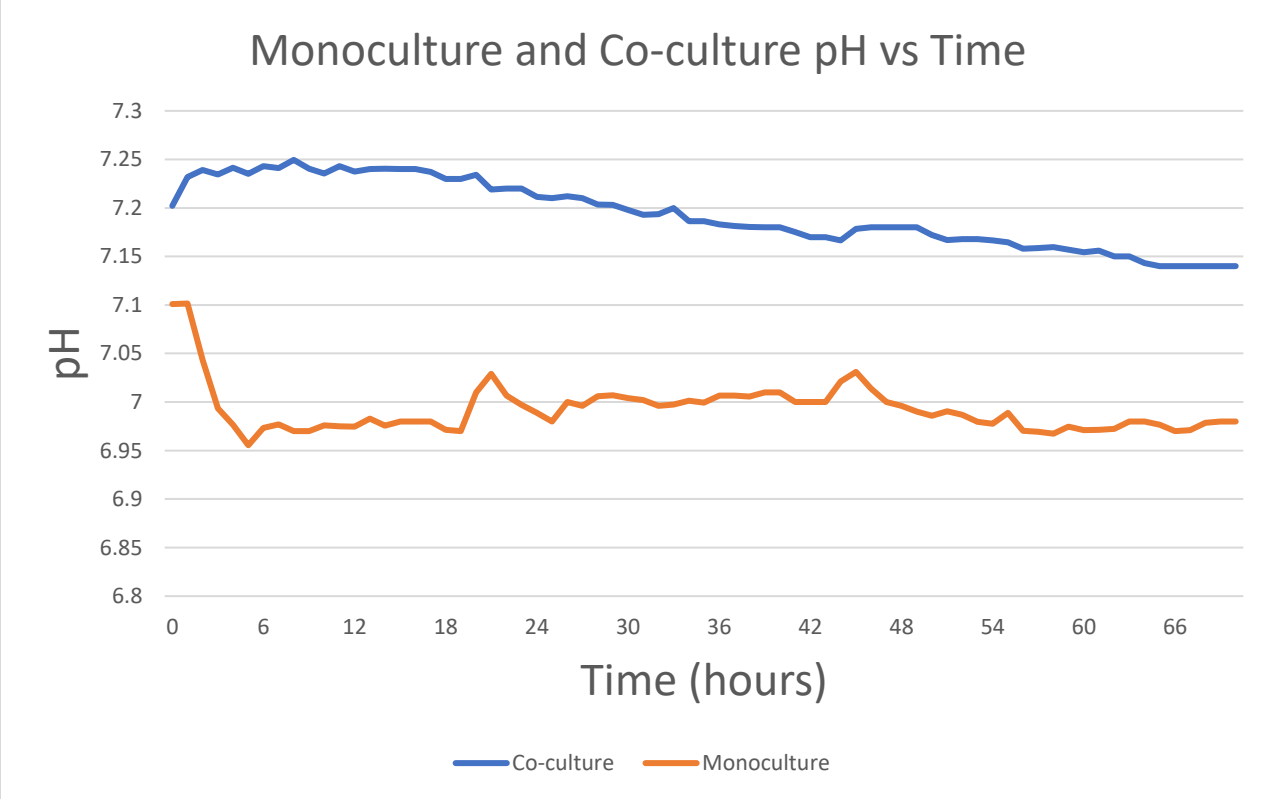
Updated total area of field of view, accounting for 8x magnification:

Updated length x updated width: 0.06755 cm x 0.090075 cm = **0.00060846 cm^2**

Total cells per area can be determined by multiplying the number of cells in an image times the total area of the experimental flask divided by 0.00060846.

Appendix J – Percentage DO and pH reader from Experiment 1





Appendix K – PBSC Media Making Protocol

Materials

- DMEM
- FBS
- Penicillin/Streptomycin
- Growth factors: FGF2, HGF, EGF, and IGF
- 50 mL conical tubes
- Sterile filter and sterile flask

Methods:

1. In a biosafety cabinet, remove 55 mL of DMEM from the flask of DMEM and store in conical tubes. Refrigerate for later use.
2. Add 50 mL FBS to 500 mL flask of DMEM for a 10% FBS concentration.
1. Add 5 mL of Pen/Strep to the media solution for a 1% Pen/Strep concentration.
2. Obtain sterile flask and sterile filter attachment. Aseptically transfer the media with DMEM, FBS, and Pen/Strep to the flask.
3. Attach the filter and use vacuum to transfer media through the filter.
4. Add 4 ng/mL of human fibroblast growth factor-2 (FGF2), 2.5 ng/mL recombinant human hepatocyte growth factor (HGF), 10 ng/mL recombinant human epidermal growth factor (EGF), and 5 ng/mL recombinant human insulin-like growth factor-1 (IGF).
5. Add cap and place in refrigerator until needed.

Appendix L - Calculations for Bioreactor

Peclet number of the fiber

$$\frac{c_{\mathrm{min}}}{c_{\mathrm{in}}} = A + \sum_{n=0}^{\infty} B_n \exp\left(-\frac{\lambda_n^2}{2Pe^*}\right) + C_n,$$

where

$$A = \frac{M}{4d^2}\left[R_e^2 - R_m^2 + \frac{2D_e}{D_m}(R_m^2 - R_e^2)\ln\left(\frac{R_m}{d}\right) + 2R_e^2\ln\left(\frac{R_m}{R_e}\right)\right] + 1 + \gamma,$$

$$B_n = E_n G_n \exp\left(\frac{\lambda_n}{2}\right)\mathrm{KummerM}\left(\frac{1}{2} + \frac{\lambda_n}{4}, 1, -\lambda_n\right), \quad C_n = \frac{E_n F_n}{\lambda_n^2}\exp\left(\frac{\lambda_n}{2}\right)\mathrm{KummerM}\left(\frac{1}{2} + \frac{\lambda_n}{4}, 1, -\lambda_n\right),$$

Cmin/cin=0.08/0.22=0.36
A = 0.8
Pe* = 0.2

Velocity inside the lumen

velocity = Pe* x k x Cp x L=0.2(1.2)1.011(98)(0.2)=0.012 m/s = 1.2 cm/s
K = Thermal Conductivity of DMEM (W/mK)
Cp = Heat Capacity of DMEM (J/kgK)

Flow rate:

Cross-sectional Area x Velocity
R^2 x 1.2 cm^2/s =0.012 x 1.2 cm/s = 0.000377 cm^3/s

Shear Rate:

$$\dot{\gamma} = \frac{2R_{\mathrm{o}}R_{\mathrm{i}}\omega}{R_{\mathrm{o}}^2 - R_{\mathrm{i}}^2}$$

Shear rate = 2RoRiw/(Ro2-Ri2)=2(200)(100)(1.2)/(200)2-(100)2=1.6 s-1

Shear Force:

Shear Force = Shear rate x viscosity (DMEM) = 1.6 s-1 x 0.9598 Pa*s = 1.536 Pa