Bioreactor Design for Bacterial Cellulose Production

A Major Qualifying Project Report

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1.0 Introduction

1.1 Problem Identification

Bacterial cellulose (BC) is a biomaterial studied for many biomedical applications. *Komagataeibacter hancenii* strains produce BC at the air/liquid interface. However, addition of nutrients and/or gases without disrupting the continuous production or homogeneity of BC is challenging. Because oxygen and other nutrients are necessary for the growth of *K. hancenii* and the formation of BC, it is desired to readily control these nutrients within the culture media.

1.2 Specific Aims

The goal of this project is to successfully design a low-cost bioreactor capable of regulating nutrients to produce BC. The regulation of specific nutrients contributes to altering production rates and characteristics of the culture. The yield of BC will be maximized, and homogeneous pellicle formation will be prioritized. The yield and properties of the BC that is produced will be compared to that produced by static standard culture. The Coburn lab has identified several carbon sources that result in unique BC properties during production. This project will specifically examine glucose as the target carbon source. Other goals of this project include the analysis of specific nutrients and environmental conditions that will result in the highest production rates of BC.

1.3 Approach

A static bioreactor provides bacteria with more consistent conditions with the goal of increasing production of BC. A static bioreactor technique allows the BC product to retain uniformity and large pellicle size, while also monitoring and sustaining specific conditions. These conditions will aim to increase production rate and total yield in the same amount of time as static

culture. This design will allow oxygen and glucose (or other carbon sources) to reach the bacterium within the pellicle without disrupting the structural integrity of the culture. This will provide better conditions for BC production by retaining production rate, pellicle uniformity, and mechanical strength.

2.0 Literature Review

2.1 Bacterial Cellulose (BC)

2.1.1 History of BC

BC was first discovered and described in 1886. Using *Bacterium aceti* to study the biochemical behaviors of the acetic fermentation process, Adrian J. Brown discovered what was commonly called "vinegar plant", a translucent jelly-like mass on the surface of the culture fluid [1]. Pellicles (thick biofilms) of *B. aceti* pure cultures were thin and cracked by the slightest agitation, but this vinegar plant remained intact for many days [2]. With a nutrient solution composed of half water and half red wine diluted with acetic acid, this growing microorganism and biofilm quickly grew until it covered the entire surface of the liquid. Upon gentle stirring, the membrane was covered with the medium and a new layer of membrane overcame the old one. The membrane was slightly denser than water [3]. In microscopical examination, the membrane of the vinegar plant showed bacteria embedded in a transparent structured film. After washing it with diluted hydrochloric acid and then with water, the final product was a colorless, semitransparent film that retained the membrane's shape and gelatinous nature. Analyses of all vinegar plant membrane samples revealed it to be pure BC [2].

A large majority of biological macromolecules produce BC. Nearly all plants, most fungi, and some algae contain this structural component [4]. Further, there are some strains of prokaryotic, non-photosynthetic organisms that can produce BC in the form of twisting ribbons of microfibril bundles [2]. Although both plant and bacterial-derived cellulose have advantageous properties that can be applied to biomedical applications, plant derived cellulose is contaminated by other molecules such as hemicelluloses, lignin, and pectin. As a result, the percentage of pure

cellulose found in plant material ranges from 50% to 90% [5]. To obtain pure plant cellulose, additional cost and production steps are required, limiting the potential of plant derived cellulose use in biomedical applications [5].

2.1.2 Chemical Composition and Mechanical Properties of BC

Unlike plant-derived cellulose, BC is made up of long, nanoscale fibrils which are uniaxially oriented and create a network structure [6]. These nanoscale fibers are formed within the bacterium and extruded as fine microfibrils (1.5 nm diameter) that combine to form fibrils with a larger diameter (30-50 nm diameter) [5]. BC possesses desirable material properties such as high BC crystallinity (60–80%) and high Young's Modulus and tensile strengths for hydrated pellicles [7]. Additionally, the size of BC fibrils is about 100 times smaller than that of plant cellulose [8]. This unique nano-morphology results in a large surface area to volume ratio that holds a large amount of water (up to 200 times its dry mass) and at the same time displays high elasticity and conformability [8]. Nimeskern et al. compared the mechanical properties of BC pellicles with varying cellulose content (2.5% to 15%) to those of native ear cartilage [9]. BC pellicle mechanical properties depend strongly on cellulose content, and researchers found that 14% cellulose produced similar stress-relaxation test results as native ear cartilage in indentation tests [9] For hydrated BC pellicles, the Young's Modulus was 10 MPa for uniaxial tension [10] and values as high as 200-500 MPa [11]. The tensile stress at failure for hydrated BC pellicles was 1 MPa for uniaxial tension [10] and 11 MPa for biaxial tension [11]. Note, however, that although BC membranes provide strong resistance to tensile deformation in directions parallel to the membrane plane, they provide little resistance to compressive deformation perpendicular to the membrane plane. As an example, a BC membrane with an in-plane Young's modulus of 2.9 MPa shows a perpendicular compression modulus of 0.007 MPa [12].

2.1.3 Cellulose Production in Bacteria

2.1.3.1 Cellulose Producing Bacteria

BC is produced extracellularly by the Gram-negative bacterial cultures including *Acetobacter (*also known as *Gluconoacetobacter*, currently classified as *Komagataeibacter)*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Sarcina*, *Azobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella and Alcaligenes* [13]. BC formation involves many enzymes and protein complexes. First, glucose is transported from the outside of the cell into the cell [14]. Next, uridine diphosphoglucose (UDPG) is created from glucose 6-phosphate and glucose 1-phosphate [14]. Then, the glucose undergoes polymerization in chains by cellulose synthase [14]. These cellulose chains are then extruded through the cell membrane and joined to form sub-fibrils, which then assemble into nanofibrils [14].

Specific microorganisms such as *Komagataeibacter* can biosynthesize β-1,4-glucan chains exopolysaccharide of cellulose. During biosynthesis, bacteria utilize various carbon compounds of the nutrition medium, polymerize single, linear β-1,4-glucan chains and then secrete outside the cells through a linear row of pores located on their outer membrane [15]. A precise, hierarchical process follows the assembly of β-1,4-glucan chains outside of the cell. Initially, they form sub fibrils (consisting of 10–15 nascent β-1,4-glucan chains), then later microfibrils, and finally bundles of microfibrils consisting of a loosely wound ribbon, which is comprised of about 1000 individual glucan chains [16] A thick, gelatinous membrane is formed in static culture conditions because of this process [7].

The formation of BC by *Komagataeibacter* occurs between the outer membrane and cytoplasmic membrane by a cellulose-synthesizing complex found on the bacterium's surface [4]. The cellulose synthase enzyme is regarded as the most important in this process. The enzyme is

partially purified and cloned [4]. Since c-di-GMP is an activator of cellulose synthase [4], the proposed biochemical pathway from glucose to BC should be linked to both cell growth and cellulose synthesis [4]. The BC produced is extruded from the transmembrane pores as fibrils and forms together with many synthesized fibrils a ribbon of crystalline cellulose.

It is possible to conduct fermentation under static, agitated, or stirred conditions for BC production [17]. Different form factors of BC are produced under these conditions. Semi-continuous processes in static conditions are typically favored over continuous processes in agitated conditions, however, sine static conditions have proven to increase BC productivity where agitated conditions cause the formation of SCP which is an irregular form of BC either in fibrous suspension, spheres, pellets or irregular masses [17]. Under static conditions, a three-dimensional interconnected reticular pellicle may form at the air-liquid interface, whereas both stirred and agitated conditions produce sphere-like BC particles (SCPs) [18].

The most studied model bacterium and most efficient BC producer belongs to the *Komagataeibacter* genus, due to its capability to make BC from a wide range of carbon and nitrogen sources such as glucose, glycerol, sucrose, fructose, mannitol, and others [14]. *Komagataeibacter* is a rod-shaped, aerobic, gram-negative bacteria that occurs as a contaminant during vinegar fermentation [19]. The *Komagataeibacter* genus is found on fruits and vegetables during the decomposition process [14]. They are defined by their ability to produce acetic acid from ethanol and oxidate acetate and lactate to carbon dioxide and water [14]. They are also able to grow in presence of 0.35% (w/v) acetic acid, without producing 2,5-diketo-D-gluconate from glucose [14].

2.1.3.2 The Influence of Carbon Sources and Other Factors

Producing BC in synthetic media using different carbon sources and growth factors such as yeast extract and peptone is expensive [20]. Researchers are investigating alternative raw materials that are inexpensive and contain high levels of sugars that serve as substrates for BC production. Many substrates have been analyzed regarding its possibility of BC production in *K. hancenii*. Studies reported by Masaoka et al. and Oikawa et al. analyzed BC production using glucose and other carbon sources. In using lower glucose concentrations, Masaoka et al. was able to show that BC production could reach up to 0.6 g/g glucose/day after 2-4 days of cultivation [21]. Oikawa et al. obtained, in comparison with other published results, better productivity for mannitol (0.233 g/g/day) and arabitol (0.155 g/g/day) [4]. Table 1 showcases the effects of carbon sources on the production of BC by *K. Hancenii* [4]. As shown, several published papers have revealed that glucose and fructose often produce the highest yields of BC.

Table 1: Effect of Carbon Sources on The Production of Cellulose by *K. Hancenii*

Carbon Source	Cellulose Yield (%)	+ or -
D-Glucose	100 ([22], [23] [24], [25])	+ [26],[27], [28]
D-Fructose	92 [24]	+ [26], [27], [28]
D-Galactose	15 [25]	+ [26] –[27]
D-Mannose	3 [24]	+ [26] –[27]
D-Xylose	11 [24]	-[26], [27], [28]
l-Arabinose	14 [24]	
L-Sorbose	11 [24]	
Lactose	16 [24]	
Maltose	7 [24]	
Sucrose	33 [24]	
Cellobiose	7-11 [25]	
Starch	18 [24]	
Ethanol	4 [24]	
Ethylene glycol	1 [24]	
Diethylene glycol	1 [24]	
Propylene glycol	8 [24]	
Glycerol	93 [24]	+ [26], [27]
Myo-inositol	17 [24]	
D-Arabitol	620 [22]	
D-Mannitol	380 [23]	
No Carbon Source	2 [24]	

*Glucose was set as 100% yield. (+) or (-) means that the referred authors found that this carbon source had a positive (+) effect on BC production or that no BC production occurred (-).

The yield and characteristics of BC production are also dependent on other factors such as pH, temperature, and dissolved oxygen content. The optimal pH resulting in the greatest yield of BC is dependent on the strain of bacteria but is typically in the range of 4-7 [29]. The pH of the bacteria culture can decrease as a function of time as secondary metabolites accumulate due to the consumption of sugars and nitrogen sources [29]. Therefore, it is important to maintain the optimal pH to produce the maximum yield of BC. The effect of temperature on the yield of BC produced by *Acetobacter* was investigated by Son et al [30]. Temperatures within the range of 20-40°C were

tested [30]. Although the optimal temperature that resulted in the greatest BC yield was 30°C, the study found that there was no significant difference in BC production at 25°C [30]. However, BC production decreased at temperatures above 35°C [30].

Since the metabolism of aerobic bacteria relies on the dissolved oxygen content, the yield and quality of BC produced during cell metabolism are dependent on the dissolved oxygen content [31]. Too high of a dissolved oxygen content results in an increase in gluconic acid concentration, diminishing cell function, which results in a lower yield of BC. However, too low of a dissolved oxygen content slows bacteria growth and therefore impedes the production of BC. In a study conducted by Hwang, the maximum BC yield was produced at 10% saturation of dissolved oxygen [32]. Cyclic diguanylmonophosphate, or c-di-GMP, is a bacterial signaling molecule that activates the formation of BC [4]. Phosphodiesterase A1, or *Ax*PDEA1 is a c-di-GMP found in *K. xylinus*. *Ax*PDEA1 contains a Per-Arnt-Sim (PAS) motif, which detects signals such as oxygen, light, voltage, redox potential, and aromatic hydrocarbons [33, 34]. Therefore, *Ax*PDEA1 can be used to measure dissolved oxygen content during BC production [33].

2.1.4 Technological Applications of Bacterial Cellulose

2.1.4.1 Food Biotechnological Applications

In ancient Southeast Asian cultures, BC has been known for its potential to reduce some risk factors for cardiovascular diseases, diabetes, diverticulitis, and obesity [35]. Being a dietary fiber, the Food and Drug Administration (FDA) approved BC as GRAS (generally recognized as safe) in 1992 [36]. Nata de coco or piña (i.e., coconut or pineapple cream) is made via *K. xylinus* fermentation of coconut water and fruits in a medium with high sucrose [14]. Likewise, a microbial consortium named Kombucha ferments black or green tea containing sucrose by BC-producing bacteria. It takes two weeks for the BC membrane to be removed, after which the liquid phase is

ready to drink [35]. Although Kombucha tea is a therapeutic infusion, its microbial consortium is still undefined and complex. Furthermore, BC has also been explored for its use as a potential gelling, thickening, suspending and stabilizing agent in the food industry [37]. For example, BC significantly increases the gel strength of tofu by providing firmness and better texture [35]. In ice cream, it also prevents flow after melting because of increased shear stress [36]. Lastly, BC also works as a food packaging as it confirms safety and increases shelf-life of products. A recent study by George et al. investigated the use of BC nanocrystals to fabricate edible, biodegradable, and high-performance gelatin nanocomposite films for food packaging [38]. Yang et al. proposed the use of BC composites as antimicrobial materials for food packages and water sterilization [39].

Table 2: Applications of Bacterial Cellulose

Type	Description	Reference
Wound Dressing	Water holding capacity, biocompatibility, and mechanical properties make BC an adequate material for wound healing on its own or enhanced with charged molecules.	[40], [41], [42],[43]
Antimicrobial Activity	After functionalization with antimicrobial molecules or mixed with other ingredients in composites can be an infection preventive material.	[40], [44], [45], [46], [47], [48] [49], [50]
Drug Delivery	Water holding capacity and drug adsorption capacity, with controlled release profiles, arising BC as potential platform of drug delivery.	[51], [52], [53], [54], [55],[56]
Tissue Regeneration	BC is a good scaffold material, improved by further physical or chemical modification, having been used as a scaffold for different cell types during in vitro growth.	[57], [14], [48], [58], [59],[29], [60]
Vascular Grafts	Water holding capacity, elasticity, biocompatibility or low inflammatory, makes BC a suitable material for artificial blood vessels.	[61],[62]
Emulsion Stabilizer	BC NPs act as an oil-in-water emulsion stabilizer.	[63]
Gastric Conditions	BC fibers prevent constipation.	[64], [65]
Enzyme Immobilization	After chemical modification for binding or by encapsulation, BC can be used for enzyme immobilization.	[66], [67],[68], [69],[70], [71], [72]
Filtration	BC can be used as a matrix for coating with other carbon-based or metal materials for specific components and high-efficiency contaminant removal.	[73], [74], [75]
Conductive Material	After functionalizing with metal NPs or other polymers, it can become a conductive material for nanowires or batteries applications.	[1],[76]
Sensors	BC nano paper can be used as sensor platform for chemicals of bioreactions.	[77], [78]
SERS	BC functionalized with NPs displays good characteristics as a platform for SERS spectroscopy.	[79], [80]
Bioethanol	BC uses as an alternative to lignocellulose from plants. BC lacks hemicellulose and lignin so that acid catalyzed hydrolysis step in bioethanol production can be eliminated.	[81]
Cosmetics	BC facial masks are moisturizing, and functionalized BC can address specific cosmetic problems	[36], [82]
Paper Manufacturing	BC as an additive increase's material strength	[83], [59]
Aerogels	BC as the primary polymer, composited with other biocompatible polymers.	[84]

2.1.4.2 Biomedical Applications

BC membranes were introduced in biomedical applications because of their high *in vivo* biocompatibility [85], ability to provide an optimal three-dimensional surface for cell attachment, and a microfibrillar structure that provides flexibility, high water retention, and gas exchange [13]. BC is potentially useful in skin repair and tissue engineering applications due to the lack of beta-glucanases (i.e., cellulases) in humans and its poor solubility in physiological media [14]. Furthermore, BC membranes prevent infections and reduce pain by maintaining a barrier that is effective at preventing bacterial invasions and allowing drug transfer into the wound site [85].

In rats, subcutaneous implantation has been studied extensively to determine BC biocompatibility. The following results were reported: No fibrosis or encapsulation was noticeable around the BC implant and there were no macroscopic signs of inflammation, such as redness, edema, or exudation, were observed [86]. In addition, cellular ingrowth penetrated the BC network and formed a new tissue integrated with the biomaterial [86]. Vascularization and collagen synthesis occurred as well [86]. Finally, the total absence of chronic inflammatory responses suggested that BC is in fact biocompatible [86]. As a blood vessel replacement material for sheep, BC has also been reported to have biostability characteristics. The researchers found good blood compatibility, minimal inflammation, and stable functional markers that compared with the control [87].

As a result of the discovery of the promising characteristics that BC presents, there has been extensive research on its potential biomedical applications. For example, BC membranes are typically used as a wound dressing device that mimics the extracellular matrix to enhance epithelialization. During wound healing, multiple cell types, extracellular matrix molecules, and soluble substances interact dynamically [88]. Commercially available products include Biofill®,

Bioprocess® Gengiflex®, and Xcell® (Table 3). Cases of second- and third-degree burns, ulcers and decubitus could be treated successfully with Biofill® and Bioprocess® [13]. Recently this biosynthetic cellulose was successfully applied in experiments with dogs to substitute the dura mater in the brain [13]. Gengiflex® was developed to recover periodontal tissues by the separation of oral epithelial cell and gingival connective tissue from the treated root surface [13]. The BC membrane displays fast epithelialization and tissue regeneration rates in several wound-healing treatments, including diabetic foot wounds, chronic wounds and burns [13]. Also, BC membranes facilitate the removal of necrotic residues by donating fluid to hydrate the dry residue [85]. When the BC dressing is removed from the wound, the tissue is not damaged because BC does not adhere to the wound surface [89].

Table 3: Commercial Products, Applications, and Effects of Bacterial Cellulose Biodevices

Brand	Utilization	Treatment	Effects	Reference
Biofill®	Temporary skin substitute	Ulcers, burns	Pain relief, reduced infection, faster healing, etc.	[85], [90], [91], [13]
Gengiflex®	Dental implants, grafting material	Recovery of periodontal tissues	Reduced inflammatory response and fewer surgical steps required	[85], [90], [91], [13]
Bionext®	Wound-dressing	Ulcers, burns, lacerations	Pain relief, reduced infection, faster healing, etc.	[85], [90], [91], [13]
Membracell®	Temporary skin substitute	Ulcers, burns, lacerations	Fast skin regeneration	[85], [90], [91], [13]
Xcell®	Wound-dressing	Venous ulcer wounds	Pain relief, reduced infection, faster healing, etc.	[85], [90], [91], [13]

2.1.5 Challenges & Future Perspectives of Bacterial Cellulose

In tissue engineering and wound repair, BC is a versatile and indispensable biomaterial in the future of regenerative medicine. The simplicity of its production, lack of contaminants, and ease of manipulation are all reasons for the wide range of BC biomedical uses - such as the

crystallinity index, aspect ratio, and the morphology that can be adjusted to meet the demands of a particular application.

The lack of functional groups for anchoring bioactive compounds with therapeutic or diagnostic potential poses a major obstacle to the successful translation of BC into other clinical applications. Due to its supramolecular architecture, organized by its hydrogen-bonding network, the material is insoluble in both water and common organic solvents and inhibits an efficient functionalization of active chemical groups while retaining its biocompatibility [92]. Thus, an overall entrapment or grafting of several bioactive compounds would be difficult under mild conditions, for example with drugs, polyelectrolytes, or proteins. This poses a challenge in adapting BC to more advanced therapeutic applications [92]. With the combination of BC characteristics in addition to specially designed chemical groups, the process of cellular differentiation, growth, and treatment of specific diseases is enhanced [13].

Future applications of BC are already envisioned in the pharmaceutical and cosmetic industry to act as emulsion and hydrogel stabilizers that reduce the need for surfactants in Pickering emulsions, enzyme and biomolecules immobilization for enhanced activity and higher stability *in vivo*, as drug-delivery systems to alleviate drug uptake by targeted cells, anchoring of immunoglobulins and translation as low cost and portable devices for nano-engineered diagnostic sensors and as smart artificial skin or wound regeneration therapies [13]. Altogether, BC offers an inestimable matrix in the development of high-tech bio-platforms to diagnose and treat a wide variety of diseases.

2.2 Bioreactors

2.2.1 Bioreactors: An Overview

The term bioreactor broadly encompasses a wide range of devices used to optimize a biological process [93]. For this BC bioreactor, the focus of the design will be to improve its functionality by increasing the quality and quantity of BC production. The quality of the BC will be determined by pellicle uniformity while quantity will be based on mass. The use of a bioreactor unit allows many characteristics of the culture environment to be adjusted, intended to increase both BC uniformity and mass. Some of the characteristics which influence this BC yield include the percentage of crystallinity, BC yield, tensile strength, and so on. Each of these characteristics are dependent on the conditions of the culture, including oxygen concentration, carbon source, and temperature regulation [94]. Through the monitoring and adjustment of the culture conditions, bioreactors aim to improve the quality of their product.

There are two broad categories that capture most BC bioreactor methodologies: static and agitated models. In a static culture, the BC is produced along the air-liquid interface [95]. To produce consistent bacterial growth, specific temperature parameters must be maintained, and a carbon source needs to be present. There are two major setbacks with static culture methods. The first is that the oxygen and carbon source is depleted through the production of the culture, resulting in reduced BC production over time. The second is that the BC must remain still and uninterrupted during its formation process to retain structural integrity. To combat these limiting factors, many static bioreactor methods aim to replenish oxygen and carbon source levels without disturbing the air-liquid interface; they also tend to regulate environmental factors of the system such as temperature and humidity. Shaking or agitated models intentionally prevent BC from developing uniformly at the surface, to optimize the delivery of oxygen to bacteria during culture

[96]. Minimal agitation systems, such as an airlift model, allow for the development of more uniform pellets in comparison to more vigorous agitation models, such as the stirred tank, which rapidly disrupts BC at the air-liquid interface [97]. In general, agitation models result in smaller pellicles of BC compared to sheet-like pellicles in static bioreactors for the sake of increased yield.

For industrial-scale production, static and submerged fermentation has been deemed unfit due to its long culture time and high production costs [98]. To overcome these limitations, a bioreactor-based approach has been utilized. As previously mentioned, the production of BC occurs of the air-liquid interface, making it crucial to maximizing the size of said interface when designing a bioreactor. Ideal bioreactors should also address other drawbacks associated with agitated cultures. These drawbacks include mutations into non-BC producing phenotypes, oxygen transfer rates, and the final shape of BC produced [98].

2.2.2 Static Fermentation

2.2.2.1 Trickle Bed

One option for static culture is a trickle bed reactor which gradually introduces nutrients through liquid without disrupting the BC interface. A trickle bed reactor possesses a favorable alternative compared to static culture methods without the major drawbacks of agitated culture. A trickle bed reactor is a tank often made of stainless steel. It consists of equally spaced inlets used for air circulation powered by a generator (43). The fermentation liquid from the collection reservoir is moved to the top of the tank via a pump. The fermentation liquid then trickles down onto packings which effectively immobilize bacteria in a specific area to concentrate BC production. The continuous trickle of media constantly recirculates bacteria and cellulose onto these packing; media then returns to the collection reservoir where it is stored and often supplemented with nutrients (43). Absorption of bacteria onto the packings has been shown to be

an effective method for immobilizing due to the exposure to the fermentation liquid and air. The finished product can be periodically removed and replaced with fresh feed (43).

This method requires some specific instruments or devices including pumps and pressure regulation [99]. Internal pumps provide continuous flow from the bottom of the vessel back to the top of the trickle bed, this provides a continuous loop that reintroduces media to the packings [100]. Alternatively, multiple reservoirs can be used, which allows the separate vessel to be a storage for media. In this case, two or more pumps would be required to transport media and retain equal flow rates, otherwise, one vessel could overflow with media while the other runs dry. To combat this, Bioreactors often include a variety of sensors that are used to determine a variety of characteristics of the bioreactor's environment [101]. Measuring flow rate of the liquid is critical to know the amount of media in circulation. Specific conditions to BC may include DO and temperature as they can be continuously monitored internally. Temperature regulation is controlled with jacketed or internal coils located underneath and unattached to the packings (43). Additionally, when using methods involving air and liquid transfer, there must be the implementation of gas release valves as the pressure can build within the system.

Lu and Jiang evaluated BC production in a trickle bed reactor, primarily used in vinegar production, in comparison to BC produced from standard static and agitated culture. They are currently the first and only to report data on BC production for this type of reactor. Lu and Jiang found that BC produced with a trickle bed reactor had higher -OH associating degree, polymerization degree, purity, water holding capacity, porosity, and thermal stability compared to

BC produced in static and agitated culture (43). These results were attributed to the trickle bed reactor's ability to increase oxygen supply and decrease shear force during fermentation.

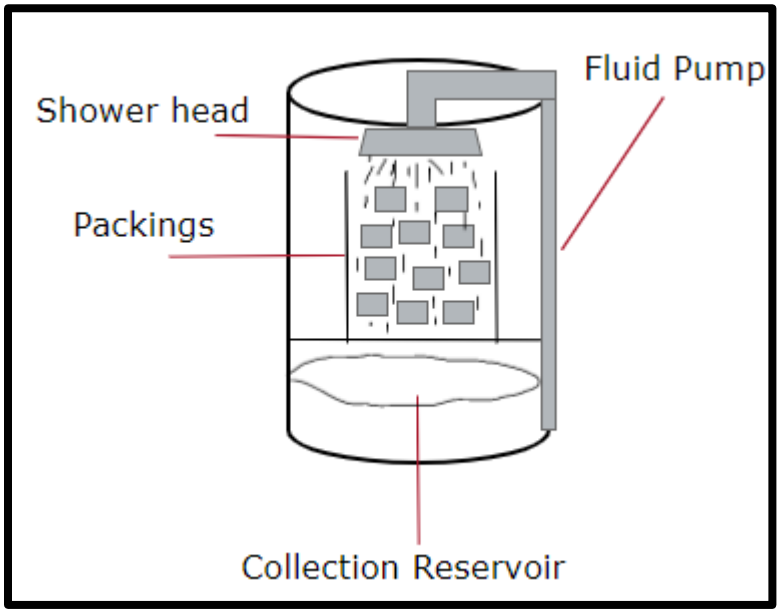

Figure 1: Trickle Bed Bioreactor

2.2.2.2 Membrane

Another type of static BC system is the membrane or biofilm method [102]. This method utilizes Plastic Composite Support (PCS), or other synthetic material comprised of polypropylene. Alternatively, silicon rubber can be used to adjust the mechanical properties of the film providing more rigidity to the system [103]. This PCS sheet provides shear strength along the fermenting surface, resulting in greater structural integrity while also providing a turgid layer to resist agitation on the air-liquid interface [102]. Finally, polypropylene allows the nutrient to be delivered to the culture surface more directly. One technique used to improve this method is by introducing oxygen from the bottom of the system (through the liquid) via a silicon membrane. The purpose of the membrane is two-fold; reduce the amount of potential agitation during BC development and provide a consistent replenishment of nutrients to the depleting system. Further application of the

membrane system aims to increase surface area of the air-liquid interface to increase BC production rate.

One example of a silicone static model uses four different vessels each paired with a variety of silicone shapes [103]. To determine how BC forms on different surfaces, the shape of the silicon membrane was manipulated. The purpose of this method is to determine how altering the membrane orientation changes the rate of absorbed oxygen and provides a change in surface area for the BC interface. The results of this method found that flat and thin sheets of silicone were preferable in the production of uniform BC pellicles [103]. Despite water retention of the BC being three percent lower than traditional static culture (98%), it was 22% greater than an agitated model [6]. What this method aims to prove is that when attempting to form a uniform sheet of BC the intended synthesizing surface must be flat and smooth, as opposed to contoured. In this example, a static model would be preferred for certain drug delivery applications for this generous increase in water retention when compared to an agitated culture [6]. A flat and smooth silicone sheet was able to provide the most advantage compared to the agitated. [103]. (Such as figure A in source [103]). Another mechanical advantage to having larger sheets of BC is the tendency to provide greater structural integrity and flexibility compared to agitated, which form BC particulates.

2.2.2.3 Aerosol

The aerosol method uses a tank of air to continuously introduce gases to the culture. Fine particles of carbon sources or oxygen are mixed into pressurized gases to make what is often referred to as a ‘nutrient broth’[104]. In one example, glucose with particle sizes of 0.5 um to 0.6um were combined with sterile air at 0.2 bar [104]. Utilizing this method allows for a slow release of nutrients, able to match the degradation of the nutrients in the culture [104]. This allows

the formation of the BC to remain linear over time rather than decreasing over time as expected if nutrients were not added.

One primary benefit of this system is a uniform distribution of nutrients throughout the entire culture body [104]. This theoretically provides the bacteria with more ideal growth conditions allowing increased growth rate, volume and provides a greater density. These outcomes would suggest desirable mechanical strength for many applications. Although, the greatest benefit of introducing the nutrients through the air is that the BC forms evenly throughout the entire interface [104]. In comparison to many other forms of bioreactors, the aerosol method provides strong formation of BC and provides manipulation of growth factors without agitation [104]. Although it can be one of the most laborious methods as constant monitoring of the nutrient broth is necessary; it also requires many initial steps such as sterilization of the pressurized vessel, dispersion of glucose and oxygen, and finally, careful calculations of nutrients release based on the expected consumption of the given culture [104].

2.2.3 Agitated Fermentation

2.2.3.1 Rotating Disk

The main goal of a rotating disk bioreactor is to produce BC with a homogeneous structure [96]. The general design consists of several circular disks connected to a central shaft with an inlet for inoculation [96]. With this design, the circular disks can rotate while interacting with the air-medium interface. BC produced with this method was able to generate a homogeneous structure, but its yield was not significantly greater than static culture methods [96]. This called for improvements in the design to be made.

The first reported BC production via a rotating disk bioreactor was in 2002. Serafica, Mormino, and Bungay produced BC with twice the water holding capacity of BC produced in

static culture. As the disk rotates between the liquid medium and air, the continuous alternation between the transport of nutrients and oxygen increases the formation of BC. They were able to have an average culture time of about 3.5 days to achieve a comparable yield to what they were able to produce with two weeks of static surface culture [61]. In this bioreactor design, various kinds of solids and fibers can be directly added to the liquid medium to form BC-based composites with ease [61]. This is an asset to this method, because it grants the ability to quickly reinforce BC composites for a variety of medical applications.

The next generation of rotating disk bioreactors utilizes plastic composite support (PCS) to aid in the production of BC [61]. The PCS was a composite material made of agricultural waste and nutrients [61]. It has been identified as an ideal surface for biofilm formation due to its ability to slowly release nutrients to attached microorganisms [61]. When used as solid support in a rotating disk bioreactor, results showed they BC can be produced in a semi-continuous process [61]. Bacteria can attach to the surface of PCS eliminating the need for inoculation. The productivity for this system averaged around 0.24 g/L/day. Compared to static culture, the BC procedure with this system shows comparable properties. It possessed a lower crystallinity of 66.9%and a young's modulus of 372.5 MPa in comparison to 88.7% and 3,955.6 MPa. Both samples had a relativity similar thermostability at 346°C and water content at 98.66 and 99.04 percent [61].

2.2.3.2 Stirred Tank

Stirred tank bioreactors are a widely used technology that offers a valuable alternative to traditional culture methods. The production of BC strongly correlates to volumetric oxygen transfer coefficients which allow for a stirred tank bioreactor to increase production rates. For example, a study testing a stirred tank reactor on *K. xylinus* was able to achieve a production rate

of 1.13 and 0.54 g/l at an agitation rate of 700rpm and 500rpm [105]. These bioreactors possess a high volumetric mass-transfer coefficient for oxygen transfer [105]. The agitation system and baffles in these bioreactors direct nutrient and oxygen mass transfer from the air-liquid interface into the bacteria cells [105]. Through the rapid mixing of the impellers within the agitation system, disperse gas bubbles can be broken down into smaller bubbles to facilitate faster production rates [105].

While rapid mixing corresponds to faster production, it possesses downsides for this project's applications. *K. xylinus*, the strain of bacteria used in our work, has been shown to be sensative to shear forces and a high stirring rate increases the shear stress. This promotes BC producing cells to turn into non- BC producing cells [105]. The mixing rate and high shear stress of a stirred tank bioreactor not only affects BC production but can also alter the physical and structural properties of the material [105].

2.2.3.3 Airlift

The airlift model prioritizes the low shear strength of the culture by gradually adding oxygen-enriched water to the lower portion of the system [106]. This process provides continuous nutrients to the system while minimizing agitation by allowing small volumes of oxygen to dissolve into the media over time. The continuous movement of the liquid caused by rising gas provides continuous mixing below the air-liquid interface without disrupting the culture production at the surface [107]. There are two methods of delivering oxygen to the bottom of the system, tubes connected to a pressurized oxygen tank or porous plates (such as wire) layered throughout the liquid both of which are designed to introduce 2 volumetric units of gas per 1 volumetric unit of gas in the system [108]. The airlift system results in BC production parallel to

that of the stirred tank model though by reducing agitation of the surface, a larger more uniform BC sheet can be produced [107].

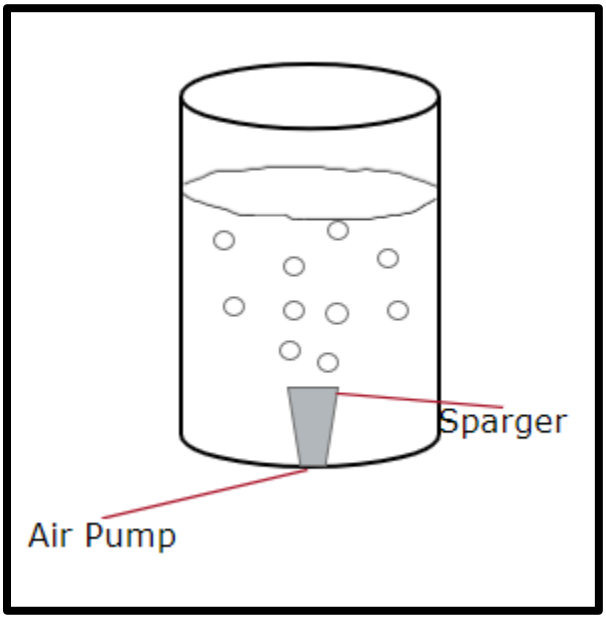

Figure 2: Airlift Bioreactor

One mechanical disadvantage to the airlift system is that it requires the liquid medium to be a low viscosity. When subject to viscous liquids, the rising gases will form channels, resulting in nonuniform air exposure at the BC surface. This results in not all the dissolved oxygen in the liquid being utilized in the system, and a reduction of structural integrity as some parts of the BC

surface will have nonuniform exposure[104]. To maintain homogenous formation, using a low viscosity will provide a uniform dispersion of diffused oxygen across the liquid surface [96].

Table 4: Summarized Comparison of Different Types of Bioreactors for BC Production

Reactor Type	Advantage	Disadvantage	References
Trickle bed Reactor	Increase oxygen supply and low shear stress	BC formed is not a homogenous pellicle	[43]
Membrane Reactor	Immobilizes cellulose and is easily integrated into static culture	Cellulose may adhere to membrane and be difficult to collect whole pellicle	[102], [103]
Aerosol	Provides DO to media increases overall BC productivity, low shear stress	High energy requirement and maintenance	[104]
Rotating Disk	High cell concentration; Good for aerobic strains	Semi-continuous production; higher risk of contamination	[109], [110]
Stirred-tank Reactor	High cell concentration and productivity; long-term production	Shear stress of cells; more mixing power needed	[60], [111], [112], [113], [114]
Airlift Reactor	High cell concentration and productivity; relatively low shear stress	High energy requirement; long biofilm establishment time	[115], [104], [116], [117], [118], [114], [119]

3.0 Project Strategy

The purpose of this chapter is to outline the considerations involved in the engineering design process and our project approach to designing a bioreactor for BC production. The team used information from the initial client statement, preliminary research, and meetings with the stakeholders to develop the design requirements. The design requirements are categorized as follows: constraints, objectives, functions, and specifications. The constraints define factors that restrict the design process. The objectives represent the "needs" and "wants" of the client in addition to those identified through background research. Functions and technical specifications were established to outline specific requirements to achieve their corresponding objectives.

3.1 Stakeholders

To ensure our bioreactor design is successful, the team must consider all the stakeholders participating in the project. These include the client, the user, and the design team. The client's words are important because they are the ones funding the project. User input is valuable given that they will constantly work with the device. The design team must take input both from the client and the user to create a feasible solution within given project constraints.

In this project, Jeannine M. Coburn, PhD, is the client and Elizabeth van Zyl is the user. The design team includes Isabelle Claude, Sawyer Fenlon, Edward Hay, and Catherine Williams. The project objective was given to the design team by Jeannine M. Coburn, PhD, given the need for an improved cultural environment which is intended to increase the production of BC. Graduate student Elizabeth van Zyl is the user of the product as a contributor to the Coburn labs which relies on BC for experimentation. Bacteria is currently limited by its inability to add nutrients and gases to the culture without disrupting the BC being produced. The goal of the design project is to

provide the user with a cultural environment that allows for the automated addition of nutrients and gases to sustain BC production without any limiting factors and interrupted cultures.

3.2 Initial Client Statement

The initial client statement is as follows: *"Design a low-cost bioreactor for automated addition of nutrients and/or gases directly into the culture medium that does not disrupt bacterial cellulose synthesis."*

The purpose of this project is to determine which characteristics of BC synthesis are most important for producing high yields of BC and designing a bioreactor that accommodates these conditions. Some of these conditions may include pH, dissolved oxygen concentration, or temperature. To achieve this, the team is required to design a vessel that can monitor the conditions of the bacteria culture and regulate them to achieve a better-suited environment. This will be accomplished by consistent and automated monitoring of the unit leading to the replenishment of the culture media. Nested within this initial statement is a variety of objectives that define how the team will measure success. The low-cost constraint is a direct correlation to the team's budget constraints, and the need for the device to be replicable for future lab uses. Automated addition of nutrients and gases requires maintenance and adjustment in culture conditions to improve the culture of BC by bacteria. This must be achieved through a static culture environment with the purpose of developing a homogeneous pellicle not interrupted by device agitation.

3.3 Constraints

As with any design project, there are a variety of constraints that limit the project. This section will take an in-depth look at some of the parameters which limit the group's research capabilities. Some of the broad categories which will be included in this section include but are not limited to the following: the stakeholder's budget for the project, the amount of time allotted

for the study, and the equipment and resources available in designated laboratory area. These constraints fall into two categories: either limiting factors during the experimental process which constrict potential avenues of design, or general restrictions which set beginning and end times for research deadlines.

Table 5: Constraints

High Level Constraints	Definition
Time	Completion of research, design and project Deliverables by April 2022
Investment	Each student is given an initial allowance of $250. $200 after Mammalian lab cost. Bringing groups total allowance of $800
Laboratory Availability	Lab spaces limited to Gateway labs for cellulose culturing and Goddard Hall for design and testing operations
Medium Level Constraints	**Definition**
Materials	Availability of components in designated lab areas, or within budget. Must be compatible with bacterial culture environment and allow visibility of culture conditions. Provides high durability and replicable, and no toxicity to culture or users.
Testing	Ability to consistently determine culture conditions using probes. Collection and testing of cellulose for mechanical properties
Sterility	Can be sterilized using existing/available methods such as UV, autoclave, or ethylene oxide.

The constraints listed in Table 5 above are broken down into two main sections: high-level and medium-level. High-level constraints are barriers to the project which are determined by WPI and stakeholders. These are limitations that are independent of the design and cannot be avoided with alternative methods. Medium level constraints are limiting factors that the team must work within, although these factors are highly dependent on the bioreactor design. Integral to the design

process, considering these constraints will help determine the function of the bioreactor and must be considered in the design comparisons to determine feasibility and convenience.

For example, materials for the project must fit within the design criteria and allow a cultural environment for the bacteria that is safe for the users. Specific materials such as glassware may be considered, as it satisfies many constraints including material availability and sterility but will also achieve many of the preferred design criteria. Although glass vessels may be less efficient in replicability and durability. These limitations will be considered in every aspect of bioreactor design with the intention of maximizing efficiency according to design analysis.

3.4 Objectives

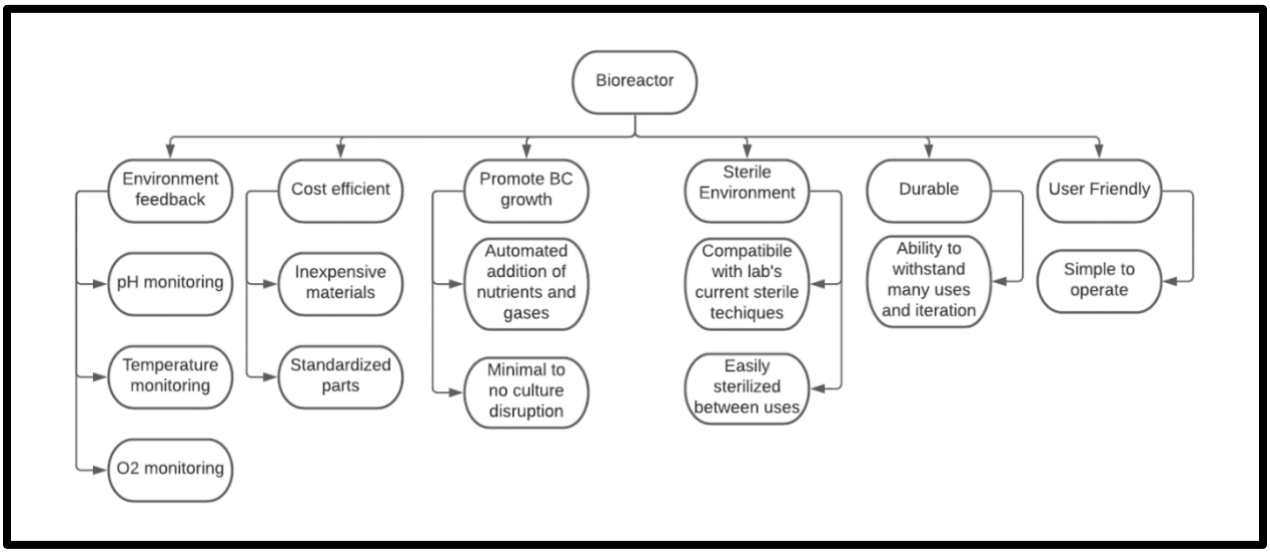

Figure 3: Objectives

As depicted in Figure 3, the six main objectives are environment feedback, cost-effective, BC Production, sterile environment, durable, and user friendly. These objectives as well as their sub-objectives are defined in the tables below.

Table 6: Main Objectives

Initial Objectives	Definition
Environment Feedback	Must be able to monitor environmental conditions within the bioreactor
Cost Efficient	Costs need to be within the designated budget and make use of inexpensive materials
Promote BC Production	Must provide a suitable environment and supplement BC production
Sterile Environment	Must provide an environment free of contaminants that can affect BC production
Durable	Must withstand many iterations of use without compromising functionality
User Friendly	Must be easy to use

Table 7: Environmental Feedback Sub-Objective Definitions

Sub-Objective Definitions: Environment Feedback	
pH monitoring	Must be able to check pH
Temperature monitoring	Must be able to store temperature data and display real-time feedback
Dissolved oxygen monitoring	Must be able to store DO data and display real time feedback

Table 8: Cost-Efficient Sub-Objective Definitions

Sub-Objective Definitions: Cost-Efficient	
Inexpensive materials	Parts required for the bioreactor must be inexpensive, capable of being purchased or already available to the user
Standardized parts	Parts required for the bioreactor must be reproduced with simplest

Table 9: Promote BC production Sub-Objective Definitions

Sub-Objective Definitions: Promote BC Production	
Automated addition of nutrients and gases	Must allow for the automated addition of nutrients and gases to aid in the production of BC
Minimal to no culture disruption	Needs to minimize or negated culture disruption so BC production is not inhibiting

Table 10: Sterile Environment Sub-Objective Definitions

Sub-Objective Definitions: Sterile Environment	
Compatible with labs sterile techniques	Design must be compatible with the sterility techniques currently available to the user
Easily sterilized between uses	Need to be simple to sterile in between uses

Table 11: Durable Sub-Objective Definitions

Sub-Objective Definitions: Durable	
Ability to withstand many uses and iterations	Materials must have sufficient properties to withstand several cultures of BC production

Table 12: User Friendly Sub-Objective Definitions

Sub-Objective Definitions: User Friendly	
Simple to operate	Design must be simple to set up (minimal prep time) and be easy for the user to work with

3.5 Quantitative Analysis of Objectives

To prioritize the objectives to determine the design specifications, a pairwise comparison was conducted by the stakeholders and the design team. The stakeholders were asked to compare each design criterion against each other and assign relative importance. A score of "1" meant that the design criterion in the column of the chart was more important than the design criterion in the row of the chart. A score of "0.5" indicated that the criteria were equally important, and a score of "0" meant that the objective in the chart column was less important than the objective in the chart row. Once these scores were assigned, the scores in each row were added together to calculate the

overall scores of each objective. The overall scores were then used to prioritize the design criteria from most important (1) to least important (6).

The final weighted objectives were calculated using the average scores of each objective that were assigned by the team, client, and user. These average scores were weighted to give greater emphasis to the user and clients' priorities. To do this, the average scores of each objective were multiplied by 0.4 for both the user and clients' responses. The average scores of the team's responses were multiplied by 0.2 to prioritize the responses of the user and client. The equation used to calculate the final weighted objectives shown in Table 17.

$$Weight = (0.4 * average\ of\ user) + (0.4 * average\ of\ client) + (0.2 * average\ of\ team)$$

Table 13: Design Team Pairwise Comparison

	Durable	Cost	User-	Promote BC	Sterile	Environment	SCORE
Durable	----------	0.5	0	0	0	0.5	**1**
Cost Efficient	0.5	----------	0	0	0	0	**0.5**
User-Friendly	1	1	----------	0	0	0	**2**
Promote BC Production	1	1	1	----------	0.5	1	**4.5**
Sterile Environment	1	1	1	0.5	----------	1	**4.5**
Environment Feedback	0.5	1	1	0	0	---------	**2.5**

Table 14: User(s) Pairwise Comparison

	Durable	Cost Efficient	User-Friendly	Promote BC Production	Sterile Environment	Environment Feedback	SCORE
Durable	---	0.5	0.5	0	0	0.5	**1.5**
Cost Efficient	0.5	---	0	0	0	0	**0.5**
User-Friendly	0.5	1	---	0	0	0	**1.5**
Promote BC Production	1	1	1	---	0.5	1	**4.5**
Sterile Environment	1	1	1	0.5	---	1	**4.5**
Environment Feedback	0.5	1	1	0	0	---	**2.5**

Table 15: Client Team Pairwise Comparison

	Durable	Cost	User-	Promote BC	Sterile	Environment	SCORE
Durable	---	0.5	0	0	0	0.5	**1**
Cost Efficient	0	---	0.5	0	0	0.5	**1**
User-Friendly	0.5	0.5	---	0	0	0.5	**1.5**
Promote BC Production	1	1	1	---	0.5	1	**4.5**
Sterile Environment	1	1	1	0.5	---	0.5	**4**
Environment Feedback	0.5	1	1	0	0	---	**2.5**

Table 16: Summary of Design Criteria Weightings

Design Criteria	Client	User(s)	Design Team	Total
Durable	1	1.5	1	3.5
Cost Efficient	1	0.5	0.5	2
User0Friendly	1.5	1.5	2	5
Promote BC Production	4.5	4.5	4.5	13.5
Sterility	4	4.5	4.5	13
Environment Feedback	2.5	2.5	2.5	7.5

Table 17: Final Weights of Design Criteria

Design Criteria	Weight
Promote BC Production	4.5
Sterility	4.3
Environment/feedback	2.5
User Friendly	1.6
Durable	1.2
Cost efficient	0.7

3.6 Functions and Specifications

Specific functions were identified to determine whether the bioreactor would successfully achieve the previously mentioned objectives. To promote BC Production, the promotion of uniformed pellicles of BC was essential. The next objective of user-friendliness was achieved by requiring a set-up time of under one hour. In addition, monitoring environmental factors for the formation of BC was prioritized as well as the ability for the bioreactor to remain sterile. Lastly, the objective of durability prioritized multiple fabrications use. Table 18 highlights a summary of the functional requirements, technical specifications, as well as the respective achieving objectives.

Table 18: Summary of Functions & Specifications

Objective	Functional Requirement	Technical Specification
Durable	**Capable of Withstanding Multiple Fabrication Use**	The materials used to manufacture the bioreactor should be able to withstand being reused up to 5 number of times.
User Friendly	**Require Under 1 Hour of Set-Up Labor**	The component parts of the bioreactor shall not exceed a total number of 100 components
Sterile Environment	**The Ability to Maintain Sterility**	Objects should be able to remain sterile for 7 days.
Promote BC Production	**Promote Uniform Formation of Bacterial Cellulose**	Produce a yield of 0.2 grams of cellulose
		90% of the produced BC is in the form of uniformed pellicles
Environmental Feedback	**Capable of Monitoring Environmental Factors for the Formation of BC**	Factors requiring passive monitoring must record respective levels and document them (via software) at least once every hour (24 times a day)
		Factors requiring iterative monitoring may only need to be documented (either via software or hand) every 12 hours (twice a day)

3.6.1 Function 1: Capable of Withstanding Multiple Fabrication Use

This bioreactor will be used for weeks at a time, cleaned, and sterilized for the commencement of a new experiment; thus, the material must be durable enough to withstand multiple uses. The material utilized for manufacturing should not degrade, rust, and be able to withstand sterilization conditions. The material is required to be thermally stable. This means it should be able to withstand high-pressure cleanings and survive undergoing sterilization techniques without material degrading or rusting and should be able to resist heat. The bioreactor should be able to withstand being reused up to five times. This means it should be able to withstand being cleaned, sterilized, and reused for experiments of up to five iterations without compromising the material integrity. The primary material utilized for the base of the bioreactor should be able

to withstand being adjusted (opened, closed, slightly moved, etc.) without fear of disrupting the static culture. In other words, the structure should be durable enough that its contents should not be easily disrupted upon careful and slight handling. Since materials do not last indefinitely, tests will be conducted to determine the number of iterations each component can withstand before needing to be replaced.

3.6.2 Function 2: Require Under One Hour of Set-Up Labor

One of the primary objectives that the bioreactor must achieve is to be determined "user-friendly". The number of parts needed to assemble the bioreactor should be kept to a relative minimum to reduce the amount of time and complexity required to disassemble and reassemble the bioreactor. An abundance of smaller pieces increases complexity significantly, as the chance of misplacing a component becomes greater. Each component used will be large enough to grab or hold easily. Being able to lay out all the components of the device will reduce the total setup time as it will be easier to keep track of each piece. Reducing component amount and increasing size should not require much effort or time to disassemble and reassemble the device. Components and program selection should be user-friendly.

3.6.3 Function 3: The Ability to Maintain Sterility

An essential functional requirement is that this bioreactor will have the ability to be and remain sterilized. Sterilization destroys all microorganisms on a part or in a fluid to remove contaminants associated with the use of that item. It is imperative that the bioreactor is made from materials that can undergo sterilization such as autoclave or ethylene oxide so that harmful microorganisms do not contaminate the culture. It is also important that the materials used to construct the bioreactor do not inherently damage the culture environment over time. Even a material that can be effectively sterilized may not be able to withstand a week or more of constant

moisture and result in leaching. The bioreactor itself, once assembled, will have to have the ability to remain sterile. All the materials used should not be porous in that there is no way for harmful microbes to enter the device once assembled. The inside of the bioreactor should be able to maintain sterility for at least the duration of each iteration (7-10 days) before it is recommended that a new experiment commences in a freshly sterilized device.

3.6.4 Function 4: Promote Uniform Formation of Bacterial Cellulose

The bioreactor must be capable of promoting the formation of large quantities of uniformed BC. To ensure that the bioreactor promotes BC production, the bioreactor would have to produce a yield of 0.2 grams of cellulose, approximately 90% of the produced BC would need to be in the formation of uniformed pellicles. This indicates that 90% of the produced pellicles must be uniformly shaped, without any holes or clumps. Yield, on the other hand, is defined as mass-produced with respect to the volume of the medium. Other technical specifications that must be considered for the promotion of uniformed production include the evaluation of the toxicity and biomaterials involved in the manufacturing of the bioreactor. The bioreactor must be made of materials that are not composed of or release any toxic substances. These materials must also be biocompatible in that they do elicit little or no negative host responses.

3.6.5 Function 5: Capable of Monitoring Environmental Factors

An imperative requirement of the design of this bioreactor is its capability to maintain and monitor specific environmental factors such as pH, temperature, oxygen, and glucose levels. The device should be able to monitor these nutrients/gases over the time of production and be able to provide feedback to the user on whether levels should be increased or decreased depending on formation rates and production needs. Technically, the bioreactor should produce continuous feedback of oxygen and temperature. Other factors of feedback such as glucose and pH may be

provided iteratively. To ensure this aspect of the bioreactor, factors requiring passive monitoring must record respective levels and document them (via software) at least once every hour (24 times a day). Factors requiring iterative monitoring may only need to be documented (either via software or hand) every 12 hours (twice a day).

3.7 Engineering Standards

To ensure the safety and functionality of the bioreactor and the produced cellulose, we will need to adhere to standards set forth by the International Organization of Standardization (ISO). ISO is an independent organization that develops standards to ensure the quality, safety, and efficiency of products. The standard 16197:2014 describes methods that can be used to assess the toxicology of engineered and manufactured nanomaterials. This standard is applicable for the device because the BC is intended to interact with cells for biomedical applications. These methods will allow us to conclude that BC is nontoxic.

To ensure the sterility of the bioreactor and the bacteria cellulose that is produced, ISO 11737 must be followed. All cell culture practices, and material handling will be performed in a sterile biosafety cabinet or in the sterile bioreactor. The tools and bioreactor will be autoclaved prior to use. In addition, any materials that encounter cells were sterilized.

Regulations relating to the instruments and sensors used in regulating the environment of the bioreactor must be followed as well. 21CFR114.90 describes methods used to determine the pH and the conditions in which to measure the pH. It also describes the accepted accuracy of the pH meter. IEC 751 defines the acceptable parameters for Platinum RTD sensors including base resistance, alpha, applicable range, resistance tolerance, and temperature deviation. IEC 751 and 21CFR114.90 both describe techniques to calibrate the sensors and ensure their proper functions.

The user manual associated with the Vernier Optical DO probe describes methods for the proper use and calibration of the device.

3.8 Revised Client Statement

The revision of the client statement ensures that the needs of the stakeholder and the constraints of the project are both being considered in the final project. After consideration of the client and the project objectives the revised client statement is as follows:

"Design a low-cost bioreactor for automated addition of nutrients and/or gases directly into the culture medium that does not disrupt bacterial cellulose synthesis. Then characterize the resulting solute concentration in the bioreactor fluid phase and the physical properties of the BC produced. Providing a greater production rate of bacterial cellulose while retaining or improving mechanical and chemical characteristics."

4.0 Design Process

After our team completely understood the needs of the client and user, our project strategy was developed. The project strategy outlined a plan for the work that needs to be done to meet the goals of the project. This started with an analysis of the needs of the client, user, and design team. Required functions and the means to reach these functions were also developed. Once all the possible means were evaluated regarding design specifications, the final design was constructed.

4.1 Needs Analysis

Chapter 3.5 outlines the ranked objectives based on their perceived importance to the success of the project by the client (Professor Coburn), the user (Elizabeth van Zyl), and the design team. The concluded percentages were calculated by dividing the determined weights of each respective objective by the total of all the weights. The objectives are listed in order of importance based on the percentage of the total points in Table 19.

Table 19: Ranked Objectives by Percentage

Objective	**Percentage**
Promote BC Production	30.41%
Sterile Environment	29.05%
Environment Feedback	16.89%
User-Friendly	10.81%
Durable	8.11%
Cost Efficient	4.73%

The needs and wants of the project were established once the objectives were quantitively analyzed. “Needs” refers to the properties that the stakeholder and user deemed essential for the integral success of the resulting project while “wants” refers to attributes that would be desirable

to have but may not be possible given some constraints. The design team determined which objectives would be converted to needs and wants based on the ranked objectives. Typically, only the top-ranked objectives would be used in creating needs but because we only have six objectives and have deemed them all important, every objective except for the last lowest-ranked objective will be translated into needs. The objective of cost-efficiency would become a want. It is important to note that needs are required while wants would significantly improve the design of the overall final project, but if not included would not depreciate the overall success of the project. Without the implementation of the wanted objective, the project would still achieve success.

4.2 Design Means

A list of design means was determined by the design team for each established function (found in section 3.6). Design means are alternative ways in which the materials utilized to manufacture the bioreactor could fulfill each functional requirement.

The process by which means were identified and evaluated is as follows: Once identified utilizing the list of functional requirements, each means was compared to the list of constraints. If the means did not pass the constraints test, then the specific means were no longer considered a viable option. The constraints test was a process of evaluating whether the usage and implementation of specific means were feasible. Appendix A depicts a list of all the means and showcases whether each has passed the constraints test. Each means was then evaluated further

pros and cons lists were developed for each. The resulting means that have passed the constraints test are depicted in Table 20 below.

Table 20: Means for Each Function

Function 1: Capable of Withstanding Multiple Fabrication Use	Function 2: Require Under 1 Hour of Set-Up Labor	Function 3: Ability to Maintain Sterility	Function 4: Promote Uniform Formation of Bacterial Cellulose	Function 5: Capable of Monitoring Environmental Factors
Stainless Steel	Arduino	Autoclave	Polyethersulfone Filter Membrane (PES)	Optical Dissolved Oxygen Probe
				Galvanic Oxygen Probe
Glass	LabQuest	Ethylene Oxide	Teflon/Nylon	Stainless Steel Temperature Probe
				Thermometer
3D Printed PLA	Logger Pro	Ethanol & PBS	Wire Mesh	pH Strips
				pH Probe

Consideration of these design mechanisms is a continuous process that occurs from beginning to end of design iterations. For example, when the team considered durability and the possible materials which could be used for the bioreactor's construction, stainless steel was the first to be considered due to its high impact and corrosion resistance; it also satisfies many other designer criteria. Despite this, it is still essential to consider other materials and their benefits in order to ensure that the design is being optimized. The remainder of this chapter will analyze each of the design mechanisms above, indicating how design means were considered and why they were ultimately replaced by a more suitable choice.

4.2.1 Means of Durability

Stainless Steel

Stainless Steel is a high-performance construction material that combines the stiffness associated with ferrous alloys with the corrosion resistance derived principally from the high chromium content [120]. The corrosion resistance of stainless steel is primarily attributable to its

chromium content [121]. On exposure to oxygen, the chromium in the steel reacts to form a thin, chromium oxide film over the surface of the material which provides resistance to corrosion [121]. The requirement of no corrosion protection leads to low-life-cycle costs, reduced environmental impact and extended design lives. Further benefits include high ductility, impact resistance, and fire resistance [122]. The principal drawback to stainless steel is the material cost – approximately four times that of carbon steel [122].

Table 21: Pros and Cons of Using Stainless Steel to Promote Durability

Pros	Cons
Corrosion resistance	Expensive
High ductility	Opaque
High impact resistance	
High fire resistance - high melting point (1510 degrees Celsius)	
Minimal maintenance	

Glass

Glass equipment provides unsurpassed corrosion resistance to saline solutions, organic substances, halogens such as chlorine and bromine, alkaline mixtures, and many acids (nitric acid, sulfuric acid, hydrochloric acid) [123]. Its resistance to chemical attack is superior to that of most metals and other materials, even during prolonged periods of exposure and at temperatures above 100 °C [123]. Glass procures an anti-adhesive and nonporous surface that resists the buildup of viscous or sticky products [123]. Due to this quality, there is much ease in cleaning glass. Lastly,

unlike most plastic and metal materials, glass equipment provides transparency [123]. An unobstructed view of the experiment which enhances the observation of the process is essential.

Table 22: Pros and Cons of Using Glass to Promote Durability

Pros	Cons
Corrosion resistance	Fragile
Transparent	
Easy to clean	
Hard & brittle	

3D Printed Polylactic Acid (PLA)

PLA is a commonly used plastic for 3D printing because it is inexpensive. One main advantage of PLA applications is that it is biocompatible, meaning that it is compatible with a living system by not being toxic or injurious and not causing immunological rejection [124]. Despite this advantageous quality of PLA, there are several disadvantages of the application of 3D printed PLA in a bioreactor application. For instance, PLA has low heat resistance, and therefore cannot be used for high-temperature applications [125]. In high temperatures, PLA can rapidly deform, especially if under stress. PLA has a higher permeability than other plastics [125]. Moisture and oxygen will go through it easier than other plastics. Lastly, PLA is not the hardest or toughest plastic. PLA is not necessarily suitable for applications where toughness and impact resistance are critical [125].

Table 23: Pros and Cons of Using 3D Printed PLA to Promote Durability

Pros	Cons
Inexpensive	Low heat resistance
Biocompatible	High permeability
	Not that hard in comparison to other plastics

Zinc Alloy

Zinc is a metal often used in many biomedical applications due to its biocompatibility. One advantage of using zinc in most biomedical applications is because of its ideal corrosive

behavior and biodegradability under physiological conditions [126]. Unfortunately, due to the nature of this project, those typically favorable characteristics present disadvantages for the design of this bioreactor. Materials that are resistant to corrosion and degradation are highly favored in this manner as they will be reused several times and for a prolonged period. Another disadvantage of zinc materials is their low mechanical properties compared to other metals. Pure zinc obtains a Young's Modulus of approximately 108 GPa compared to stainless steel's value of 189-205 GPa [126]. Its tensile strength is also significantly lower, acquiring a value of 88 MPa compared to stainless steel's tensile strength of 490-695 MPa [126].

Table 24: Pros and Cons of Using Zinc to Promote Durability

Pros	Cons
Biocompatible	Highly corrosive
	Highly biodegradable
	Low mechanical properties

Polymethyl-methacrylate (PMMA) or Acrylic

Acrylic Polymethyl-methacrylate (PMMA) is a common polymer used in many biomedical applications due to its favorable working characteristics including mechanical strength, biocompatibility, and the fact that it is inexpensive [127]. Acrylic is a strong ad lightweight material. The density of acrylic ranges between 1.17-1.20 g/cm^3 which is half less than that of glass [128]. The impact strength of acrylic is greater than that of glass and polystyrene [128]. The primary purpose that it serves is to provide good quality transparency in various glass components. Acrylic can transmit up to 92% of visible light when 3 mm in thickness (128). Other advantages

of acrylic include the fact that it is easily processed, optical clarity, UV resistance, surface hardness, and good chemical resistance [128].

Table 25: Pros and Cons of Using Acrylic PMMA to Promote Durability

Pros	Cons
Transparent (Excellent Aesthetics)	
Favorable Working Characteristic	
Adequate Mechanical Strength	
Inexpensive	
Biocompatible	
Lightweight	
Shatter Resistance	
Hydrophobic	

4.2.2 Means of User-Friendliness

Arduino Circuitry

An important advantage of utilizing circuitry is that there is a multitude of resources available on how to set up and use circuits for basic functions [125]. Using these resources such as an Arduino database provides instruction and guidance on circuits for a specific purpose such as controlling the rate increments of pumps and having safety triggers to turn off pumps or other motors. For our bioreactor design, the team will focus on the flow rate of the media transport which can be modified primarily with voltage and resistance split between the pumps. Another benefit of using breadboard circuits is that they are inexpensive and can be modified with just a few simple

components. Some of the limitations to the circuitry is the reliability and durability of the components, especially when used for extended periods of time.

Table 26: Pros and Cons of Using an Arduino for Minimal Set Up Labor

Pros	Cons
Inexpensive	Difficult to find codes and set ups for specific processes and interfaces
Easy to set up	
Provided manuals for use	
Abundant online resources	

LabQuest

LabQuest is a data collection interface that has a built-in graphing and analysis application. This interface is simple to use in that it only requires the effort of connecting it to a sensor and a commuter device and running compatible analysis software. The definition of "plug and play". The LabQuest devices are also affordable. The only disadvantage proposed by the LabQuest interface is that it needs to be connected to a device (computer, tablet, or any other mobile device through a LabQuest interface), for it to monitor and provide continuous feedback.

Table 27: Pros and Cons for Using LabQuest for Minimal Set Up Labor

Pros	Cons
"Plug and Play"	Requires computer device for continuous monitoring
Inexpensive	

Logger Pro

Logger Pro is a data collection and analysis software that is used in conjunction with other device interfaces. For example, Logger Pro is one of the programs that is compatible with LabQuest. An advantage of this specific software is that it is available at no cost because the university has a subscription. Continuous temperature and dissolved oxygen concentration data can be obtained and recorded via Logger Pro. Continuous monitoring requires a one-time set up and therefore minimal set up labor. Familiarity with the software combined with continuous

monitoring satisfies the user-friendly objective. Like LabQuest, since this program is run via software, a device will need to be acquired for monitoring and continuous feedback.

Table 28: Pros and Cons of Using Logger Pro for Minimal Set Up Labor

Pros	Cons
Available for no cost through university	Requires additional computer for continuous monitoring
Continuous monitoring requires a one-time set up	

4.2.3 Means of Sterility

Autoclave

An autoclave is a machine that uses high temperature and pressurized steam to kill harmful bacteria, viruses, fungi, and spores on items that are placed inside a pressure vessel [129]. The items are heated to an appropriate sterilization temperature for a given amount of time [129]. The moisture in the steam efficiently transfers heat to the items to destroy the protein structure of the bacteria and spores [129]. Autoclaves use a combination of steam and pressure at high temperatures to sterilize most items in 3 to 15 minutes [130]. A limitation of an autoclave is that it cannot be overloaded. If overloaded, steam can't reach all surfaces, and microbes and spores can survive [130]. Safety can also be an issue when opening the autoclave due to the intense heat and pressure inside [130]. Lastly, although the high pressure and temperature of the autoclave is an advantage in killing off bacteria, it is a disadvantage in terms of the materials that can be placed within the autoclave for sterilization. Materials that obtain a decomposition temperature of that of the temperature used in autoclaves cannot be sterilized via an autoclave. While utilizing the

autoclave sterilization method, it is essential to ensure the material will not be compromised by the heat and temperature.

Table 29: Pros and cons for using autoclaving to sterilize the bioreactor and its components

Pros	Cons
Can sterilize almost all materials	If an autoclave is overloaded, it can be ineffective
Only takes about >15 minutes	Safety
Inexpensive	Only instruments and plastics which can bear the heat can be sterilized

Ethylene Oxide

Ethylene oxide (EO) is a low-temperature gaseous process widely used to sterilize a variety of medical devices [131]. Its lethality is driven by a chemical reaction (alkylation) with the DNA of bacteria, viruses, molds, and yeasts [131]. The benefits of EO are that it can sterilize heat-sensitive or moisture-sensitive devices, meaning it does not cause any deleterious effects on the materials used that otherwise be unable to handle autoclave sterilization [131]. EO has also been proven to have high efficiency rates as well as being a large chamber to hold large volumes of items that need to be sterilized [131]. In contrast to autoclave sterilization, EO requires a long cycle of about 2.5 hours excluding aeration time and is expensive to maintain and service [131]. Additionally, EO presents several safety concerns as it is carcinogenic to humans [131].

Table 30: Pros and cons for using ethylene oxide to sterilize the bioreactor and its components

Pros	Cons
Sterilize heat-sensitive or moisture sensitive devices (non-corrosive to plastic)	Excessively long cycle
High efficiency	Expensive cost
Large sterilizing volume	Potential hazards to handlers - carcinogenic

Ethanol and PBS

Ethanol is an effective disinfectant at concentrations between 60% and 80%. The benefit of using a combination of ethanol and PBS to disinfect a surface or instrument is that it is fast-acting against bacteria and fungi [132]. It is also non-corrosive to most materials [132].

Unfortunately for this mean, the cons outweigh the pros, in that ethanol and PBS do not kill spores, it is not registered by the environmental protection agency (EPA) as a safe and effective sterilization method, and lastly it evaporates very quickly [132]. Disinfectants rely on contact time to be effective. Alcohol solutions tend to evaporate quickly when applied to surfaces which then limits the contact time [132].

Table 31: Pros and cons for using ethanol and PBS to sterilize the bioreactor and its components

Pros	Cons
Fast acting	Not sporicidal
Non-corrosive	Not EPA registered
	Evaporates rapidly

4.2.4 Means of Uniformed Formation of BC

Filter Membrane (PES)

Polyethersulfone (PES) membranes are hydrophilic and exhibit low protein binding [133]. They are resistant to high temperatures and acids and bases [133]. Their pore uniformity allows for predictable flow rate and particle retention, which facilitates uniform pellicle formation with predictable properties [133]. PES membranes are durable and can withstand the automated culture process [133].

Table 32: Pros and cons for using PES to promote uniform formation of bacterial cellulose

Pros	Cons
Nontoxic	Disposable
Inexpensive	
Hydrophilic	

Teflon/Nylon

Hydrophilic polytetrafluorethylene (PTFE) filter membranes such as Teflon and nylon are effective in collecting bacteria for the formation of BC [134]. PTFE filter membranes are available in a variety of pore sizes and are inexpensive. Furthermore, they can endure the weight of the BC and moisture of the bioreactor without degrading [134]. PTFE filter membranes can absorb more

bacteria than PES filters, which will result in a greater yield of BC [134]. However, PTFE is more expensive than PES filters and has a higher potential for toxicity because it can release mildly toxic fumes when overheated [135].

Table 33: Pros and cons for using Teflon/nylon to promote uniform formation of bacterial cellulose

Pros	Cons
Available in a variety of pore sizes	Cannot be sterilized via gamma radiation
Durable	More expensive
Can be autoclaved	Can release mildly toxic fumes when overheated
Hydrophilic	Disposable

Wire Mesh

Wire mesh is a durable material that is resistant to deterioration and can be reused [121]. However, the large pore size is not favorable for the formation of BC because bacteria will not accumulate on the wire mesh, it will flow through. This would result in a low yield of BC.

Table 34: Pros and cons for using wire mesh to promote uniform formation of bacterial cellulose

Pros	Cons
Durable	More expensive
Reusable	Hydrophobic
	Too porous

4.2.5 Means Monitoring Environmental Factors

Optical Dissolved Oxygen Probe

Optical dissolved oxygen sensors are luminescence-based sensors that consist of an oxygen-sensitive membrane and an optical system [136]. Since they don't consume oxygen, optical dissolved oxygen sensors do not require a reference electrode [136]. Therefore, no calibration or filling solution is required, ultimately requiring little maintenance [136]. Optical

dissolved oxygen sensors are affected by stirring speed or flow rate, so they do not require constant stirring and will not be affected by fluctuations in flow rate of the bioreactor [136].

Table 35: Pros and cons for using an Optical Dissolved Oxygen Probe to allow for the monitoring of environmental factors

Pros	Cons
No calibration	Slower response time
No warmup time	More expensive
No filling solution	
No stirring	
Less maintenance required	

Galvanic Oxygen Probe

Galvanic oxygen sensors are electrochemical sensors that use two dissimilar metal electrodes (typically silver and lead) which are consumed during oxygen measurement [136]. Although the optical dissolved oxygen probe and the galvanic oxygen probe are both similarly accurate at peak performance, the accuracy of the galvanic oxygen probe diminishes over time as the electrolytes are depleted [136]. The galvanic oxygen probe requires more maintenance as the electrolytes need to be replenished every few weeks to ensure accuracy [136]. In addition, the process of using the galvanic oxygen sensor is more time consuming as constant stirring is required during measurement [136].

Table 36: Pros and cons of using a Galvanic Oxygen Probe to allow for the monitoring of environmental factors

Pros	Cons
Doesn’t require voltage source	More maintenance required
No warmup time	Electrolytes deplete over time
Faster Response time	Less accurate over time
	Requires stirring
	More time consuming

Stainless Steel Temperature Probe

The resistance temperature detector (RTD) probe measures temperature by changing resistance proportional to the temperature [137]. RTDs have a positive temperature coefficient,

which means that as temperature rises, resistance rises [137]. Since it is made of stainless steel, it is durable and unlikely to corrode or rust. There are three types of RTDs: platinum, nickel, and copper [137]. Platinum RTDs are corrosion resistant, have long term stability, and can measure a wide range of temperatures [137]. Nickel RTDs are less expensive than platinum and have good corrosion resistance [137]. However, nickel ages more quickly over time and is sensitive to higher temperatures [137]. Copper RTDs are the most resistant to temperature linearity and low cost [137]. However, copper oxidizes at higher temperatures [137].

Table 37: Pros and cons for using continuous temperature measurements to allow for the monitoring of environmental factors

Pros	Cons
Time efficient	More expensive than manual monitoring
More accurate	

Thermistors

Like RTDs, thermistors operate based on the idea that resistance changes with temperature [138]. However, thermistors employ metal oxide and have a negative temperature coefficient [138]. Thermistor digital thermometers are an inexpensive method of measuring temperature [138]. However, thermistors are less accurate than RTDs [138]. Thermistors have a higher sensitivity than RTDs, so they can measure changes in temperature in smaller increments [138].

Table 38: Pros and cons for using manual temperature measurements to allow for the monitoring of environmental factors

Pros	Cons
Less expensive than continuous monitoring	Time consuming
Durable	Less accurate
High sensitivity	

pH Strips

pH strips are an inexpensive way to measure pH at a point in time [139]. When the strip is placed in the medium, it changes color based on the acidity of the solution [139]. This color can be compared to a chart that indicates what color corresponds to what numerical value of pH [139].

Because the color is relative, it is up to interpretation of the individual measuring the pH [140]. This results in lower accuracy than a pH meter. pH strips can only be used for a single measurement, so they are not suitable for continuous pH monitoring [140].

Table 39: Pros and Cons for Using Manual pH Measurement to Allow for the Monitoring of Environmental Factors

Pros	Cons
Inexpensive	Time consuming
Does not require calibration	Less accurate
	Disposable

pH Probe

pH probes are more accurate than pH strips and can be used for continuous measurements [139]. Continuous pH monitoring with a pH meter is less labor demanding than manual pH monitoring with pH strips. The pH probe has an attached electrode that measures the voltage of the solution and converts it to a numerical pH value [139]. However, they are more expensive and require periodic calibration and cleaning to ensure their accuracy [139].

Table 40: Pros and Cons for Using Passive pH Measurement to Allow for the Monitoring of Environmental Factors

Pros	Cons
Time efficient	More expensive
More Accurate	Requires Calibration
Reusable	More maintenance

4.3 Evaluation of Design Means

An assessment and consideration of the design criteria above determined the final means implemented into the final prototype. To satisfy function 1, the body and vessels of the bioreactor will be constructed from glass. Glass provides many of the basic physical needs of the design including reusability, visibility, and ease of sterilization. One of the greatest advantages to using glass is that there is a large variety of sizes of glass vessels, many of which with universal openings such as mason jars; this means that the rest of the constructed materials can function on widely available glassware and allows the construction of the device to be tailored to a specific though

widely available size vessel. In comparison to PLA, glass is the most ideal because of its ability to resist wear and can be easily sterilized. In comparison to metals such as zinc alloy or stainless steel, a glass vessel improves visibility allowing for observational analysis of the media. Thick glassware can be used to easily compensate for glass' tendency to be fragile.

In addition to the glassware, scalable and custom parts will be constructed from PLA. These components are designed to fix other parts in place and allow for a low-cost construction. Constructing the lids from glassware would not be a viable option, as the ability to manipulate and shape small glass components is difficult and costly as compared to 3D printing PLA. For a more permanent setup, custom machined stainless-steel lids could be used to improve the durability of the design. Another viable option for custom or machine parts is acrylic as it provides greater durability and can be easily sterilized. One of the greatest benefits of acrylic is its relative hydrophilicity, which could lead to strong adhesion of media to a BC production surface.

When considering the user-friendliness of the design, the main consideration is the data interface between the user and the sensors. Interfaces have high variability in terms of complexity and cost, optimizing this function is critical for giving the client a functional bioreactor that provides consolidated feedback while staying within an acceptable price range. Other considerations with the interface mechanism are sensitivity and setup time for all the components necessary. To optimize the motorized components, testing, coding, and installment of the Arduino boards can be predesigned, becoming plug and play for the client. Once a code has been installed on an Arduino, it no longer requires any adjustments unless desired. Recycling or adjusting pre-scripted code requires little background experience. For the use of motorized components, the use of breadboard circuitry is relatively simple in construction and provides effective utility in transporting media. When considering the two primary sensors, the dissolved oxygen (DO) and

temperature probe with a display are more effective as it provides a more accurate depiction of the cultural environment and retains long-term storage of the collected data. For this interface, the use of a LabQuest mini and LabView display software provides continuous updates on the bioreactor's internal environment. It records and stores data over long periods. These technologies require minimal setup time and automatically calibrate to the sensor, providing automated feedback at designated time intervals during the entire duration of each test.

Another type of environmental feedback which could be installed on either the breadboard or work independently is pH monitoring. Background research and client need indicated that these factors are less critical than temperature and DO. They can be manually measured and tested intermittently throughout a culture experimentation to measure internal conditions effectively and quickly. For lower priority environmental conditions, it is more ideal to limit external components permanently installed on the bioreactor, if they are only going to be used sparingly. For these factors, it is suggested to use daily pH strips and add glucose through the external ports.

Finally, to capture and reliably develop cellulose pellicles, there must be a uniform surface that collects the bacteria in the media. A variety of organic and inorganic methods were considered although the reliability and sterility of all other options were too significant to overlook. A porous membrane such as PES provides highly regulated pores, allowing liquid and media to drain, although tiny particles of cellulose mixed. Additionally, this surface provides a smooth layer which should give easier detachment of cellulose in comparison to the alternatives or PLA. Table 41 illustrated below is a finalized version of all the design means, color coded to indicate which

materials will be considered for use (green), which will be considered (yellow), and which will not be used at all (red).

Table 41: Finalized List of Design Means and Their Feasibility Criteria

Function 1: Capable of Withstanding Multiple Fabrication Use	**Function 2:** Require Under 1 Hour of Set-Up Labor	**Function 3:** Ability to Maintain Sterility	**Function 4:** Promote Uniform Formation of Bacterial Cellulose	**Function 5:** Capable of Monitoring Environmental Factors for the Formation of BC
Stainless Steel	Arduino	Autoclave	Filter Membrane (PES)	Optical Dissolved Oxygen Probe
				Galvanic Oxygen Probe
Glass	LabQuest	Ethylene Oxide	Teflon/Nylon	Stainless Steel Temperature Probe
			Wire Mesh	Thermometer
3D Printed PLA	Logger Pro	Ethanol & PBS	Corn Husks	pH Strips
				pH Probe

4.4 Feasible Designs

Once the team created the project strategy by compiling the constraints, objectives, functions, and specifications according to the goals of the project, preliminary designs were created. First, the team evaluated which bioreactor types would be suitable for the project. Once the ideal bioreactor type was identified, several iterations of prototypes were designed and created to produce our final design.

4.4.1 Evaluation of Feasible Bioreactor Types

In conjunction with the means evaluations, an analysis of the types of bioreactors was conducted to determine which type of bioreactor would be best suited to explore. This analysis will provide specific aspects of existing bioreactor models to be inherited into this design. The

following bioreactor types were analyzed for their feasibility in manufacturing as well as their likelihood of fulfilling the established objectives: trickle bed, aerosol, membrane, rotating disk, stirred tank, and airlift. In analyzing all the respective advantages and disadvantages (explained in detail in sections 2.2.2 & 2.2.3) for each bioreactor type, the team has determined that to achieve our core objectives the design of a combination of an airlift, trickled bed, and the membrane bioreactor should be explored. The airlift bioreactor inspired this design as it provides a sufficient method of delivering dissolved oxygen to the media. The filter membrane inspired the idea of using a PES membrane.

The main influence of trickle bed bioreactors was in its ability to cycle media and nutrients throughout the system. The trickling reduces agitation to the BC produced in comparison to other moving models, but still allows continuous regulation of media and nutrient exposure. The main advantage of this method is that it allows nutrients to be supplemented without disrupting the air-liquid interface. In a simple comparison to static culture, this should be an immediate benefit, as the pellicle formation method is the same, though it has the potential to produce a larger yield as a result of more nutrients exposure. Rather than pumping a pool of media into the bottom of a single reservoir, the team utilized a two-vessel design. The first vessel (the collection vessel) is the primary bioreactor component; here, cellulose forms on designated tiers, as the media is dripped from above through the trickle shower head. The second reservoir is the monitoring station where the conditions of the system are evaluated. Dissolved oxygen is added through an air pump and monitored with a sensor. The temperature is monitored using a thermometer. Having two reservoirs allows for manipulation in one reservoir (monitoring vessel) without agitating the BC being produced in the second reservoir (collection vessel). To transport transported between vessels, an Arduino breadboard which controls two pumps. Each pump will be operated using the

same board and power supply, which helps to ensure that the flow rate between vessels is the same. By doing so, the volume of media in each vessel should remain constant, allowing media flow to be constant during the entire production cycle. This device is customizable and allows voltage to be safely transmitted from a power source to a series of motors.

The membrane bioreactor inspired the idea of using a filter membrane to immobilize the bacteria for BC production. Initially, the membrane a variety of materials including mesh, film, and nylon were considered. Eventually, this progressed into using a more sophisticated filtering process. Filter membranes are typically used to isolate contaminants away from a liquid, although in this case, we want to capture the particles to promote cohesive and uniform cellulose formation. Finally, the airlift bioreactor influenced the designed introduction of dissolved oxygen to the system. Since the team is using two vessels, the airlift portion was included in the monitoring portion, as the oxygen can still be supplemented into the media which is being pumped to the tiers/cellulose collection points. In this way, the team intends to obtain the largest benefits of both the trickle bed and airlift and retain the properties of static culture through membrane stabilization.

4.4.2 Design Iteration Analysis

This series of design iterations represents the construction of the main body of the bioreactor responsible for the immobilization and collection of cellulose. These designs consist of various shaped caps which are intended for glass collection vessels. Through an iterative process, the design evolved over time as new issues were identified. Through each iteration, the design improved to fit the needs of the client, user, and design team.

4.4.2.1 Collection Vessel Design Iterations

The first iteration of our collection vessel top consisted of two pieces: a cap to interface with the glass bioreactor bottle and a shower head attached to the cap. This design had several issues. The purpose of this design was to allow the nutrient solution to gradually fall on the cellulose below, providing constant replenishment. The image on the left shows support rings on the inner diameter of the assembly; these were intended to be support for bacterial cellulose. These rings would support some type of film or mesh where the bacteria would form. These supports were insufficient to support BC production. The design team could not surmise a way to fix a membrane for BC production Second the formation of BC could not be monitored without disassembling the top. Given that the prototype was printed using opaque, blue PLA, a visual

assessment was impossible with this design. Lastly, the threading for the showerhead and the cap possesses a lot of friction making it difficult to open and close.

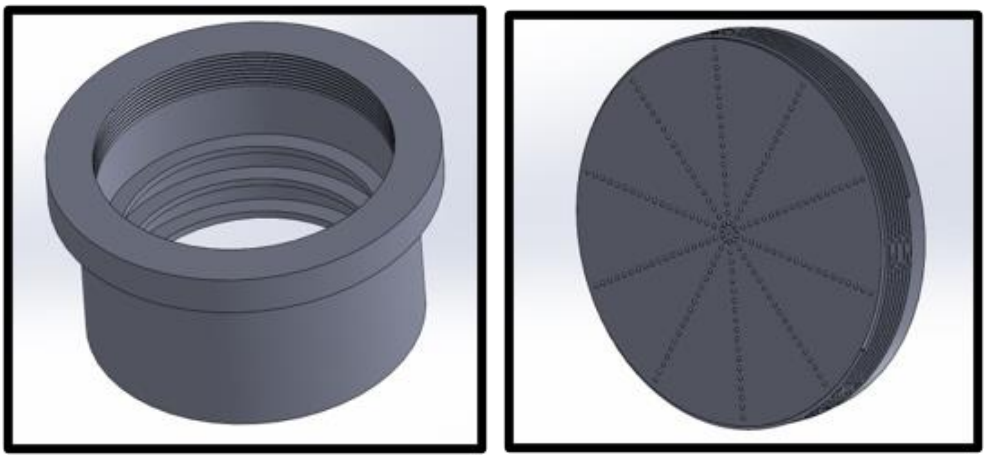

Figure 4: 1st Iteration of Collection Vessel Top

To address the issues of the first iteration the design for the bioreactor top was reimagined. The main differences between the first and second iteration are seen in its open concept and one-piece design. First, the shower head is built into the cap eliminating the threading issue seen in the first iteration. The design also utilized four pillars instead of a surrounding wall for visual assessment of BC production. Lastly, the design utilizes a circular tiered system so a membrane can be easily fixed to support BC production. Each tier increases in diameter from bottom to top, this is intended to provide continuous cascade of nutrients flowing from the top tier to the bottom tier, resulting in potentially larger pellicles at each subsequent tier. While this design addressed all the issues seen in the first iteration, it unfolded new issues. The main issue with this iteration was found in its production. To 3D print material with this design, support material must be used. Normally, support material can be taken off easily, however, this was not the case with this design. The amount of support material made it unusable.

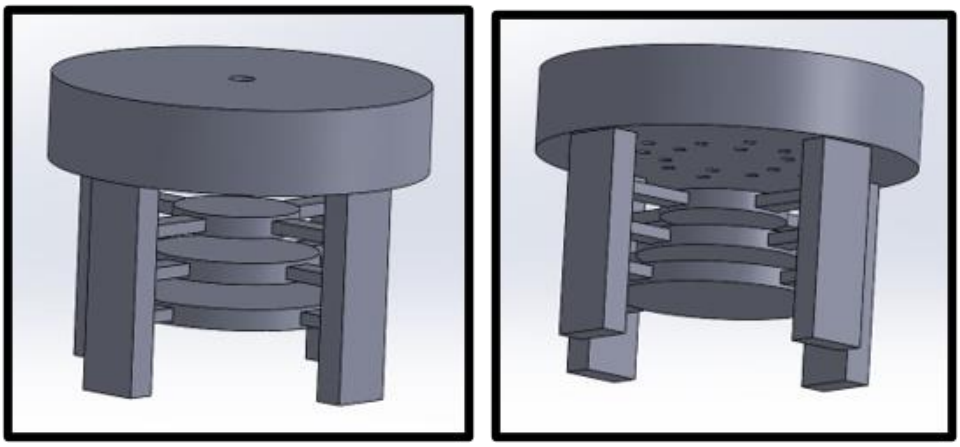

Figure 5: 2nd Iteration of Vessel Top

The third iteration only slightly altered the second to address the issues associated with the support material. In the four columns, slots were cut out to match the width of the posts of each of the tiers. This design allows the tiers to be 3D printed separately from the cap and slide right into their desired location. In this design there was a problem with tier stability. The addition of tiers to the showerhead assembly causes the pillars to flare out due to the pressure. Due to this effect friction was insufficient to hold the lower tiers in place. At this point in the design process, the team realized the 3D printed PLA is not compatible with any of the sterilization techniques available at WPI. This led to the third design being revamped with materials that can be sterilized.

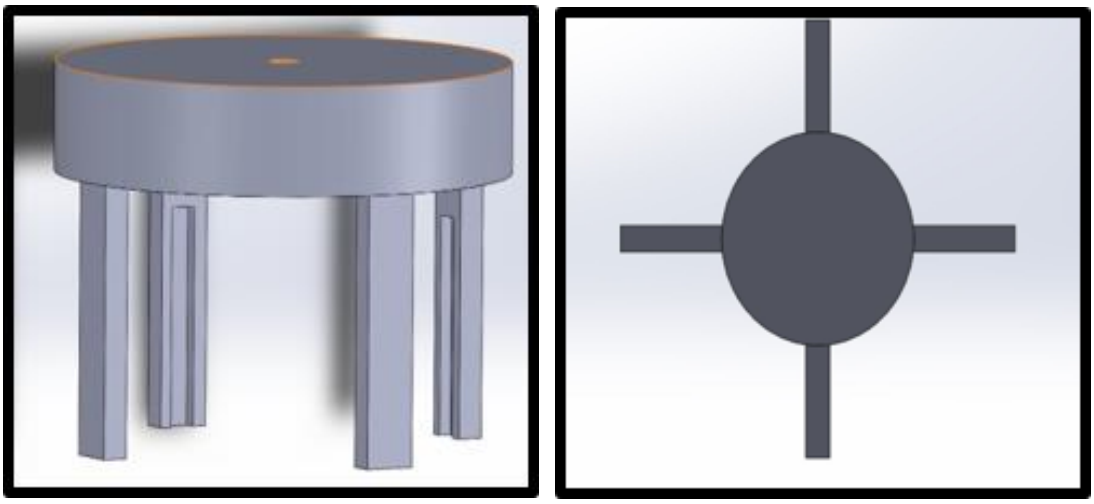

Figure 6: 3rd Iteration of Collection Vessel Top

The fourth iteration of the design kept essentially the same concept as the third with the biggest change being the material used. First, the shower head top was made of aluminum with an acrylic topper. Each of the tiers were made with two acrylic pieces that held in place a PES membrane. The PES membrane was added to immobilize the bacteria on the tiers surface, so BC can be produced. The tiers were held in place by zinc screws and bolts fixing the tier stability issues. The screws can thread into the aluminum top making a one-piece cap.

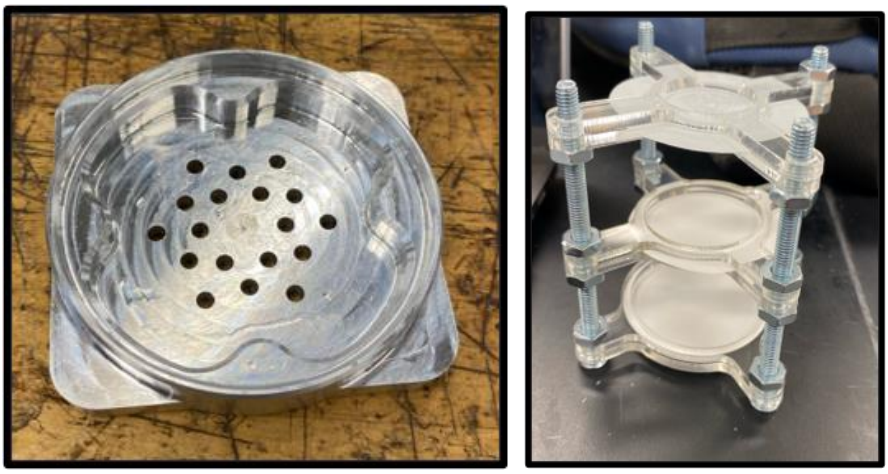

Figure 7: 4th Iteration of Collection Vessel Top

The final iteration of the showerhead top for the collection saw the addition of longer screws and three more acrylic tiers. This was done in essential to increase the surface area available

for BC production, ergo increasing the potential yield. In total, this design utilizes 6 acrylic tiers, ranging from 30 to 55 mm in diameter with increments of 5 mm. For each tier, there are two pieces of acrylic, the bottom layer has the entire diameter of the tier, this is where the PES membrane will rest as the accumulation point of cellulose. The upper layer of acrylic is a frame of the tier diameter, which is designed to secure the PES membrane to the lower tier. Each tier of acrylic is stacked on 5-inch zinc screws spaced about 1 cm apart via zinc bolts. The 5-inch screws are threaded into the trickle bed compartment to secure the entire assembly to lid. The aluminum collection vessel shower head is secured in place with a rubber seal which provides a tight connection between the aluminum and glass. The shower head also has a thick acrylic topper, which allows visibility through the top of the shower head. Since it is likely that the shower head may be an unintended cellulose accumulation point, it is required to have visibility into this portion to see any obstructions without compromising sterility.

Figure 8: Final Iteration of Collection Vessel Top

4.4.2.2 Monitoring Vessel Design Iterations

The first iteration of our monitoring vessel top consisted of one cap made of PLA. It contained five ports to house the DO probe, temperature probe, tubing for media in, tubing for media out, and tubing for air in. This cap was descended into the mason jar for the probes to able

to reach the bottom of the mason jar. Similarity seen with the collection vessel top; PLA is not a compatible material with any of the sterility methods that are available at WPI causing the team to remake the design with compatible materials.

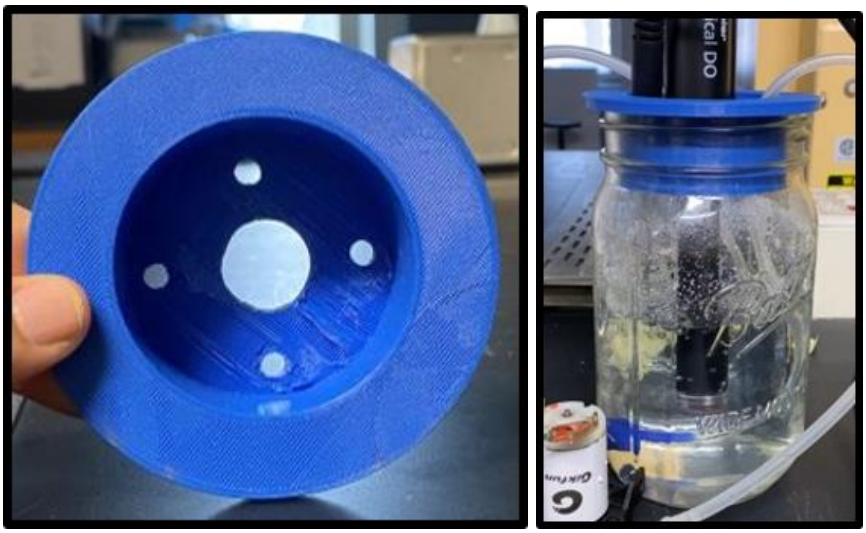

Figure 9 :1st Iteration of Monitoring Vessel Top

The second iteration of our monitoring vessel top consisted of a two-piece cap made of an acrylic piece and an aluminum outer ring to hold it in place. These materials made sterility possible. This design contained seven ports to house the DO probe, temperature probe, tubing for media in, tubing for media out, tubing for air in, a valve for air out, and a septum for the addition of nutrients into the system. The air out valve was added to ensure there is a pressure release for the system. The septum (butyl self-healing rubber stopper) was added to provide an easy way to add nutrients into the culture environment without disturbing pellicle formation via a syringe.

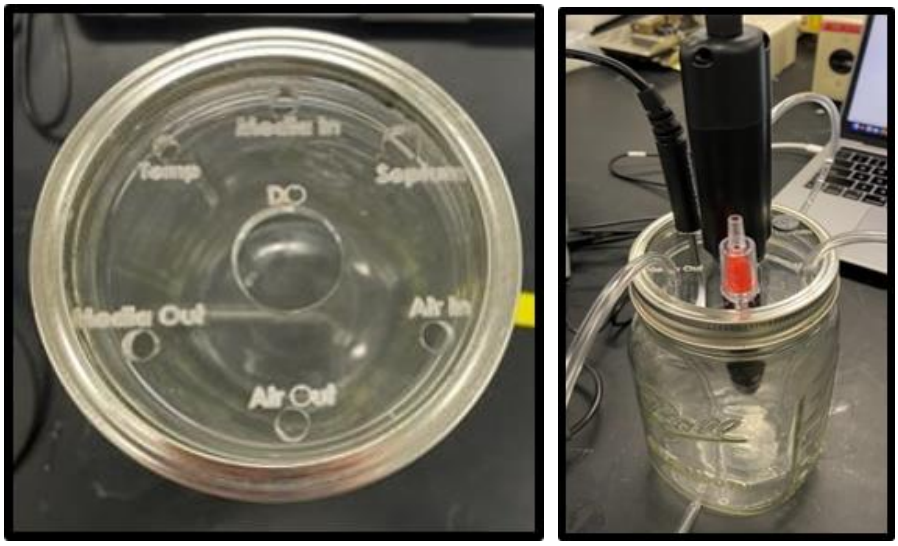

Figure 10: 2nd Iteration of Monitoring Vessel Top

4.4.2.3 Breadboard Pumping Circuit Design Iterations

The purpose of designing breadboard circuit was to incorporate an effective method to control the flow rate of the pumps transporting media between the collection vessel and the monitoring vessel. Since the media is being supplemented with nutrients in the monitoring vessel, most of its volume will be stored there at any given time. In the first iteration, a single pump was included in the bioreactor design so that we had a baseline understanding of the flow rate and duration of the motor's longevity. The voltage source is a 12V outlet adapter which plugs directly into the Arduino driver. The driver can reduce voltage down to 9V, 5V or 3.3V based on pins used. Initially, the group intended to use code to control the pumping speed. With this iteration, code was uploaded to the Arduino driver with the intention of controlling voltage sent to the system. This iteration functioned as proof on concept, to ensure that pumps could run for extended durations of time. In order to transfer media between two vessels would require two pumps; the

addition of another pump would require an adjustment of board components as well as an increase in input voltage in order to ensure synchronized flow rates.

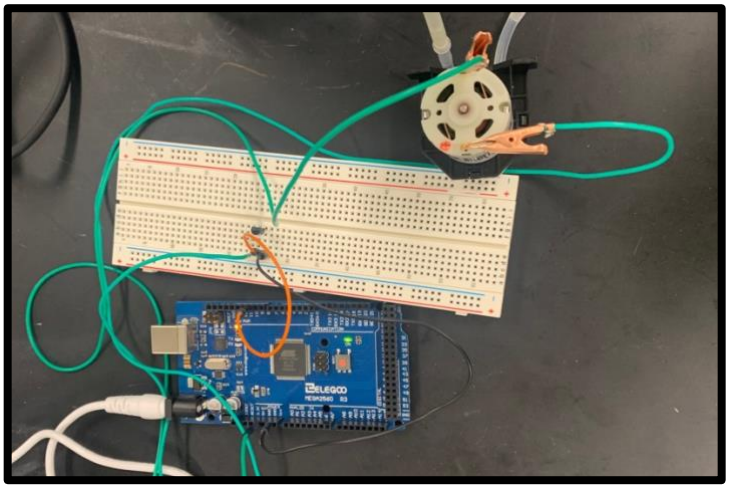

Figure 11: 1st Iteration of Breadboard Pumping Circuit

The second iteration of the breadboard circuitry included a potentiometer which is manual control of the resistance applied to the voltage. The addition of a potentiometer allows the user to adjust the flow rate, by increasing or decreasing the amount of voltage applied to the pumps. The advantage of this interaction is that there is no need for code as users can manually adjust the potentiometer. In this iteration, two pumps were installed on the board to replicate the required setup to transfer media between two vessels. Since the potentiometer is placed between the pumps, the voltage and resistance are simultaneously applied to each, ensuring that the flow rate is equal in both pumps. To reiterate, the resistance is being applied to the voltage, and the voltage is being divided by two pumps. At this point, duration tests were conducted on the pumps to ensure they could withstand long-term, sustained usage at the driver's highest limit voltage of 9V. Each pump had flow rates of 15-30 mL/minute (at the highest and lowest potentiometer resistances, respectively). The limiting factor in the duration of the test was the high amperage from the pumps which caused a temperature increase in the potentiometer. After a 1 and 2-day trial, the system did not fail, although it was reconstructed to accommodate for better power distribution. Following

this test, the first trial of the bioreactor was conducted, where the pumps ran for one week. For this trial, the Arduino driver supplied 9V of power to pumps, with the potentiometer adjusted to provide its minimum resistance. The pumps retained voltage for this test though were operating at too high of a flow rate.

The main issue with this design is that the voltage was too high for effective trickle into the collection vessel. Even at the highest resistance, the 15 mL/min flow rate caused agitation to the cellulose tiers. Additionally, there was no way to regulate spikes and drops in voltage from the wall outlet power source. This makes fluctuations in voltage high-risk as the resistor, pumps could overheat and terminate a trial.

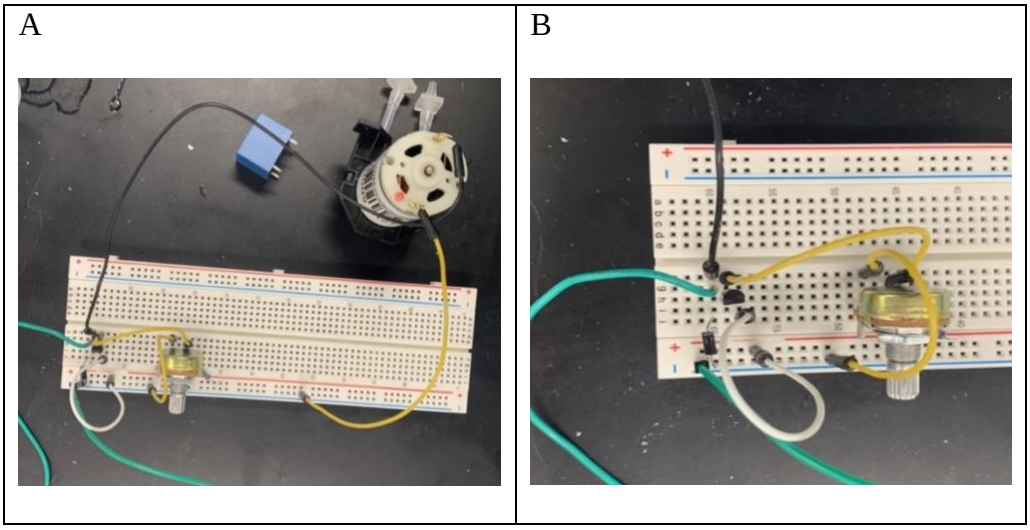

Figure 12: 2nd Iteration of Breadboard Pumping Circuit
A) The complete circuitry layout including the pump. B) Close up of the specific wring placements on the breadboard.

In the third iteration, the 3.3 V to 5 V driver (black box on the right) was applied to the circuit, this driver solves two of the discussed issues in the previous iteration. First, it reduces the voltage from 9 V to either 3.3 V or 5 V (user can choose). This ensures that the pumps can run at their lowest spin rate and be adjusted faster using the potentiometer and voltage options. In this case, the potentiometer can manually stop the system when resistance is maximized, and gradually

increases flow rate as the potentiometer resistance is reduced. This still allows the voltage distributed to each pump to be identical. This is a key safety feature, because rather than terminating a trial, the user can manually increase the resistance to stop the motors, rather than disassembling the breadboard and pumps. Additionally, the driver provides an internal safety which only supplies the designated amount of voltage from the power source to the board. This means that a short on the board will cause the power supply to stop, preventing a burnout of the device. Manual adjustment between 3.3 V and 5 V occurs on the yellow pins located on the right on the picture below allowing a finite power supply to be constantly applied to the system. What this provides is an additional safety measure between the wall outlet and the board. Finally, the driver can be manually turned on and off using the white switch, it correlates to a green LED (light emitting diode) indicator showing if the driver is supplying power to the board. This iteration aimed to provide users with high user friendliness by providing simple manual components that can just with an outlet, supply two simultaneous pumps from the same board, and give live feedback on how the board is active or not.

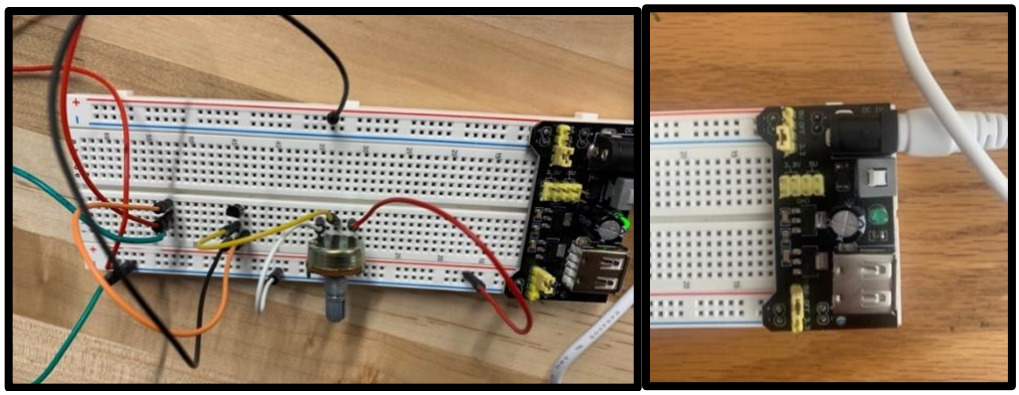

Figure 13: 3rd Iteration of Breadboard Pumping Circuit with Driver

The 4th iteration is identical to 3rd iteration, although cables were replaced for wires allowing loops and excess wiring to be reduced. The wiring now lay flush with the board reducing

incidental removal of wires from their designated locations. 3.3V to 5V is shown in the final image of this iteration. The yellow pins allow manual adjustment between voltage options.

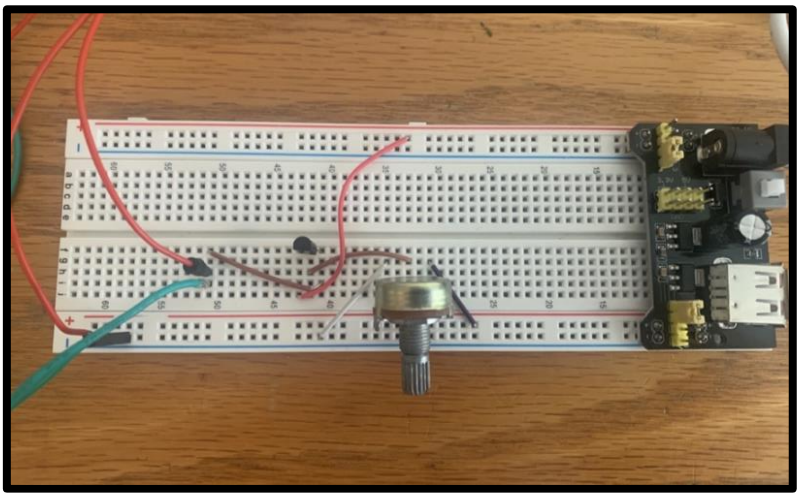

Figure 14: 4th Iteration of Breadboard Pumping Circuit

4.5 Design Conclusion

The main components of this bioreactor system include the collection vessel, the monitoring vessel, the air pump, the breadboard pumping circuit, and the data collection system. In an overview on how the system functions, the media starts in the monitoring vessel so data can be gathered about culture conditions. The media is then pumped out via the pumps controlled by the breadboard circuit and brought to the top of the collection vessel. The media then trickles down through the circular tiers and reaches the bottom of the collection vessel. After reaching the bottom the media is pumped out and brought back to the monitoring vessel making it a closed system. The full assembly can be seen in Figure 15 below. For an in-depth look at each of these components please refer to Chapter 6 of this paper.

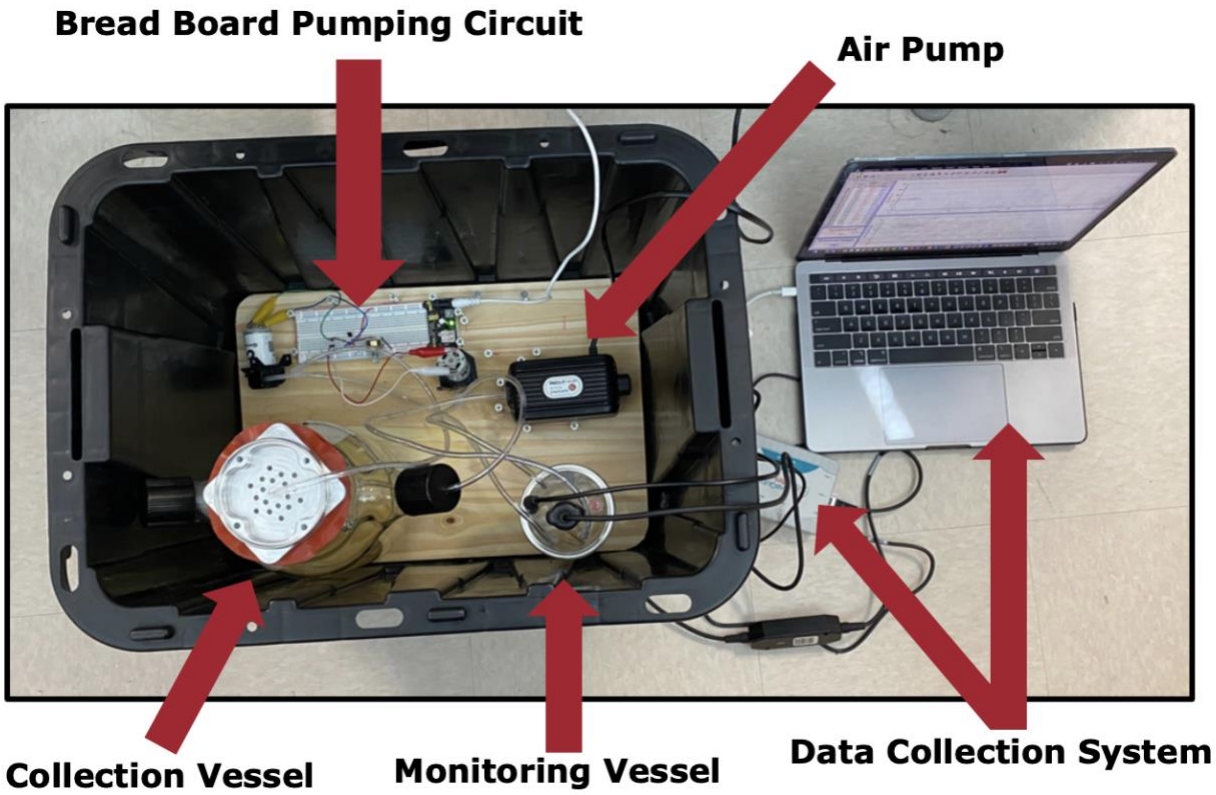

Figure 15: Final Bioreactor System Design

4.6 Static Culture Bench Standard Development

The testing conducted in this chapter was done on our first two control samples which were produced via static culture methods. The results obtained from these tests will be used as benchmark protocols and values that will be used to conduct and analyze testing on BC samples obtained by the bioreactor in future work. Uniformity, tensile strength, and yield testing were conducted on the control samples for the criteria of uniformity, mechanical properties, and percent yield that should be obtained by the produced pellicles of the bioreactor.

4.6.1 Uniformity Testing: ImageJ Analysis

Function 4: Promote Uniform Formation of BC

The fourth objective of this bioreactor was to promote the production of uniformly shaped BC pellicles. BC particles can be formed as irregular masses such as granule, stellate, and fibrous

strands, but this is not the criteria of formation that is desired of this bioreactor [141]. This uniform criterion specifies that the produced BC pellicles will appear as uniform circular structures and will not appear irregularly shaped or in the form of clumps. The uniform formation of BC is highly favored by the clients because it has certain advantages such as well-controlled spherical morphology, larger surface area, and higher porosity [141]. The higher porosity feature obtained by most uniform pellicles, for example, causes BC to be favorable for applications such as slow drug release, enzyme immobilization, and heavy metal adsorption [141].

To analyze the shape and formation of the BC produced by the bioreactor, ImageJ was used to analyze images of each sample of BC placed on a dark background. The uniformity of the sample was quantified by their gray values, which depict the intensity of the gray. A larger gray value corresponds to a darker grey. Since the samples were placed on a dark background, a transparent sample should have a higher mean gray value because the dark background will be observed through the sample. The standard deviation obtained of all the average grey scale values

indicates the variation in relative transparency within the sample. A smaller standard deviation for a pellicle, for example, meant a particular sample was more uniform.

In order to obtain the mean and standard deviation, the BC was isolated within the image by selecting the BC and clearing the background. The image was converted to 32-bit RGB, which allowed us to accurately measure the intensity of the gray values. The mean and standard deviation of the gray values was then measured using the measure tool. Once the gray values of each sample were measured, throughout the BC sample, the range of values will be evaluated, determining what overall uniformity of the sample. The standard operating procedure to conduct this analysis is located in Appendix C. The results of the samples from culture 1 and 2 are demonstrated in Table 42 and Table 43 respectively. A two-tailed two sample T-test was performed for both the mean transparency values and the mean standard deviation values between cultures 1 and 2. The results of the T-tests indicate that both the means and standard deviations of the mean gray values were statistically significant as the p-values were both less that 0.001.

Table 42: Uniformity Data of Culture 1 Samples

Sample (#)	Mean Transparency Value (%)	Mean Standard Deviation Value (%)
1	74.96	11.45
2	74.72	11.57
3	71.47	12.23
4	70.29	11.40
5	73.87	9.35
6	74.08	11.16
7	77.89	7.55
8	75.17	12.62
9	77.76	9.03
10	72.92	10.20
11	73.52	12.96
12	71.34	8.65
Average	29.02	4.19
Standard Deviation	0.92	0.67

Table 43: Uniformity Data of Culture 2 Samples

Sample (#)	Mean Transparency Value (%)	Mean Standard Deviation Value (%)
1	70.00	10.72
2	71.41	9.54
3	70.90	8.53
4	70.02	12.39
5	74.15	8.52
6	69.59	10.62
7	67.06	5.64
8	73.65	9.26
9	74.78	5.23
10	67.61	10.19
11	66.96	6.18
12	67.46	9.26
13	53.73	8.97
14	59.30	8.82
15	71.17	7.43
16	70.43	8.72
17	74.96	6.57
18	71.39	7.71
19	72.54	9.06
20	75.48	7.94
21	68.42	8.14
22	68.01	4.26
23	64.81	7.20
24	69.85	8.01
25	58.94	7.55
26	56.39	7.07
27	73.81	6.36
28	67.19	7.04
29	69.45	7.58
30	70.07	7.56
31	69.50	6.09
32	73.84	5.60
33	76.48	6.59
34	80.75	5.99
Average	27.22	3.07
Standard Deviation	2.22	0.69

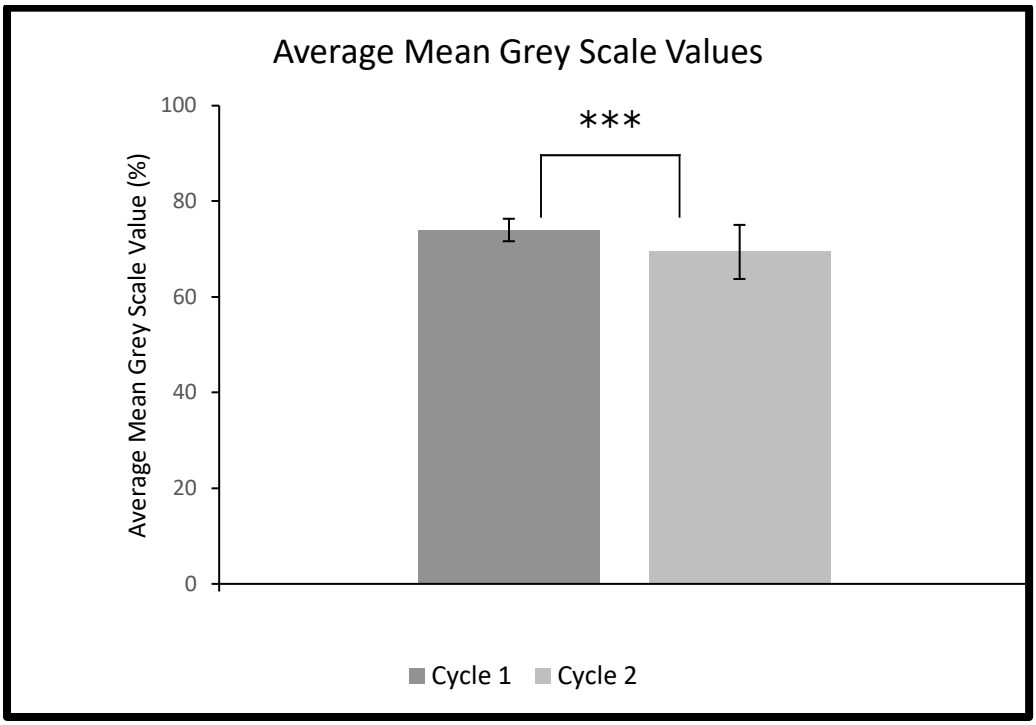

Figure 16: Transparency of Samples 1 and 2
Data presented as ± SD of a minimum of three independent experiments. Asterisk indicates statistical significance between the groups (***p<0.001). T-test.

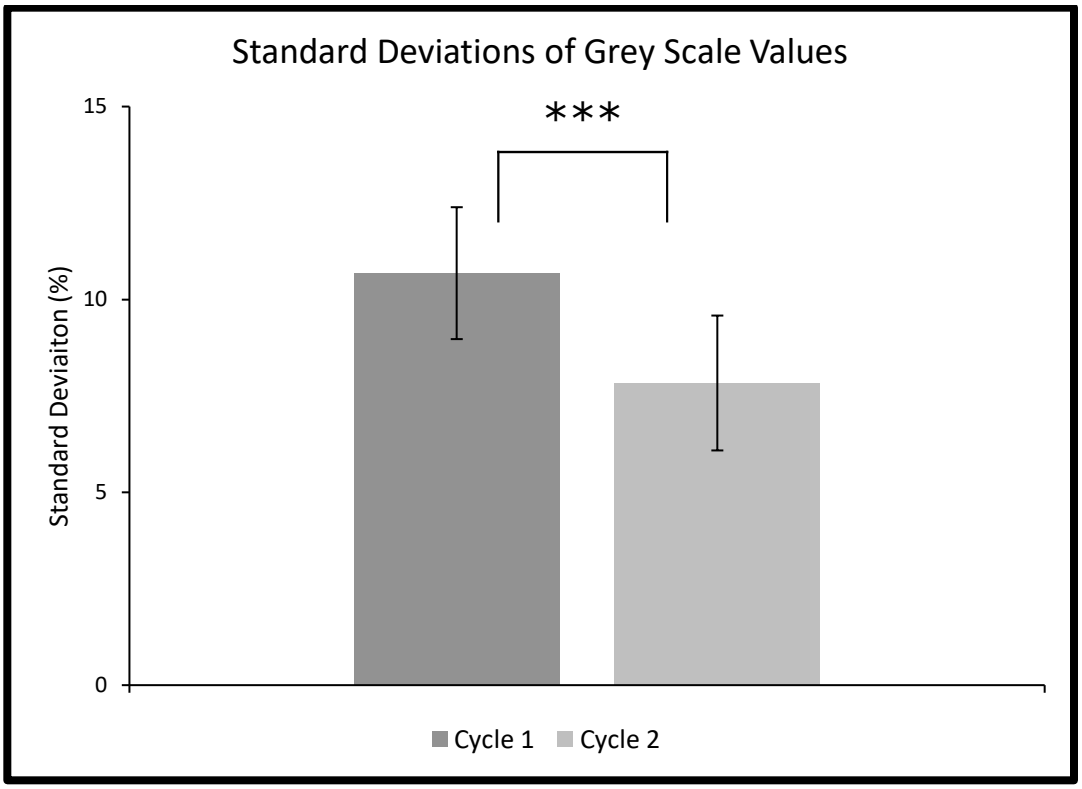

Figure 17: Uniformity of Culture 1 and Culture 2 Samples
Data presented as ± SD of a minimum of three independent experiments. Asterisk indicates statistical significance between the groups (***p<0.001). T-test.

4.6.2 Tensile Testing: Uniaxial Instron Tensile Test

Function 4: Promote Uniform Formation of BC

A common method of analyzing and comparing the mechanical properties of BC is tensile testing. Additionally, tensile tests are commonly used to analyze new materials for engineering applications to compare the properties of different materials. The measurement of an object's tensile strength is of fundamental importance, as it can be expressed either as the stress required to cause significant plastic deformation or as the maximum stress it can withstand [142]. This testing is imperative to determine whether the BC fabricated by the bioreactor is of high-quality, meaning it obtains approximately the same mechanical properties as those stated in literature and those of the control samples. Following geometrical measurements of the samples (Figure 18) the values of stress and strain were calculated based on the force measured by the Instron, as a function

of the gage length. The force, along with the stress, strain, and Young's modulus were calculated as follows:

- $F = k\Delta L$, where ΔL is the amount of deformation (the change in length) produced and k is a proportionality constant that depends on the shape and composition of the object and the direction of the force
- Engineering stress (σ)= (F/A_0), where A_0 is the initial cross-sectional area inside the gage sector, and F is the force in Newtons registered by the machine [142].
- Engineering Strain (ε) = $(\Delta L/L_0)$, where $L_0$is the extension of the gage-length section at the starting point, and ΔL is the amount of deformation (the change in length) and L_0 [142].
- The modulus of elasticity (Young's Modulus) = Stress/Strain ; indicates the stiffness of the material in the linear region of the stress-strain curve, where the material exhibits elastic response [142].

Following the Instron testing protocol in Appendix C, a series of tensile testing was completed on control BC samples and samples that were fabricated by the bioreactor. Table 44 and

Table 45 depict the measurements obtained via a calibrator for each experiment which was implemented into the Bluehill Universal program to determine the tensile strength of each given sample. Bluehill Universal is a testing software that is used in conjunction with an Instron machine to obtain data. Bluehill provides functions for several mechanical tests such as tension, fatigue, tensile tests and more. Instrons are signaled to conduct certain tests and protocols via the Bluehill software. In making a Bluehill test, specific information such as inputs and outputs are chosen. The purpose of our testing strategy is to determine the ultimate tensile strength and Young's modulus of our BC samples, thus in order to receive this information, the following geometrical measurements of the wall thickness and the outer diameter (Figure 18) were inputted into the

Bluehill program at the beginning of each test for each corresponding sample. Figure 18 showcases the measurements of the wall thickness and the outer diameter obtained via a calibrator for each bioreactor sample which was implemented into the Bluehill program to determine the tensile strength of each given sample for our first culture, while

Table 45 showcases the same data for our second culture. These two tables also showcase the resulting ultimate tensile strength and Young's modulus for each sample for both cultures. The Stress-Strain Curves of each sample in each culture are depicted by Figure 19 and Figure 20.

- Engineering stress (σ)= (F/A_0) , where A_0 is the initial cross-sectional area inside the gage sector, and F is the force in Newtons registered by the machine (141).
- Engineering Strain (ε) = $(\Delta L/L_0)$, where $L_0$is the extension of the gage-length section at the starting point, and ΔL is the amount of deformation (the change in length) and L_0 (141).
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samples, thus in order to receive this information, the following geometrical measurements of the wall thickness and the outer diameter (Figure 18) were inputted into the Bluehill program at the beginning of each test for each corresponding sample. Table 44 showcases the measurements of the wall thickness and the outer diameter obtained via a calibrator for each bioreactor sample which was implemented into the Bluehill program to determine the tensile strength of each given sample for our control cycle 1, while Table 45 showcases the same data for our control cycle 2. These two tables also showcase the resulting ultimate tensile strength and Young's modulus for each sample for control cycles 1 and 2. The Stress-Strain Curves of each sample in each cycle are depicted by Figures 19 and 20.

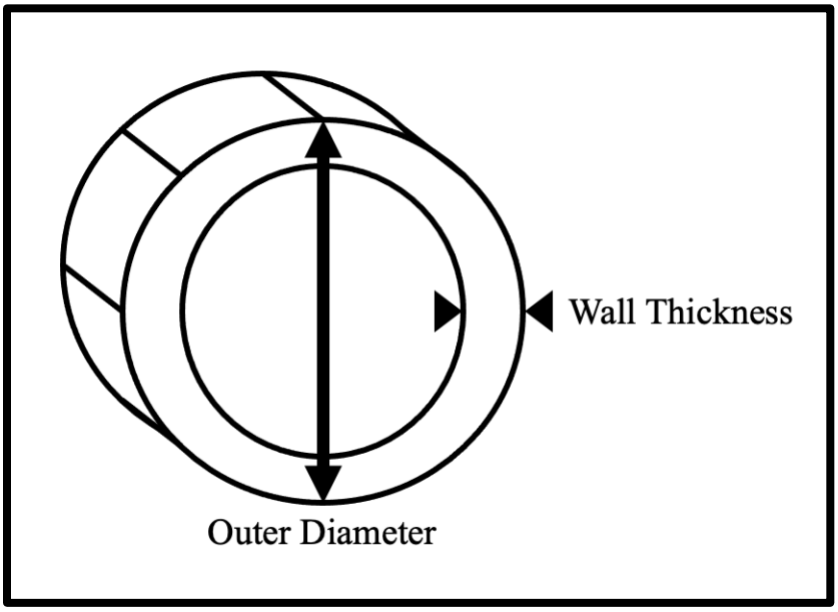

Figure 18: Geometrical measurements of each BC sample that inputted into the Bluehill software

Table 44: Tensile Testing Inputs and Outputs of Culture 1 Samples of BC

Sample (#)	Wall Thickness (mm)	Outer Diameter (mm)	Ultimate Tensile Strength (kPa)	Young's Modulus (kPa)
1	1	42	17.9	23030.0
2	1.5	35	26.4	21300.0
3	1.5	34	20.3	3680.0
4	1	31	38.4	1450.0
5	1	33	19.6	500.0
6	1	36	23.6	2450.0
7	1	40	24.2	2810.0
8	1	36	27.1	480.0
9	1	34	30.7	6040.0
10	1	42	17.9	560.0
11	1.16	34	22.6	3180.0

Table 45: Tensile Testing Inputs and Outputs of Culture 2 Samples of BC

Sample (#)	Wall Thickness (mm)	Outer Diameter (mm)	Ultimate Tensile Strength (kPa)	Young's Modulus (kPa)
1	6.28	27.86	5.9	2207.9
2	7.20	28.42	2.6	971.2
3	5.12	36.59	2.6	14666.5
4	6.83	31.91	4.0	1012.6
5	4.9	32.03	7.2	1559.9
6	5.61	31.50	8.4	2198.2
8	6.15	31.11	5.5	2522.7
9	6.97	31.33	4.8	1428.1
10	6.39	32.94	6.8	1052.2
11	5.57	33.87	4.0	1774.8
12	6.65	33.66	4.8	7480.4

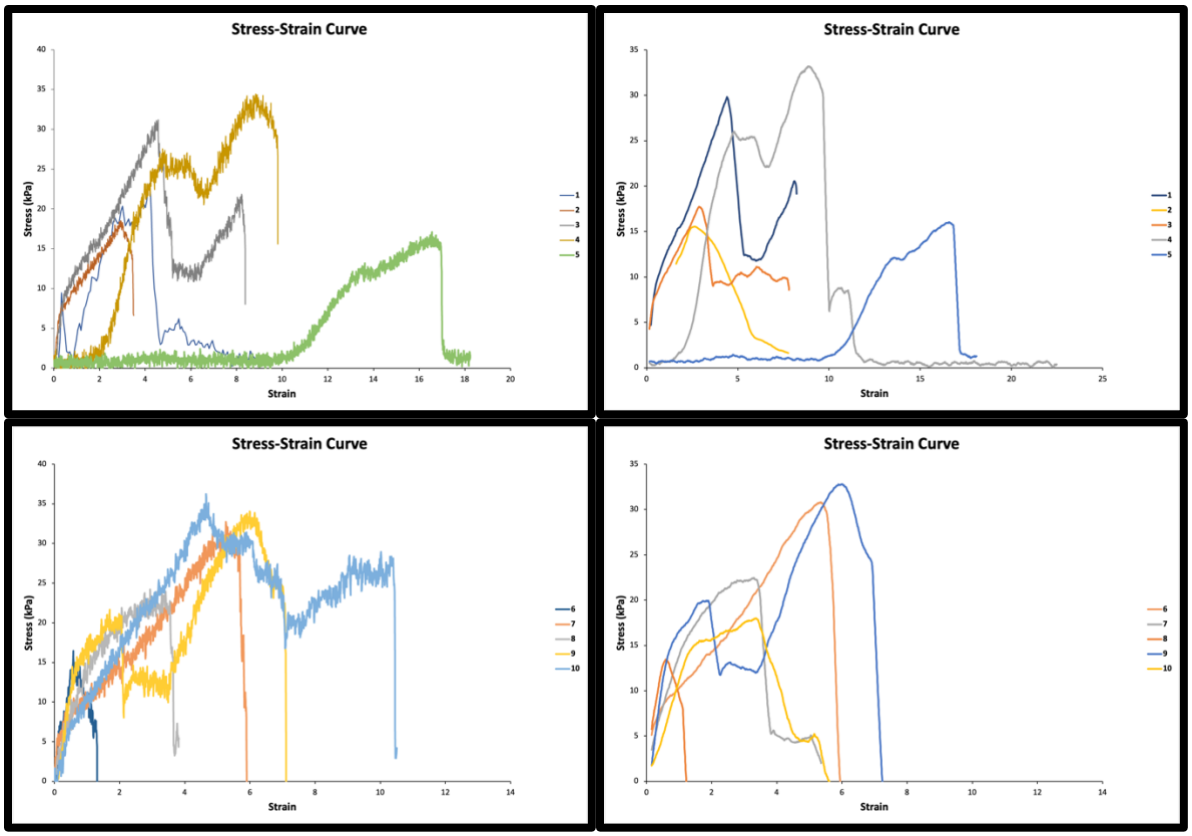

Figure 19: Experiment 1 Tensile Testing Samples
(Images on the left are raw data, images on the right are moving average)

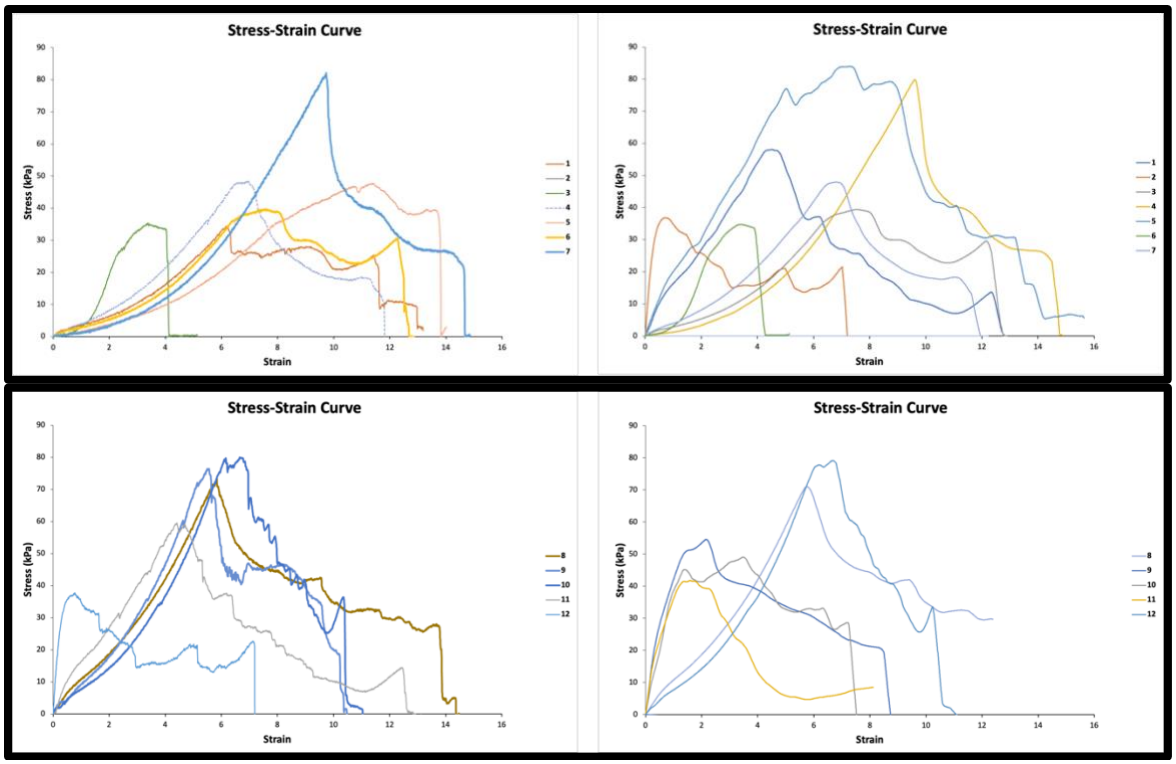

Figure 20: Experiment 2 Tensile Testing Samples
(Images on the left are raw data, images on the right are moving average)

4.6.3 Yield Testing: Gravimetrical Test

Function 4: Promote Uniform Formation of Bacterial Cellulose

One key characteristic that was aimed for this bioreactor was the ability to produce either the same or higher yields of BC compared to that of the standard approach. To verify that this criterion was met, gravimetrical tests were conducted to analyze the percentage yield of BC produced. The standard operating procedure on this gravimetrical test can be found in Appendix C. In order to conduct this gravimetrical test, the samples we first lyophilized using a FreeZone Triad Benchtop Freeze Dryer supplied by Labconco. The samples were lyophilized until a constant weight was reached. This process typically took about two days. Once finished, the dried samples were weighed. Each dry weight of each sample was acquired and recorded. Table 46 showcases the dry weights of the BC pellicles formed in grams and the calculated yield percent of each of

those samples from the very first experiment. Table 47 showcases the same data but for the second experiment. The formula utilized to calculate the percent yield of each sample is as follows:

- $Yield\ (\%) = \frac{W_{dry}}{C} \times 100$, where W_{dry} is the dry weight of a given BC sample in grams and C is the weight of the carbon source added to the whole system in grams.

The carbon source indicates how much glucose was added into the system; thus the yield percent formula quantifies the percent of cellulose produced in comparison to the amount of carbon absorbed within the culture. Table 48 depicts the statistical analysis of the data. A T-test was performed to determine whether the average yield of both cycles was statistically significant. The p-value was statically significant for an alpha of 0.001. Obtaining information on the percent yield of the bioreactor is imperative to ensure that the bioreactor is producing a yield that is comparable to the yield of our control cycles.

One key characteristic that was aimed for this bioreactor was the ability to produce either the same or higher yields of BC compared to that of the standard approach. To verify that this criterion was met, gravimetrical tests were conducted to analyze the percentage yield of BC produced. The standard operating procedure on this gravimetrical test can be found in Appendix C. In order to conduct this gravimetrical test, the samples we first lyophilized. This lyophilizer freeze-dries the samples by means of low temperature dehydration processes that involve freezing the samples, lowering pressure, then removing the ice by sublimation. The samples were lyophilized until a constant weight was reached. This process typically took about two days. Once finished, the dried samples, were then weighed. Each dry weight of each sample was acquired and recorded. Table 46 showcases the dry weights of the BC pellicles formed in grams and the calculated yield percent of each of those samples from the very first control cycle. Table 47 showcases the same data but

for the second cycle. The formula utilized to calculate the percent yield of each sample is as follows:

- $Yield\ (\%) = \frac{W_{dry}}{C} \times 100$, Where W_{dry} is the dry weight of a given BC sample in grams and C is the weight of the carbon source added to the whole system in grams.

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of the bioreactor is imperative to ensure that the bioreactor is producing a yield that is comparable to the yield of our control cycles.

Table 46: Gravimetrical Test Data for Samples Produced by Static Culture (Experiment 1)

Sample (#)	Dry Weight (g)	Calculated Yield (%)
1	0.037	4.1
2	0.043	4.8
3	0.035	3.9
4	0.038	4.2
5	0.039	4.4
6	0.034	3.8
7	0.035	3.9
8	0.035	3.9
9	0.035	3.9
10	0.033	3.7
11	0.033	3.6
12	0.034	3.7
13	0.034	3.7
14	0.033	3.7
15	0.037	4.1
16	0.036	4
17	0.040	4.4
18	0.033	3.7
19	0.033	3.7
20	0.035	3.8
21	0.036	4
22	0.039	4.3
23	0.038	4.2
24	0.036	4
25	0.036	4
26	0.035	3.8
27	0.035	3.9
28	0.032	3.5
29	0.031	3.4
30	0.034	3.8
31	0.034	3.7
32	0.033	3.6
33	0.043	4.8
34	0.034	3.8
35	0.041	4.5

Table 47: Gravimetrical Test Data for Samples Produced by Static Culture (Experiment 2)

Sample (#)	Dry Weight (g)	Calculated Yield (%)
1	0.254	28.2
2	0.121	13.5
3	0.104	11.6
4	0.095	10.6
5	0.107	11.9
6	0.099	10.9
7	0.266	29.5
8	0.346	38.4
9	0.100	11.1
10	0.108	12
11	0.090	10
12	0.148	16.4
13	0.117	13
14	0.115	12.7
15	0.099	11
16	0.100	11.1
17	0.191	21.2
18	0.098	10.8
19	0.098	10.8
20	0.099	10.9
21	0.097	10.8
22	0.097	10.8
23	0.098	10.9
24	0.098	10.9
25	0.098	10.9
26	0.092	10.3
27	0.096	10.7
28	0.108	12
29	0.094	10.4
30	0.096	10.7
31	0.093	10.3
32	0.086	9.5
33	0.092	10.2
34	0.099	11
35	0.121	13.4
36	0.112	12.5

Table 48: Average and Standard Deviations of Yield (%)

Experiment (#)	Average Yield (%)	Standard Deviation (%)
1	3.95	0.32
2	13.36	6.21

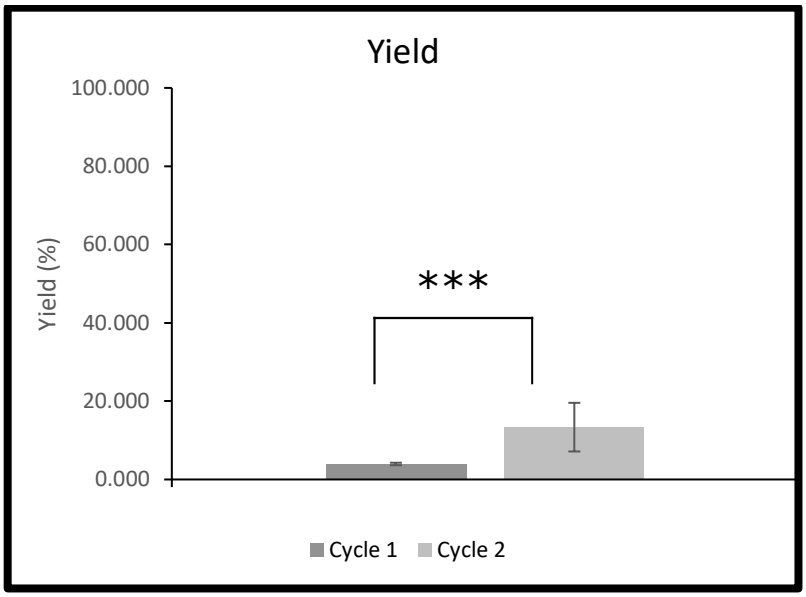


Figure 21: Average Yield (%) of Samples Produced by Static Culture Experiments 1 and 2
Data presented as ± SD of a minimum of three independent experiments. Asterisk indicates statistical significance between the groups (***p<0.001). T-test.

5.0 Bioreactor Design Verification

This chapter includes all the results of the verification testing for this project as well as an overview of the tests performed. The functionality of the bioreactor itself was evaluated using several tests. This section highlights these tests and their results. A visual observation test was conducted to make sure the bioreactor is durable and capable of withstanding multiple cycles of production and sterilization. A timed set-up test was completed to verify that the bioreactor is not too complicated to set up and requires minimal labor. A coverslip microscopic analysis was performed to establish the bioreactor's ability to maintain an aseptic environment. Testing was conducted on the bioreactor to ensure that it is durable, capable of maintaining a sterile environment, and user friendly.

5.1 Durability Testing: Visual Observation Test

Function 1: Capable of Withstanding Multiple Fabrication Use

The bioreactor system is designed to be used for multiple cycles. The system consists of reusable components and single use components as depicted in Table 49 below.

Table 49: Multiple Use and Single Use Components

Multiple Use Components	Single-Use Components
Monitoring Vessel	Wires
Collection Vessel	PES membranes
Screws and bolts	Air filter
Acrylic tiers	
Aluminum Cap	

Although certain components are intended to be used once, most of the components are designed to be used for multiple cycles, so the durability testing only focuses on the components that are used multiple times.

Ideally, to determine how many iterations of cultures and sterilization the bioreactor can be used for, the team would run cycles of cultures and sterilization until individual components

started to degrade. As the team completed each cycle, they took note of the condition of each component to ensure that they could be used for additional cultures. The standard operating procedure for the durability testing is included in Appendix C. The teams' observations after each cycle as well as their comments for the potential reasoning and planned adaptations are illustrated in Table 50.

Table 50: Observed Degradation and Analysis of Durability After Each Cycle

Cycle (#)	Observed Degradation	Potential Reasoning	Adaptions Made for the Next cycle
1	Corrosion was visually evident on the zinc screws and nuts after the first cycle	The screws and nuts were not washed directly after the first wash; thus the corrosion could have been due to them being unwashed and left out	First running another cycle with different zinc screws and washing them and drying them right after the cycle is done running. If corrosion is present after this second cycle, then the team will explore utilizing stainless steel screws and nuts instead

5.2 User-Friendly Testing: Timed Set-Up Test

Function 2: Require Under 1 Hour of Set-Up Labor

To determine how long it would take to set up the bioreactor, the team measured how long it took to set up each of the components of the bioreactor. The standard operating procedure for the set-up process is in Appendix C. The set-up process was completed by the same team member three times each and these times were averaged.

The assembly process began by placing the collection vessel and the monitoring vessel on the lab bench and assembling the aluminum cap and tiers. The environmental monitoring system was then set up by plugging the components (thermometer, Arduino, and DO) into the laptop and placing the DO probe and thermistor into the cap. The necessary tubing to transfer media from the monitoring vessel to the collection vessel was then assembled. Media was then added to the monitoring vessel and the pump and data collection was initiated. Table 51 illustrates the duration

of each set-up time trial and their average. Figure 22 illustrates the results of the set- up analysis. An ANOVA test was conducted to see if there was statistical significance between any of the individuals. The p-value from the ANOVA test indicated that at least two of the average times were significant, so a post hoc Tukey test was conducted to see which averages were significant. The only significance was between individuals 1 and 2.

Table 51: Set-up time trials & durations

Person (#)	Trial (#)	Duration (Minutes)
1	1	47
	2	40
	3	38
	Average	42
	Standard Deviation	5
2	1	30
	2	29
	3	28
	Average	29
	Standard Deviation	2
3	1	41
	2	36
	3	34
	Average	37
	Standard Deviation	4

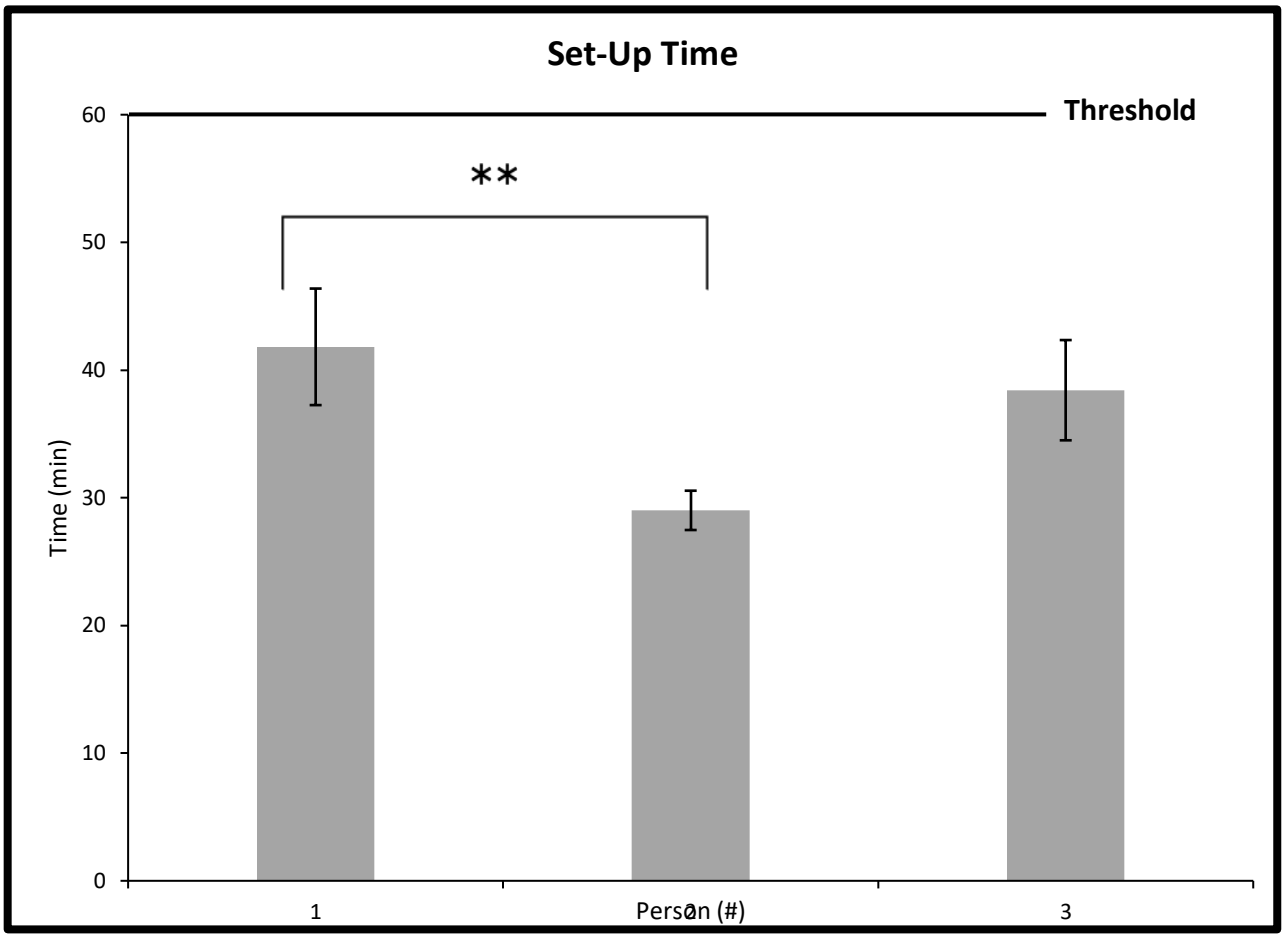


Figure 22: Set-Up Time Analysis
Data presented as ± SD of a minimum of three independent experiments. Asterisk indicates statistical significance between the groups (**p<0.01). ANOVA and post hoc Tukey test.

5.3 Sterility Testing: Coverslip Microscopic Analysis

Function 3: The Ability to Maintain Sterility

Although it is possible to observe contamination of the HS media as discoloration and odor during the bioreactor cycle, a sterility test was performed to ensure that the bioreactor can maintain a sterile environment for the duration of each culture cycle. The standard operating procedure is in Appendix C. First, the bioreactor and all its components were sterilized using the necessary sterilization methods. The following components were sterilized via the autoclave: the PES filter membranes, the media, zinc screws, nuts, the aluminum showerhead, the glass collection and the

monitoring vessels. The following components were sterilized via EO: tubing components, rubber seal, acrylic components, air valve and tubing connectors. Once each of the components were sterilized, the monitoring vessel was filled with media and the system was set to run for two days. On day two, fluid from the monitoring vessel was placed on a coverslip. The coverslip was then analyzed for bacterial presence. A bioburden test was performed on the coverslips in accordance with ISO 11737-1. This protocol calls for the observation and analysis of the media using coverslips and a microscope. Due to time constraints, one cycle was conducted in which media was taken from the monitoring vessel in three different iterations and three images were captured of each trial, to ensure that the results are consistent throughout. A Nikon Eclipse E600 upright microscope was used to conduct this testing protocol. As seen in Figure 23 and Table 52, there were no bacteria seen on the coverslip after two days, on each coverslip. The results of the sterility test affirm that the bioreactor can maintain an aseptic environment for one typical cycle. This sterility test was only performed once and up to the first benchmark of two days instead of for the full duration of a typical cycle which is seven days. The results of this test are depicted in Table 52 below.

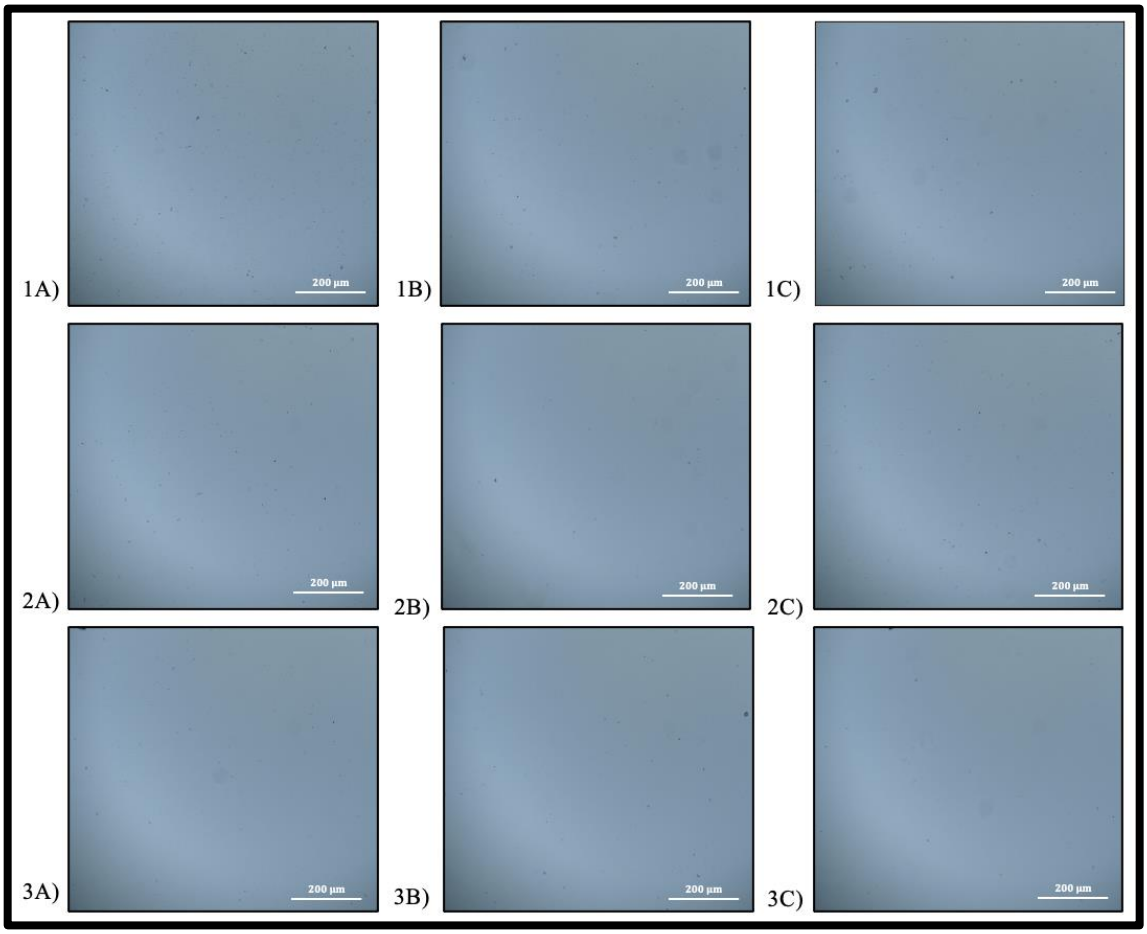


Figure 23: The captured images of the sterility testing after 2 days using 4X magnification 1A) Coverslip 1, Image 1. 1B) Coverslip 1, Image 1. 1C) Coverslip 1, Image 1. 2A) Coverslip 2, Image 1. 2B) Coverslip 2, Image 1. 2C) Coverslip 2, Image 1. Image 3. 3A) Coverslip 3, Image 1. Image 1. 3B) Coverslip 3, Image 1. Image 2. 3B) Coverslip 3, Image 1. Image 3.

Table 52: Sterility Testing Results

Coverslip (#)	Bacterial Presence? (Yes/No)
1	No
2	No
3	No

6.0 Final Design and Validation

6.1 Overview of Final Design

The following section will outline the production of BC using our bioreactor system. The information described in this section can be used to replicate our system and mimic the design. In depth protocols will be outlined in the Appendix.

Our bioreactor system consists of several main components: a collection vessel, a monitoring vessel, breadboard pumping system, air pump, and laptop for data collection. The following sections will describe each of the main components in more detail.

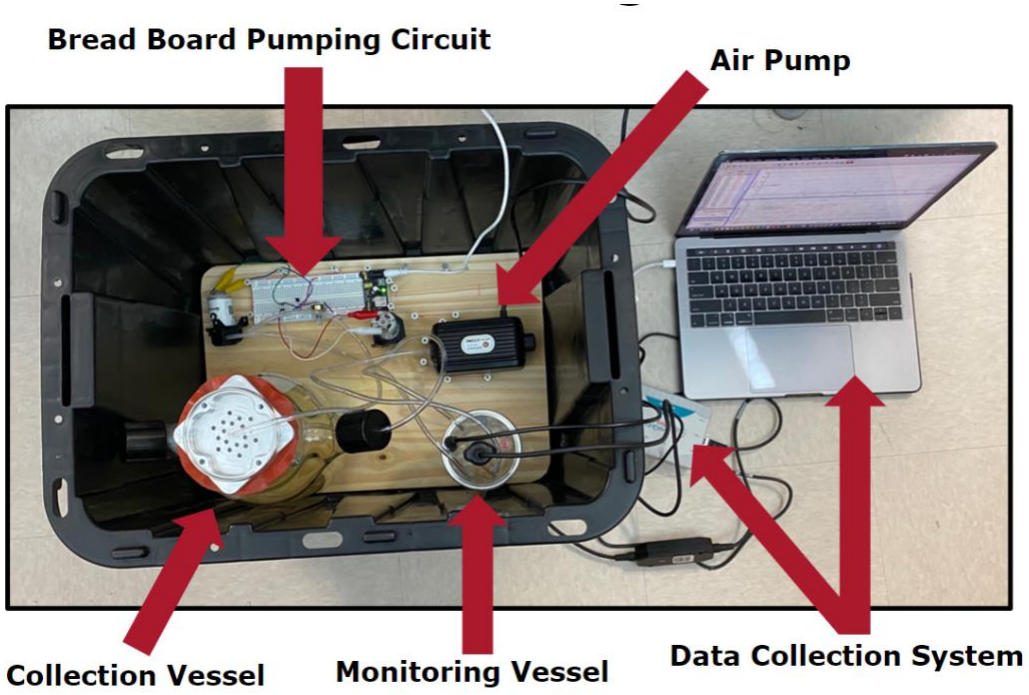


Figure 24: Final Design Layout

For the formation of BC in Coburn lab HS media, a carbon source, and inoculate starter are utilized. The HS media used is a solution composed of distilled water as the solvent and peptone, citric acid, disodium phosphate, and yeast extract as the solutes. This mixture provides nutrients to the bacteria strain to aid in BC production. Glucose was used as the carbon source for

BC production acting as the main energy source. The inoculate starter introduces the bacteria strain into the culture environment. In our bioreactor system, these three components will be initially stored in the monitoring vessel. The monitoring vessel's main body is a 16oz wide-mouth mason jar with a custom acrylic top and aluminum outer ring to hold it in place. The custom laser-cut acrylic top possesses ports that allow for the insertion of our Vernier Optical DO Probe (Item# ODO-BTA), Vernier Stainless Steel Temperature Probe (Item# TMP-BTA), media intake, media outtake, air intake, air release, and a septum for media addition.

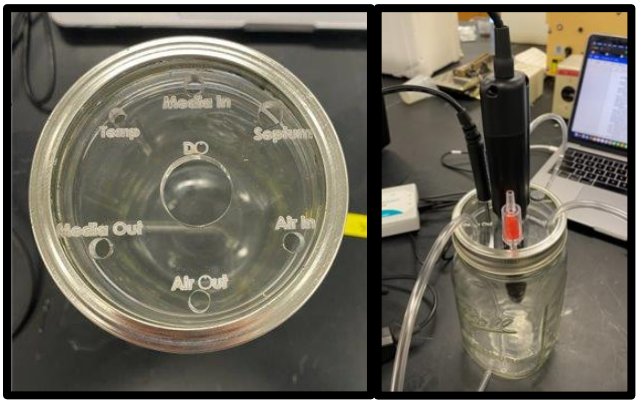


Figure 25: Monitoring Cap

The two probes gather data about cultural conditions and relay that information to a laptop via a Vernier LabQuest mini (Item # LQ-MINI). The data is stored on the laptop via the program Vernier's data collection program Logger Pro 3.14.1 (Appendix D). Collecting this data provides valuable information that can be used to fine-tune the collection process. The media intake and outtake ports are used via the pumping system, these utilize tubing with size of 3mm inner and 5mm outer. Our pumping systems consist of two Gikfun peristaltic pumps (Item # LYSB01IUVHB8E-ELECTRNCS) connected to a breadboard circuit. The breadboard circuit was made via components found in ELEGOO Uno project starter kit. The circuit controls the pump's rotations per minute by utilizing a potentiometer the applies variable resistance. This allows for the flow rate of the pumps to be adjustable during a culture period.

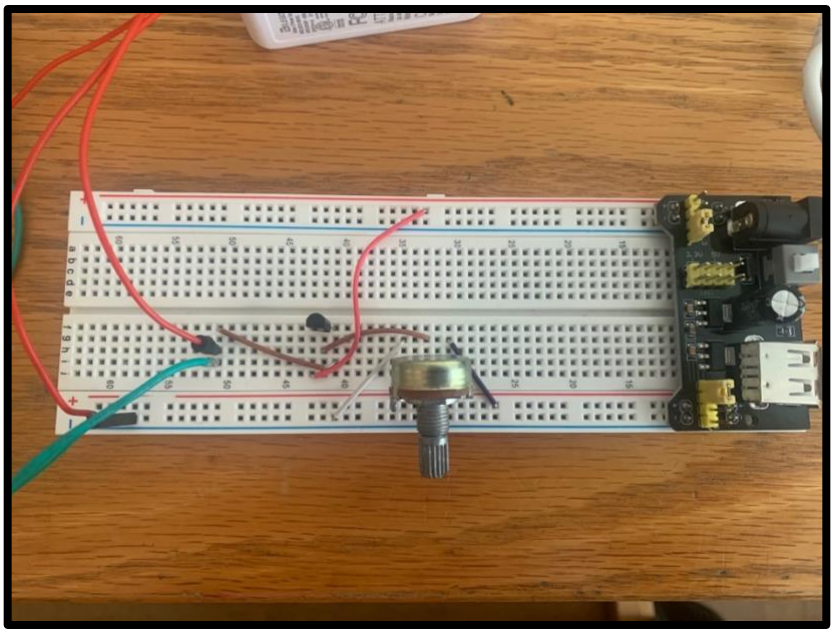


Figure 26: Breadboard

The ports for air intake and air release are utilized via an air pump and a one-way air valve (Figure 25). The air pump supplies oxygen to the culture through the air intake port. To ensure sterility a filter is used in line with the air pump. The one-way air valve is placed in the air release port to ensure unidirectional flow of the air release. The last port in the monitoring vessel top is for the septum (Figure 25). The septum is a material that can be perforated for media addition without compromising sterility. This can be done with a syringe.

The purpose of the collection vessel is to immobilize the bacteria, allowing the production of cellulose to occur on each tier. The collection vessel's main body is a glass jar repurposed from a spinner flask (patent #3622129) with three openings, a top, and two side ports. The two side ports have premade caps. One of the caps had a 5mm hole drilled into it to act as an opening for media to be pumped out of the collection vessel. The top consists of three parts. The first layer is an acrylic piece with a port for media to be pumped into the collection vessel. The first layer press fits into the top of the second layer. The second layer is an aluminum shower head that causes the media to trickle down. The third layer consists of six tiers of the increasing diameter of acrylic and

PES membrane. The acrylic provides a steady base for the PES membrane to effectively immobilize the bacteria strain so BC can be produced as the media trickle downs each tier from the showerhead. The third layer attaches to the second via 10-32 (M5 for metric units) threaded zinc alloy screws. The tiers are held in place via 10-32 zinc alloy nuts.

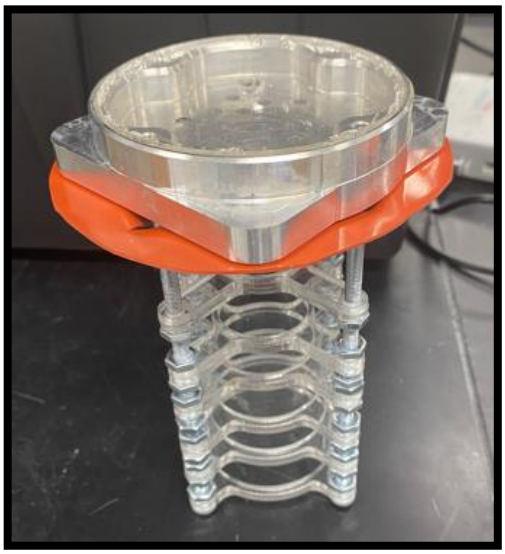


Figure 27: Vessel Collection Assembly

In summary, after placing media into monitoring vessels it is pumped into the collection vessel for BC to form on each of the individual tiers. The media is then pumped back into the monitoring vessel to be re-oxygenated by the air pump in a closed system. BC should be formed on each tier in the collection vessel after a seven-day culture period.

6.2 Objectives Achieved

Our team set out to design a low-cost bioreactor system for the automated addition of nutrients and/or gases directly into the culture medium with minimal disruption to BC fermentation. With these goals in mind, the team was able to craft a bioreactor system capable of the addition of media and gases into the environment with minimal to no disruption of the BC fermentation. While the addition of extra media in our system is not automated and must be done by an individual, we were able to achieve the automated addition of gases into the system. With a

functioning system in place the team also developed a SOP for use of the bioreactor. This will allow future users of the system to accurately operate it.

6.3 Impact Analysis of Device

The purpose of the following sections is to discuss the impacts the device has on areas including economics, environment, society, politics, ethics, health and safety, manufacturing, and sustainability. Although this design is a prototype and in its first iteration, it still has the potential to affect the world.

6.3.1 Economic Impact

Extensive cultivation times, low production yields, and the limited yield of cellulose layers are major obstacles in the conventional production of BC, which limits its commercial application. The commercial mass production of high-quality BC membranes could allow for more and new applications of this material. The Coburn Lab is focused on producing BC for the advanced research of its applications in wound dressings. For this reason, the team has created a low-cost bioreactor system for BC production by means of continuous, multi-tiered culture. This method should produce a high yield of high-quality BC which is necessary to expedite experimental research and processes involving the application of BC. Thus, a possible economic advantage is that this approach could allow for developed BC applications to become FDA approved and commercially available faster, resulting in treatments becoming more available, which would increase the number of treated patients. If implemented, the applications of BC could increase the cost of media, since there would be a larger demand for BC. This, in turn, would promote the creation of companies that would focus on providing BC and its required materials.

6.3.2 Environmental Impact

The designed bioreactor system and the fabricated BC could have an impact on the environment. Because BC is made from the fermentation of bacteria, leftover media, any unused BC, and any other waste products left behind in the bioreactor must be disposed of as biohazard waste. Once biohazard waste is brought to the appropriate facility, it is incinerated and dumped into landfills. Incinerators are one of the country's leading emissions sources of toxic and bio accumulative pollutants [143]. The diesel trucks which travel far distances to deliver medical waste to incinerators also emit toxic pollutants **[**143**]**. These pollutants that are released into the atmosphere threaten the public's health [143].

The primary method of sterilization of this bioreactor's components includes autoclaving and ethylene oxide treatments. Autoclave machines do not present much of a negative environmental impact, especially those that re-circulate their water via an integral tank. Power consumption can be an issue for many autoclaves. Autoclaves with the lowest power consumption would be those which use a site steam supply as no heating is involved or those with separate steam generators kept on constant standby which use relatively little electricity **[**144**]**. The overall negative environmental impacts associated with autoclaving such as water waste and power consumption can be kept to a minimum and controlled. The same cannot be done for ethylene oxide sterilization techniques. There are severe hazards associated with the emission of EO **[**131**]**. If released into the atmosphere, EO has the potential to cause cancer, birth defects, and other severe health issues associated with EO toxicity **[**131**]**. The mass production of this bioreactor would require the need for EO sterilization of certain materials, which would in turn present negative environmental effects.

The final prototype is comprised of seven main materials: aluminum alloy, zinc alloy, glass, PES filter membranes, acrylic, rubber, and PVC vinyl tubing. If the bioreactor becomes commercially available, then these main materials will be required for the manufacturing of the bioreactor. The showerhead of the main vessel and the top for the monitoring vessel of the bioreactor are both composed of aluminum. Regarding aluminum, there are no environmental impacts that exist. Natural aluminum occurs in the soil and makes up about 8% of the surface of the earth and it the most environmentally friendly metal on the planet [145]. In fact, it is the most recycled of any industrial metal. As this element is mostly found in nature, it is not dangerous, nor does it present any harm to nature [145].

The 5-inch screws are made of zinc, which is the most commonly found in the Earth's crust. Zinc presents negative toxicity effects as it can easily contaminate the soil and water in areas where it is naturally present or mined. When ingested in excess, it can cause various health problems including copper deficiency and organ damage. If this bioreactor is mass produced, then more zinc will need to be acquired which could call for more mining, thus more contamination of soil and water [146].

The manufacturing of the main collection vessel, which is made of glass, may have some negative impacts on the environment, since glass is difficult to recycle. As a result of the commitment to meeting the quality criteria for glass remanufacturing, there have been limits to the re-use of glass [147]. Thus, the proportion of non-recyclable materials in landfills has increased further. In many developed countries, landfill volume has increased to accommodate around 200 million tons of glass waste per year, despite very low recycling rates [147]. In the next few years, unless more suitable recycling methods for glass are introduced, acceptance of this bioreactor

would result in more glass waste. However, glass is reusable and easy to clean so it is an attractive material for our bioreactor.

The filter membranes used as the packing material for the BC to fabricate on is made from polyethersulfone (PES). Semipermeable polymetric membranes such as PES and polysulfone (PSF) are environmentally friendly, because they are produced by eco-friendly solvents and materials such as glass fibers, thus the implementation of this material should not present any major adverse environmental impacts [148]. The tiers that hold the PES membranes in place are made of PMMA or acrylic. The highest environmental impact presented with the textile manufacturing industry is from acrylic [149]. The reason is that production of acrylic substances is a high consumer of fossil fuels [149]. Human health and ecosystem quality are also subjected to negative impacts with the increased production of acrylic. These impacts are due to the inorganic chemicals used during the manufacturing process of the product and the acidification impact on the environment [149]. Rubber is also used in the design of the bioreactor to provide a tight connection between the aluminum and glass showerhead. Rubber stoppers are also used to introduce nutrients into the system. Synthetic rubber is a product of the petroleum industry. The environmental impact from the production and use of synthetic rubber derives primarily from energy consumption, use of fossil raw materials, emissions to air and water, and waste products [150]. The positive environmental aspects of synthetic rubber are associated with products that contribute to energy saving and reduction of noise and vibration [150].

6.3.3 Societal Influence

The project could pose a substantial impact on society in the future. For instance, if the bioreactor was deemed effective in producing high-quality BC which could be used to further advance the research and development of BC applications, then treatments would become more

readily available for patients. These treatments would enhance the quality of life of patients. If the results of this project were to be optimized in the future, the use of BC could likely extend beyond the current applications, calling for an increase in productivity and efficiency in many fields of research and development.

6.3.4 Political Ramifications

This project has insignificant political ramifications, as there would be no direct effects on the industry or commercial market. If commercialized, it is possible that well developed countries would implement this device first, since these countries would have access to the research, materials, and funding to provide them. The increase in standard of living would make living in these countries more favorable, which could potentially lead to migration of people from less developed countries. The bioreactor system design is in the initial stages of research and development, which means significant work needs to be done before it becomes marketable and accessible to the public.

6.3.5 Ethical Concerns

There are minimal ethical concerns associated with the results of the project. Since the goal of the bioreactor is to produce BC which will be used for research and development of BC applications, there are no direct ethical concerns associated with it. The only ethical concern that could be raised is the fact that BC is fabricated by the fermentation of bacteria. Some potential users may feel uncomfortable using BC derived materials from a bacteria source, even if the way the materials were obtained was ethical.

6.3.6 Health & Safety Impact

The BC produced by this bioreactor has many potential biomedical applications such as wound dressings due to its biocompatibility and mechanical strength. Therefore, increasing the

yield of BC without compromising its properties or uniformity makes it more widely available for these applications. By making BC more widely available, more patients will be able to access treatments that involve BC. In addition, the biocompatible and nontoxic nature of BC reduces the risk complications that are commonly associated with other existing treatments.

6.3.7 Manufacturability

This project aims to successfully design a low-cost bioreactor capable of regulating nutrients for the formation of BC while maximizing yield and ensuring homogeneous pellicle formation. Our novel design aims to preserve the advantages of a static culture including uniform pellicle formation and mechanical properties while maximizing the overall yield of BC. If the project is successful, BC will be produced on a larger scale with less manual labor due to the autonomous delivery of nutrients. The tiered cap design has the potential to become the gold standard for bioreactors to formulate BC as it incorporates elements of existing agitated bioreactors that increase the overall yield without disrupting the culture, resulting in uniform pellicles. The bioreactor system was designed to be user-friendly and durable. The entire system takes less than one hour to set up and consists of minimal components. The autonomous delivery of nutrients requires less manual labor to conduct the culture, which saves time and money. The overall bioreactor system is comprised of low-cost components that are easy to replace, if necessary, as the tiered cap design is easy to replicate on the university campus if necessary and the automated addition of nutrients requires less manual labor than the current static cultures. In the future, the

environmental monitoring system can become fully continuous if the demand is great enough. A fully continuous monitoring system would minimize the manufacturing impact even more.

6.3.8 Sustainability

A large amount of energy is required to produce BC via the bioreactor. To run the pumps, a constant supply of power is required. To formulate the media required for the culture and ensure sterility, energy is expended to power the biosafety cabinet and incubator. Repeated autoclave sterilizations require a significant amount of energy as well.

7.0 Discussion

Due to unforeseen circumstances, our bioreactor system was unable to produce BC on any measurable level. In the four cultures run within our system different issues were brought to light. In the first culture, the team realized that the flow rate on our pumping system was too high to promote BC growth on each of the tiers. The air-liquid interface was consistently being disturbed making pellicle formation difficult. The team attempted to mitigate this issue by reducing the overall resistance of the breadboard circuit to allow for greater control over the pump's rotations per minute. While this solution improved upon control of the pump, the overall flow rate was not ideal. The pumps and the circuit itself also proved to be unreliable. For instance, there would be multiple occurrences in all the cultures run where the pumps would stop working throughout the seven-day period. This was either due to a loose wire within the circuit or the static friction within the pumps prevented motion. In the third culture run, a mistake was made during the cell culture process. An old inoculate starter with a low bacteria concentration was used. Given that the initial concentration of bacteria was minimal, the possibility of BC production was reduced. In the last culture, run attempted the bioreactor was contaminated. This contamination was first indicated by the monitoring system before any visual signs with the media showed. It was shown by a rapid drop in dissolved oxygen content within the media. While this was not the intended use for this monitoring system, it is a useful feature to have. Despite not being able to produce a measurable amount of BC within the bioreactor system, specific benchmarks for the bioreactor system were determined by producing and testing BC from static culture.

7.1 User Friendly Testing

The results of the user-friendly testing showed that bioreactor system is capable of being assembled within the stated functional requirement. This means that the system can be setup and

fully functioning within an hour of labor. The team recognizes that throughout the course of designing the bioreactor system, a familiarity with all the systems components were gained making the assembly process easier. This can be seen with the improvement in assembly times between the three trial runs conducted by teammates. The team's one way ANOVA analysis indicates that the null hypothesis was rejected meaning at least one of the means measured is statistically different from the rest. This may be due to some team members having more experience than others in assembling the bioreactor system. Overall, this analysis does not put the functional requirement in question.

7.2 Durability Testing

The purpose of durability testing was to understand the number of cycles each component could withstand before needing to be replaced. Some components such as PES membranes are disposable, meaning they require replacement for each cycle. The inline air filter should also be replaced every iteration of testing to prevent a buildup of potential contaminants in the filter. Replacement of PES membrane and inline air filter should occur regardless of observational degradation. Other components such as zinc alloy nuts and screws to hold in place the acrylic tiers show corrosion following 2 cycles. This would require a replacement of zinc alloy components between every run to ensure rust or corrosion of the metal would not contaminate the culture. To improve this aspect of the design's durability, future experimentation with stainless steel screws and nuts would improve this component's durability. The tubing of the bioreactor shows no apparent degradation, although it should be thoroughly observed between runs for any damage to tubing at connection points. This includes locations where tubing is inserted into vessels, and where tubing is connected to pumps. Breadboard components and pumps were durable and sustained functionality through all testing cycles, although they should be frequently monitored

for damage to component pins. Most notably, potentiometers became easily shifted out of place, for a more permanent setup solder of the breadboard components could help secure them in place to prevent damage to pins.

The glassware used for the collection and monitoring vessels was exceptionally durable. The team did not observe any physical degradation of the material or cracking throughout several prototypes, trials, and several sterility cycles. The collection vessel constructed out of machined aluminum also showed good durability, no corrosion of the metal was detected throughout experimentation or sterilization. Monitoring components including the optical DO sensor, and the thermometer did not experience any corrosion or physical degradation. Acrylic components should be replaced on an as-need basis. After several trials with acrylic tier, one minor crack was observed in a piece of acrylic, although it did not influence its functionality in the system. Acrylic components should be checked frequently to detect any damage prior to assembly of the device.

7.3 Sterility Testing

The results of the sterility testing show that the bioreactor is capable of maintaining an aseptic environment for the duration of one cycle, as there were no bacteria observed under the microscope after the testing. Therefore, our bioreactor meets our sterility objective. It is essential that the bioreactor can maintain a sterile environment as any contamination will alter the properties of the BC that is produced. Furthermore, BC produced from a contaminated culture would not be able to be used in biomedical applications. While during this testing process the bioreactor was able to maintain a sterile environment, some instances of contamination did occur while running a cycle.

7.4 Uniformity Testing

The results of the uniformity testing indicate relative uniformity and transparency of BC produced from static culture. These values provide benchmarks that the BC produced by our bioreactor should be able to meet. Ideally, the bioreactor should be able to produce BC with the same or less relative variation compared to that of static culture, as the lower variation corresponds to a more uniform pellicle. The bioreactor should be able to produce BC with similar relative transparency values to that of BC produced by static culture.

7.5 Tensile Strength Testing

The results of our tensile strength testing on our two sets of BC samples produced by static culture serve as a benchmark for our samples produced by the bioreactor to meet. Specifically, the bioreactor should be able to meet or exceed the average values obtained for Young's Modulus and ultimate tensile strength, as high elasticity and mechanical strength are advantageous properties of BC.

7.6 Yield Testing

The results of our yield testing on our two sets of BC samples produced by static culture serve as a benchmark for our samples produced by the bioreactor to meet. The yield of BC obtained from our bioreactor should be greater yield than the yield of BC produced by our two static cultures, as one of our goals was to increase the overall yield of BC produced from bioreactor culture. The significant difference in average yield from each cycle is likely attributed to the fact that we used an older starter for the first cycle and a newer starter for the second cycle.

8.0 Conclusions and Recommendations

The task proposed to the design team was to design a low-cost bioreactor capable of the addition of nutrients and/or gases without disrupting the culture process. Given the revised client statement, the bioreactor must be durable, user-friendly, maintain a sterile environment, promote the production of BC, and effectively monitor environmental conditions. The design team was successful in designing and creating this bioreactor for BC production. Through specified testing methods the design objectives of being user-friendly, maintaining a sterile environment, and effectively monitoring environmental conditions were met.

However, two of the functional objectives were not met. In terms of durability, even though most bioreactor components can be sustained for an entire culture cycle, there needs to be a significant improvement in design durability to ensure the long-term function of the design. Observed corrosion to zinc components compromised the durability of the culture environment. Additionally, the bioreactor did not result in the production of uniform BC. This was due to minimal control over the flow rate of the pumping system, corrosion of the zinc components negatively affecting the culture environment, and unintended contamination. The production of uniform BC in the bioreactor system was discouraged to the minimal control over the flow rate. The pumps were operating too fast causing a constant interruption to the air-liquid interface on each of the tiers. This issue made our bioreactor's production process more agitated than static. The corrosion observed on the zinc components may have negatively affected the culture environment discouraging BC production. A portion of the contamination instances were accounted for due to human error during the culture process, however, not all. The team believes the small openings at the interface between the collection vessel and its cap or in the ports of the monitoring vessel may be allowing contaminates to get into the system. The flow rate of the air

pump may also be too strong for the inline filter causing a tear to occur. Evaluating these areas further will provide more insight. While the team could not conclude that the bioreactor promotes the production of BC, specific benchmarks were developed as standards for the bioreactor to meet in future development. Testing protocols for this analysis were optimized.

Future work will require more cultures to run within the developed bioreactor system. Due to unforeseen complications, our team was unable to determine how the bioreactor system compares to standard static culture. Before running these cultures within the system, our team would recommend that some improvements be made. First, the final showerhead design did not distribute the liquid as intended. After running the cultures, the team noticed that the holes for the showerhead were too large and spread apart. Remaking these holes with a smaller diameter and closer together will optimize the dispersion of media throughout each of the tiers. Second, the pumps used in our system proved to be unreliable. While the team was able to control the rotations per minute of these pumps using a potentiometer, we believe a higher initial investment into pumps that already have these features will be worthwhile. It will offer better control over the flow rate of the system ergo more influence over BC production. Lastly, we suggest replacing the zinc screws and bolts with stainless steel versions to avoid issues with corrosion. The overall goal of this project is to produce a bioreactor system capable of overcoming the limitation associated with standard static culture.

References

[1] M. L. Foresti, A. Vázquez, and B. Boury, "Applications of bacterial cellulose as precursor of carbon and composites with metal oxide, metal sulfide and metal nanoparticles: A review of recent advances," *Carbohydrate Polymers,* vol. 157, pp. 447-467, 2017/02/10/ 2017, doi: https://doi.org/10.1016/j.carbpol.2016.09.008.

[2] A. J. Brown, "XLIII.—On an acetic ferment which forms cellulose," *Journal of the Chemical Society, Transactions,* 10.1039/CT8864900432 vol. 49, no. 0, pp. 432-439, 1886, doi: 10.1039/CT8864900432.

[3] J. D. Fontana *et al.*, "Chapter 7 - New Insights on Bacterial Cellulose," in *Food Biosynthesis*, A. M. Grumezescu and A. M. Holban Eds.: Academic Press, 2017, pp. 213-249.

[4] R. Jonas and L. F. Farah, "Production and application of microbial cellulose," *Polymer Degradation and Stability,* vol. 59, no. 1, pp. 101-106, 1998/01/03/ 1998, doi: https://doi.org/10.1016/S0141-3910(97)00197-3.

[5] E. M. van Zyl and J. M. Coburn, "Hierarchical structure of bacterial-derived cellulose and its impact on biomedical applications," *Current Opinion in Chemical Engineering,* vol. 24, pp. 122-130, 2019.

[6] W. Czaja, D. Romanovicz, and R. m. Brown, "Structural investigations of microbial cellulose produced in stationary and agitated culture," *Cellulose,* vol. 11, no. 3, pp. 403-411, 2004/09/01 2004, doi: 10.1023/B:CELL.0000046412.11983.61.

[7] M. Roman, A. P. Haring, and T. J. Bertucio, "The growing merits and dwindling limitations of bacterial cellulose-based tissue engineering scaffolds," *Current Opinion in Chemical Engineering,* vol. 24, pp. 98-106, 2019/06/01 2019, doi: https://doi.org/10.1016/j.coche.2019.03.006.

[8] P. R. Chawla, I. B. Bajaj, S. A. Survase, and R. S. Singhal, "Microbial cellulose: fermentative production and applications," *Food Technology & Biotechnology,* vol. 47, no. 2, 2009.

[9] L. Nimeskern, H. Martínez Ávila, J. Sundberg, P. Gatenholm, R. Müller, and K. S. Stok, "Mechanical evaluation of bacterial nanocellulose as an implant material for ear cartilage replacement," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 22, pp. 12-21, 2013/06/01/ 2013, doi: https://doi.org/10.1016/j.jmbbm.2013.03.005.

[10] B. A. McKenna, D. Mikkelsen, J. B. Wehr, M. J. Gidley, and N. W. Menzies, "Mechanical and structural properties of native and alkali-treated bacterial cellulose produced by Gluconacetobacter xylinus strain ATCC 53524," *Cellulose,* vol. 16, no. 6, pp. 1047-1055, 2009/12/01 2009, doi: 10.1007/s10570-009-9340-y.

[11] E. Chanliaud, K. M. Burrows, G. Jeronimidis, and M. J. Gidley, "Mechanical properties of primary plant cell wall analogues," *Planta,* vol. 215, no. 6, pp. 989-996, 2002/10/01 2002, doi: 10.1007/s00425-002-0783-8.

[12] A. Nakayama *et al.*, "High Mechanical Strength Double-Network Hydrogel with Bacterial Cellulose," *Advanced Functional Materials,* vol. 14, no. 11, pp. 1124-1128, 2004, doi: https://doi.org/10.1002/adfm.200305197.

[13] G. F. Picheth *et al.*, "Bacterial cellulose in biomedical applications: A review," *International Journal of Biological Macromolecules,* vol. 104, pp. 97-106, 2017/11/01/ 2017, doi: https://doi.org/10.1016/j.ijbiomac.2017.05.171.

[14] M. L. Cacicedo *et al.*, "Progress in bacterial cellulose matrices for biotechnological applications," *Bioresource Technology,* vol. 213, pp. 172-180, 2016/08/01/ 2016, doi: https://doi.org/10.1016/j.biortech.2016.02.071.

[15] W. Czaja, A. Krystynowicz, S. Bielecki, and R. M. Brown, "Microbial cellulose—the natural power to heal wounds," *Biomaterials,* vol. 27, no. 2, pp. 145-151, 2006/01/01/ 2006, doi: https://doi.org/10.1016/j.biomaterials.2005.07.035.

[16] P. Ross, R. Mayer, and M. Benziman, "Cellulose biosynthesis and function in bacteria," (in eng), *Microbiol Rev,* vol. 55, no. 1, pp. 35-58, Mar 1991, doi: 10.1128/mr.55.1.35-58.1991.
[17] F. Esa, S. M. Tasirin, and N. A. Rahman, "Overview of Bacterial Cellulose Production and Application," *Agriculture and Agricultural Science Procedia,* vol. 2, pp. 113-119, 2014/01/01/ 2014, doi: https://doi.org/10.1016/j.aaspro.2014.11.017.
[18] S. Tanskul, K. Amornthatree, and N. Jaturonlak, "A new cellulose-producing bacterium, Rhodococcus sp. MI 2: Screening and optimization of culture conditions," *Carbohydrate Polymers,* vol. 92, no. 1, pp. 421-428, 2013/01/30/ 2013, doi: https://doi.org/10.1016/j.carbpol.2012.09.017.
[19] K. V. Ramana, A. Tomar, and L. Singh, "Effect of various carbon and nitrogen sources on cellulose synthesis by Acetobacter xylinum," *World Journal of Microbiology and Biotechnology,* vol. 16, no. 3, pp. 245-248, 2000/04/01 2000, doi: 10.1023/A:1008958014270.
[20] S. Gorgieva and J. Trček, "Bacterial Cellulose: Production, Modification and Perspectives in Biomedical Applications," (in eng), *Nanomaterials (Basel),* vol. 9, no. 10, Sep 20 2019, doi: 10.3390/nano9101352.
[21] S. Masaoka, T. Ohe, and N. Sakota, "Production of cellulose from glucose by Acetobacter xylinum," *Journal of Fermentation and Bioengineering,* vol. 75, no. 1, pp. 18-22, 1993/01/01/ 1993, doi: https://doi.org/10.1016/0922-338X(93)90171-4.
[22] S. Li *et al.*, "Fabrication of pH-electroactive Bacterial Cellulose/Polyaniline Hydrogel for the Development of a Controlled Drug Release System," *ES Materials & Manufacturing,* journal article vol. 1, pp. 41-49, 2018, doi: 10.30919/esmm5f120.
[23] P. Singhsa, R. Narain, and H. Manuspiya, "Bacterial Cellulose Nanocrystals (BCNC) Preparation and Characterization from Three Bacterial Cellulose Sources and Development of Functionalized BCNCs as Nucleic Acid Delivery Systems," *ACS Applied Nano Materials,* vol. 1, no. 1, pp. 209-221, 2018/01/26 2018, doi: 10.1021/acsanm.7b00105.
[24] M. L. Cacicedo *et al.*, "Bacterial cellulose hydrogel loaded with lipid nanoparticles for localized cancer treatment," *Colloids and Surfaces B: Biointerfaces,* vol. 170, pp. 596-608, 2018/10/01/ 2018, doi: https://doi.org/10.1016/j.colsurfb.2018.06.056.
[25] C. S. Szot, C. F. Buchanan, J. W. Freeman, and M. N. Rylander, "3D in vitro bioengineered tumors based on collagen I hydrogels," *Biomaterials,* vol. 32, no. 31, pp. 7905-7912, 2011/11/01/ 2011, doi: https://doi.org/10.1016/j.biomaterials.2011.07.001.
[26] S. R. Iyer, N. Udpa, and Y. Gao, "Chitosan selectively promotes adhesion of myoblasts over fibroblasts," *Journal of Biomedical Materials Research Part A,* vol. 103, no. 6, pp. 1899-1906, 2015, doi: https://doi.org/10.1002/jbm.a.35075.
[27] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2017," *CA: A Cancer Journal for Clinicians,* vol. 67, no. 1, pp. 7-30, 2017, doi: https://doi.org/10.3322/caac.21387.
[28] B. Ma, M. Zhang, C. He, and J. Sun, "New binary ionic liquid system for the preparation of chitosan/cellulose composite fibers," *Carbohydrate Polymers,* vol. 88, no. 1, pp. 347-351, 2012/03/17/ 2012, doi: https://doi.org/10.1016/j.carbpol.2011.12.020.
[29] K.-Y. Lee, G. Buldum, A. Mantalaris, and A. Bismarck, "More Than Meets the Eye in Bacterial Cellulose: Biosynthesis, Bioprocessing, and Applications in Advanced Fiber Composites," *Macromolecular Bioscience,* vol. 14, no. 1, pp. 10-32, 2014, doi: https://doi.org/10.1002/mabi.201300298.
[30] H.-J. Son, M.-S. Heo, Y.-G. Kim, and S.-J. Lee, "Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated Acetobacter," *Biotechnology and Applied Biochemistry,* vol. 33, no. 1, pp. 1-5, 2001, doi: https://doi.org/10.1042/BA20000065.

[31] A. Shirai *et al.*, "Biosynthesis of a novel polysaccharide by Acetobacter xylinum," *International Journal of Biological Macromolecules,* vol. 16, no. 6, pp. 297-300, 1994/12/01/ 1994, doi: https://doi.org/10.1016/0141-8130(94)90059-0.

[32] J. W. Hwang, Y. K. Yang, J. K. Hwang, Y. R. Pyun, and Y. S. Kim, "Effects of pH and dissolved oxygen on cellulose production by Acetobacter xylinum BRC5 in agitated culture," (in eng), *J Biosci Bioeng,* vol. 88, no. 2, pp. 183-8, 1999, doi: 10.1016/s1389-1723(99)80199-6.

[33] A. L. Chang *et al.*, "Phosphodiesterase A1, a Regulator of Cellulose Synthesis in Acetobacter xylinum, Is a Heme-Based Sensor," *Biochemistry,* vol. 40, no. 12, pp. 3420-3426, 2001/03/01 2001, doi: 10.1021/bi0100236.

[34] J. T. Henry and S. Crosson, "Ligand-binding PAS domains in a genomic, cellular, and structural context," (in eng), *Annu Rev Microbiol,* vol. 65, pp. 261-286, 2011, doi: 10.1146/annurev-micro-121809-151631.

[35] Z. Shi, Y. Zhang, G. O. Phillips, and G. Yang, "Utilization of bacterial cellulose in food," *Food Hydrocolloids,* vol. 35, pp. 539-545, 2014/03/01/ 2014, doi: https://doi.org/10.1016/j.foodhyd.2013.07.012.

[36] S.-P. Lin, I. Loira Calvar, J. M. Catchmark, J.-R. Liu, A. Demirci, and K.-C. Cheng, "Biosynthesis, production and applications of bacterial cellulose," *Cellulose,* vol. 20, no. 5, pp. 2191-2219, 2013/10/01 2013, doi: 10.1007/s10570-013-9994-3.

[37] X. Shi, Y. Zheng, G. Wang, Q. Lin, and J. Fan, "pH- and electro-response characteristics of bacterial cellulose nanofiber/sodium alginate hybrid hydrogels for dual controlled drug delivery," *RSC Advances,* 10.1039/C4RA09640A vol. 4, no. 87, pp. 47056-47065, 2014, doi: 10.1039/C4RA09640A.

[38] J. George and Siddaramaiah, "High performance edible nanocomposite films containing bacterial cellulose nanocrystals," *Carbohydrate Polymers,* vol. 87, no. 3, pp. 2031-2037, 2012/02/14/ 2012, doi: https://doi.org/10.1016/j.carbpol.2011.10.019.

[39] G. Yang, J. Xie, Y. Deng, Y. Bian, and F. Hong, "Hydrothermal synthesis of bacterial cellulose/AgNPs composite: A “green” route for antibacterial application," *Carbohydrate Polymers,* vol. 87, no. 4, pp. 2482-2487, 2012/03/01/ 2012, doi: https://doi.org/10.1016/j.carbpol.2011.11.017.

[40] X. Fan *et al.*, "Production of nano bacterial cellulose from beverage industrial waste of citrus peel and pomace using Komagataeibacter xylinus," *Carbohydrate Polymers,* vol. 151, pp. 1068-1072, 2016/10/20/ 2016, doi: https://doi.org/10.1016/j.carbpol.2016.06.062.

[41] F. Çakar, I. Özer, A. Ö. Aytekin, and F. Şahin, "Improvement production of bacterial cellulose by semi-continuous process in molasses medium," *Carbohydrate Polymers,* vol. 106, pp. 7-13, 2014/06/15/ 2014, doi: https://doi.org/10.1016/j.carbpol.2014.01.103.

[42] A. Cavka, X. Guo, S.-J. Tang, S. Winestrand, L. J. Jönsson, and F. Hong, "Production of bacterial cellulose and enzyme from waste fiber sludge," *Biotechnology for Biofuels,* vol. 6, no. 1, p. 25, 2013/02/16 2013, doi: 10.1186/1754-6834-6-25.

[43] H. K. Uzyol and M. T. Saçan, "Bacterial cellulose production by Komagataeibacter hansenii using algae-based glucose," *Environmental Science and Pollution Research,* vol. 24, no. 12, pp. 11154-11162, 2017/04/01 2017, doi: 10.1007/s11356-016-7049-7.

[44] A. F. Jozala *et al.*, "Bacterial cellulose production by Gluconacetobacter xylinus by employing alternative culture media," *Applied Microbiology and Biotechnology,* vol. 99, no. 3, pp. 1181-1190, 2015/02/01 2015, doi: 10.1007/s00253-014-6232-3.

[45] J.-M. Wu and R.-H. Liu, "Thin stillage supplementation greatly enhances bacterial cellulose production by Gluconacetobacter xylinus," *Carbohydrate Polymers,* vol. 90, no. 1, pp. 116-121, 2012/09/01/ 2012, doi: https://doi.org/10.1016/j.carbpol.2012.05.003.

[46] C. Huang *et al.*, "Using wastewater after lipid fermentation as substrate for bacterial cellulose production by Gluconacetobacter xylinus," *Carbohydrate Polymers,* vol. 136, pp. 198-202, 2016/01/20/ 2016, doi: https://doi.org/10.1016/j.carbpol.2015.09.043.

[47] B. V. Mohite, B. K. Salunke, and S. V. Patil, "Enhanced Production of Bacterial Cellulose by Using Gluconacetobacter hansenii NCIM 2529 Strain Under Shaking Conditions," *Applied Biochemistry and Biotechnology,* vol. 169, no. 5, pp. 1497-1511, 2013/03/01 2013, doi: 10.1007/s12010-013-0092-7.

[48] C. Molina-Ramírez *et al.*, "Effect of Different Carbon Sources on Bacterial Nanocellulose Production and Structure Using the Low pH Resistant Strain Komagataeibacter Medellinensis," *Materials,* vol. 10, no. 6, p. 639, 2017. [Online]. Available: https://www.mdpi.com/1996-1944/10/6/639.

[49] S. Abbasi-Moayed, H. Golmohammadi, and M. R. Hormozi-Nezhad, "A Nanopaper based Artificial Tongue: A Ratiometric Fluorescent Sensor Array on Bacterial Nanocellulose for Chemical Discrimination Applications," *Nanoscale,* vol. 10, pp. 2492-2502, 01/15 2018, doi: 10.1039/C7NR05801B.

[50] S. Abbasi-Moayed, H. Golmohammadi, A. Bigdeli, and M. R. Hormozi-Nezhad, "A Rainbow Ratiometric Fluorescence Sensor Array on Bacterial Nanocellulose for Visual Discrimination of Biothiols," *The Analyst,* vol. 143, 05/28 2018, doi: 10.1039/C8AN00637G.

[51] L. Urbina *et al.*, "By-products of the cider production: an alternative source of nutrients to produce bacterial cellulose," *Cellulose,* vol. 24, no. 5, pp. 2071-2082, 2017/05/01 2017, doi: 10.1007/s10570-017-1263-4.

[52] S.-P. Lin, Y.-H. Huang, K.-D. Hsu, Y.-J. Lai, Y.-K. Chen, and K.-C. Cheng, "Isolation and identification of cellulose-producing strain Komagataeibacter intermedius from fermented fruit juice," *Carbohydrate Polymers,* vol. 151, pp. 827-833, 2016/10/20/ 2016, doi: https://doi.org/10.1016/j.carbpol.2016.06.032.

[53] C.-H. Kuo, J.-H. Chen, B.-K. Liou, and C.-K. Lee, "Utilization of acetate buffer to improve bacterial cellulose production by Gluconacetobacter xylinus," *Food Hydrocolloids,* vol. 53, pp. 98-103, 2016/02/01/ 2016, doi: https://doi.org/10.1016/j.foodhyd.2014.12.034.

[54] N. Saichana, K. Matsushita, O. Adachi, I. Frébort, and J. Frebortova, "Acetic acid bacteria: A group of bacteria with versatile biotechnological applications," *Biotechnology Advances,* vol. 33, no. 6, Part 2, pp. 1260-1271, 2015/11/01/ 2015, doi: https://doi.org/10.1016/j.biotechadv.2014.12.001.

[55] M. Liu *et al.*, "Enhanced bacterial cellulose production by Gluconacetobacter xylinus via expression of Vitreoscilla hemoglobin and oxygen tension regulation," *Applied Microbiology and Biotechnology,* vol. 102, no. 3, pp. 1155-1165, 2018/02/01 2018, doi: 10.1007/s00253-017-8680-z.

[56] C. Castro *et al.*, "In situ production of nanocomposites of poly(vinyl alcohol) and cellulose nanofibrils from Gluconacetobacter bacteria: effect of chemical crosslinking," *Cellulose,* vol. 21, no. 3, pp. 1745-1756, 2014/06/01 2014, doi: 10.1007/s10570-014-0170-1.

[57] S.-P. Lin, C.-T. Liu, K.-D. Hsu, Y.-T. Hung, T.-Y. Shih, and K.-C. Cheng, "Production of bacterial cellulose with various additives in a PCS rotating disk bioreactor and its material property analysis," *Cellulose,* vol. 23, no. 1, pp. 367-377, 2016/02/01 2016, doi: 10.1007/s10570-015-0855-0.

[58] S. M. A. S. Keshk, "Vitamin C enhances bacterial cellulose production in Gluconacetobacter xylinus," *Carbohydrate Polymers,* vol. 99, pp. 98-100, 2014/01/02/ 2014, doi: https://doi.org/10.1016/j.carbpol.2013.08.060.

[59] R. V. Augimeri and J. L. Strap, "The Phytohormone Ethylene Enhances Cellulose Production, Regulates CRP/FNRKx Transcription and Causes Differential Gene Expression within the Bacterial

Cellulose Synthesis Operon of Komagataeibacter (Gluconacetobacter) xylinus ATCC 53582," (in English), *Frontiers in Microbiology,* Original Research vol. 6, 2015-December-22 2015, doi: 10.3389/fmicb.2015.01459.

[60] Y. Chao, M. Mitarai, Y. Sugano, and M. Shoda, "Effect of Addition of Water-Soluble Polysaccharides on Bacterial Cellulose Production in a 50-L Airlift Reactor," *Biotechnology Progress,* vol. 17, no. 4, pp. 781-785, 2001, doi: https://doi.org/10.1021/bp010046b.

[61] G. Serafica, R. Mormino, and H. Bungay, "Inclusion of solid particle in bacterial cellulose," *Applied microbiology and biotechnology,* vol. 58, pp. 756-60, 06/01 2002, doi: 10.1007/s00253-002-0978-8.

[62] P. Zhang, L. Chen, Q. Zhang, L. J. Jönsson, and F. F. Hong, "Using in situ nanocellulose-coating technology based on dynamic bacterial cultures for upgrading conventional biomedical materials and reinforcing nanocellulose hydrogels," *Biotechnology Progress,* vol. 32, no. 4, pp. 1077-1084, 2016, doi: https://doi.org/10.1002/btpr.2280.

[63] K. L. Hong and L. J. Sooter, "Single-Stranded DNA Aptamers against Pathogens and Toxins: Identification and Biosensing Applications," *BioMed Research International,* vol. 2015, p. 419318, 2015/06/23 2015, doi: 10.1155/2015/419318.

[64] M. Zaborowska, A. Bodin, H. Bäckdahl, J. Popp, A. Goldstein, and P. Gatenholm, "Microporous bacterial cellulose as a potential scaffold for bone regeneration," *Acta Biomaterialia,* vol. 6, no. 7, pp. 2540-2547, 2010/07/01/ 2010, doi: https://doi.org/10.1016/j.actbio.2010.01.004.

[65] M. A. Naeem, M. Alfred, P. Lv, H. Zhou, and Q. Wei, "Three-dimensional bacterial cellulose-electrospun membrane hybrid structures fabricated through in-situ self-assembly," *Cellulose,* vol. 25, no. 12, pp. 6823-6830, 2018/12/01 2018, doi: 10.1007/s10570-018-2084-9.

[66] N. F. Vasconcelos *et al.*, "Bacterial cellulose nanocrystals produced under different hydrolysis conditions: Properties and morphological features," *Carbohydrate Polymers,* vol. 155, pp. 425-431, 2017/01/02/ 2017, doi: https://doi.org/10.1016/j.carbpol.2016.08.090.

[67] J. A. Ávila Ramírez, C. Gómez Hoyos, S. Arroyo, P. Cerrutti, and M. L. Foresti, "Acetylation of bacterial cellulose catalyzed by citric acid: Use of reaction conditions for tailoring the esterification extent," *Carbohydrate Polymers,* vol. 153, pp. 686-695, 2016/11/20/ 2016, doi: https://doi.org/10.1016/j.carbpol.2016.08.009.

[68] S. C. M. Fernandes *et al.*, "Bioinspired Antimicrobial and Biocompatible Bacterial Cellulose Membranes Obtained by Surface Functionalization with Aminoalkyl Groups," *ACS Applied Materials & Interfaces,* vol. 5, no. 8, pp. 3290-3297, 2013/04/24 2013, doi: 10.1021/am400338n.

[69] C.-N. Wu *et al.*, "TEMPO-Oxidized Bacterial Cellulose Pellicle with Silver Nanoparticles for Wound Dressing," *Biomacromolecules,* vol. 19, no. 2, pp. 544-554, 2018/02/12 2018, doi: 10.1021/acs.biomac.7b01660.

[70] E. Morales-Narváez *et al.*, "Nanopaper as an Optical Sensing Platform," *ACS Nano,* vol. 9, no. 7, pp. 7296-7305, 2015/07/28 2015, doi: 10.1021/acsnano.5b03097.

[71] P. Paximada, E. A. Dimitrakopoulou, E. Tsouko, A. A. Koutinas, C. Fasseas, and I. G. Mandala, "Structural modification of bacterial cellulose fibrils under ultrasonic irradiation," *Carbohydrate Polymers,* vol. 150, pp. 5-12, 2016/10/05/ 2016, doi: https://doi.org/10.1016/j.carbpol.2016.04.125.

[72] K. Fijałkowski *et al.*, "Increased water content in bacterial cellulose synthesized under rotating magnetic fields," *Electromagnetic Biology and Medicine,* vol. 36, no. 2, pp. 192-201, 2017/04/03 2017, doi: 10.1080/15368378.2016.1243554.

[73] D. Dehnad, H. Mirzaei, Z. Emam-Djomeh, S.-M. Jafari, and S. Dadashi, "Thermal and antimicrobial properties of chitosan–nanocellulose films for extending shelf life of ground meat," *Carbohydrate Polymers,* vol. 109, pp. 148-154, 2014/08/30/ 2014, doi: https://doi.org/10.1016/j.carbpol.2014.03.063.

[74] F. Mohammadkazemi, M. Faria, and N. Cordeiro, "In situ biosynthesis of bacterial nanocellulose-CaCO3 hybrid bionanocomposite: One-step process," *Materials Science and Engineering: C,* vol. 65, pp. 393-399, 2016/08/01/ 2016, doi: https://doi.org/10.1016/j.msec.2016.04.069.

[75] T. D. Lopes, I. C. Riegel-Vidotti, A. Grein, C. A. Tischer, and P. C. d. S. Faria-Tischer, "Bacterial cellulose and hyaluronic acid hybrid membranes: Production and characterization," *International Journal of Biological Macromolecules,* vol. 67, pp. 401-408, 2014/06/01/ 2014, doi: https://doi.org/10.1016/j.ijbiomac.2014.03.047.

[76] R. Poonguzhali, S. Khaleel Basha, and V. Sugantha Kumari, "Novel asymmetric chitosan/PVP/nanocellulose wound dressing: In vitro and in vivo evaluation," *International Journal of Biological Macromolecules,* vol. 112, pp. 1300-1309, 2018/06/01/ 2018, doi: https://doi.org/10.1016/j.ijbiomac.2018.02.073.

[77] I. Sulaeva, U. Henniges, T. Rosenau, and A. Potthast, "Bacterial cellulose as a material for wound treatment: Properties and modifications. A review," *Biotechnology Advances,* vol. 33, no. 8, pp. 1547-1571, 2015/12/01/ 2015, doi: https://doi.org/10.1016/j.biotechadv.2015.07.009.

[78] O. Shezad, S. Khan, T. Khan, and J. K. Park, "Production of bacterial cellulose in static conditions by a simple fed-batch cultivation strategy," *Korean Journal of Chemical Engineering,* vol. 26, no. 6, pp. 1689-1692, 2009/11/01 2009, doi: 10.1007/s11814-009-0232-5.

[79] L. Lamboni, Y. Li, J. Liu, and G. Yang, "Silk Sericin-Functionalized Bacterial Cellulose as a Potential Wound-Healing Biomaterial," *Biomacromolecules,* vol. 17, no. 9, pp. 3076-3084, 2016/09/12 2016, doi: 10.1021/acs.biomac.6b00995.

[80] S. Napavichayanun, P. Amornsudthiwat, P. Pienpinijtham, and P. Aramwit, "Interaction and effectiveness of antimicrobials along with healing-promoting agents in a novel biocellulose wound dressing," *Materials Science and Engineering: C,* vol. 55, pp. 95-104, 2015/10/01/ 2015, doi: https://doi.org/10.1016/j.msec.2015.05.026.

[81] M. Balat, "Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review," *Energy Conversion and Management,* vol. 52, no. 2, pp. 858-875, 2011/02/01/ 2011, doi: https://doi.org/10.1016/j.enconman.2010.08.013.

[82] S. Bottan *et al.*, "Surface-Structured Bacterial Cellulose with Guided Assembly-Based Biolithography (GAB)," *ACS Nano,* vol. 9, no. 1, pp. 206-219, 2015/01/27 2015, doi: 10.1021/nn5036125.

[83] M. Rouabhia, J. Asselin, N. Tazi, Y. Messaddeq, D. Levinson, and Z. Zhang, "Production of Biocompatible and Antimicrobial Bacterial Cellulose Polymers Functionalized by RGDC Grafting Groups and Gentamicin," *ACS Applied Materials & Interfaces,* vol. 6, no. 3, pp. 1439-1446, 2014/02/12 2014, doi: 10.1021/am4027983.

[84] N. Pircher, S. Veigel, N. Aigner, J. M. Nedelec, T. Rosenau, and F. Liebner, "Reinforcement of bacterial cellulose aerogels with biocompatible polymers," *Carbohydrate Polymers,* vol. 111, pp. 505-513, 2014/10/13/ 2014, doi: https://doi.org/10.1016/j.carbpol.2014.04.029.

[85] M. H. Kwak *et al.*, "Bacterial cellulose membrane produced by Acetobacter sp. A10 for burn wound dressing applications," *Carbohydrate Polymers,* vol. 122, pp. 387-398, 2015/05/20/ 2015, doi: https://doi.org/10.1016/j.carbpol.2014.10.049.

[86] G. Helenius, H. Bäckdahl, A. Bodin, U. Nannmark, P. Gatenholm, and B. Risberg, "In vivo biocompatibility of bacterial cellulose," *Journal of Biomedical Materials Research Part A,* vol. 76A, no. 2, pp. 431-438, 2006, doi: https://doi.org/10.1002/jbm.a.30570.

[87] M. Scherner *et al.*, "In vivo application of tissue-engineered blood vessels of bacterial cellulose as small arterial substitutes: proof of concept?," *Journal of Surgical Research,* vol. 189, no. 2, pp. 340-347, 2014/06/15/ 2014, doi: https://doi.org/10.1016/j.jss.2014.02.011.

[88] S. A. Eming, H. Smola, and T. Krieg, "Treatment of Chronic Wounds: State of the Art and Future Concepts," *Cells Tissues Organs,* vol. 172, no. 2, pp. 105-117, 2002, doi: 10.1159/000065611.

[89] F. M. Portela da Gama and F. Dourado, "Bacterial NanoCellulose: what future?," (in eng), *Bioimpacts,* vol. 8, no. 1, pp. 1-3, 2018, doi: 10.15171/bi.2018.01.
[90] N. Lin and A. Dufresne, "Nanocellulose in biomedicine: Current status and future prospect," *European Polymer Journal,* vol. 59, pp. 302-325, 2014/10/01/ 2014, doi: https://doi.org/10.1016/j.eurpolymj.2014.07.025.
[91] H. Ullah, F. Wahid, H. A. Santos, and T. Khan, "Advances in biomedical and pharmaceutical applications of functional bacterial cellulose-based nanocomposites," *Carbohydrate Polymers,* vol. 150, pp. 330-352, 2016/10/05/ 2016, doi: https://doi.org/10.1016/j.carbpol.2016.05.029.
[92] T. Heinze and T. Liebert, "Unconventional methods in cellulose functionalization," *Progress in Polymer Science,* vol. 26, no. 9, pp. 1689-1762, 2001/11/01/ 2001, doi: https://doi.org/10.1016/S0079-6700(01)00022-3.
[93] A. Kaasi and A. L. Jardini, "Bioreactors," in *Reference Module in Materials Science and Materials Engineering*: Elsevier, 2016.
[94] S. S. Muthu and R. Rathinamoorthy, "Characteristics of Bacterial Cellulose," in *Bacterial Cellulose: Sustainable Material for Textiles*, S. S. Muthu and R. Rathinamoorthy Eds. Singapore: Springer Singapore, 2021, pp. 61-130.
[95] C. Zhong, "Industrial-Scale Production and Applications of Bacterial Cellulose," (in English), *Frontiers in Bioengineering and Biotechnology,* Review vol. 8, no. 1425, 2020-December-22 2020, doi: 10.3389/fbioe.2020.605374.
[96] J. Wang, J. Tavakoli, and Y. Tang, "Bacterial cellulose production, properties and applications with different culture methods – A review," *Carbohydrate Polymers,* vol. 219, pp. 63-76, 2019/09/01/ 2019, doi: https://doi.org/10.1016/j.carbpol.2019.05.008.
[97] K. Watanabe, M. Tabuchi, Y. Morinaga, and F. Yoshinaga, "Structural Features and Properties of Bacterial Cellulose Produced in Agitated Culture," *Cellulose,* vol. 5, no. 3, pp. 187-200, 1998/09/01 1998, doi: 10.1023/A:1009272904582.
[98] F. G. Blanco Parte *et al.*, "Current progress on the production, modification, and applications of bacterial cellulose," *Critical Reviews in Biotechnology,* vol. 40, no. 3, pp. 397-414, 2020/04/02 2020, doi: 10.1080/07388551.2020.1713721.
[99] G. D. Najafpour, "CHAPTER 6 - Bioreactor Design," in *Biochemical Engineering and Biotechnology*, G. D. Najafpour Ed. Amsterdam: Elsevier, 2007, pp. 142-169.
[100] S. Mitra and G. S. Murthy, "Bioreactor control systems in the biopharmaceutical industry: a critical perspective," (in eng), *Systems Microbiology and Biomanufacturing,* vol. 2, no. 1, pp. 91-112, 2022, doi: 10.1007/s43393-021-00048-6.
[101] M. P. Selker, Barbara, "Single-Use Solutions for Scale-Up and Technology Transfer," in "Innovations in Pharmaceutical Technology," Finesse Solutions.
[102] K.-C. Cheng, J. M. Catchmark, and A. Demirci, "Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis," *Journal of Biological Engineering,* vol. 3, no. 1, p. 12, 2009/07/24 2009, doi: 10.1186/1754-1611-3-12.
[103] M. Onodera, I. Harashima, K. Toda, and T. Asakura, "Silicone rubber membrane bioreactors for bacterial cellulose production," *Biotechnology and Bioprocess Engineering,* vol. 7, no. 5, p. 289, 2002/10/01 2002, doi: 10.1007/BF02932838.
[104] Y. Chao, Y. Sugano, and M. Shoda, "Bacterial cellulose production under oxygen-enriched air at different fructose concentrations in a 50-liter, internal-loop airlift reactor," (in eng), *Appl Microbiol Biotechnol,* vol. 55, no. 6, pp. 673-9, Jun 2001, doi: 10.1007/s002530000503.
[105] I. Reiniati, A. N. Hrymak, and A. Margaritis, "Kinetics of cell growth and crystalline nanocellulose production by Komagataeibacter xylinus," *Biochemical Engineering Journal,* vol. 127, pp. 21-31, 2017/11/15/ 2017, doi: https://doi.org/10.1016/j.bej.2017.07.007.

[106] C.-C. Fu, W.-T. Wu, and S.-Y. Lu, "Performance of airlift bioreactors with net draft tube," *Enzyme and Microbial Technology,* vol. 33, no. 4, pp. 332-342, 2003/09/10/ 2003, doi: https://doi.org/10.1016/S0141-0229(03)00151-0.
[107] M. Shoda and Y. Sugano, "Recent advances in bacterial cellulose production," *Biotechnology and Bioprocess Engineering,* vol. 10, no. 1, p. 1, 2005/02/01 2005, doi: 10.1007/BF02931175.
[108] S.-C. Wu and M.-H. Li, "Production of bacterial cellulose membranes in a modified airlift bioreactor by Gluconacetobacter xylinus," *Journal of Bioscience and Bioengineering,* vol. 120, no. 4, pp. 444-449, 2015/10/01/ 2015, doi: https://doi.org/10.1016/j.jbiosc.2015.02.018.
[109] A. Krystynowicz, W. Czaja, A. Wiktorowska-Jezierska, M. Gonçalves-Miśkiewicz, M. Turkiewicz, and S. Bielecki, "Factors affecting the yield and properties of bacterial cellulose," *Journal of Industrial Microbiology and Biotechnology,* vol. 29, no. 4, pp. 189-195, 2002, doi: 10.1038/sj.jim.7000303.
[110] S. Gonzalo Cruz, "Production of bacterial cellulose using a rotating disk film bioreactor by Acetobacter xylinum," Chemical Engineering, Rensselaer Polytechnic Institute, 1997.
[111] K.-C. Cheng, J. M. Catchmark, and A. Demirci, "Effects of CMC Addition on Bacterial Cellulose Production in a Biofilm Reactor and Its Paper Sheets Analysis," *Biomacromolecules,* vol. 12, no. 3, pp. 730-736, 2011/03/14 2011, doi: 10.1021/bm101363t.
[112] J. Y. Jung, T. Khan, J. K. Park, and H. N. Chang, "Production of bacterial cellulose by Gluconacetobacter hansenii using a novel bioreactor equipped with a spin filter," *Korean Journal of Chemical Engineering,* vol. 24, no. 2, pp. 265-271, 2007/03/01 2007, doi: 10.1007/s11814-007-5058-4.
[113] W. J. Zinnanti *et al.*, "Dual mechanism of brain injury and novel treatment strategy in maple syrup urine disease," *Brain,* vol. 132, no. 4, pp. 903-918, 2009, doi: 10.1093/brain/awp024.
[114] N. Noro, Y. Sugano, and M. Shoda, "Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by Acetobacter xylinum," *Applied Microbiology and Biotechnology,* vol. 64, no. 2, pp. 199-205, 2004/04/01 2004, doi: 10.1007/s00253-003-1457-6.
[115] Y. p. Chao, Y. Sugano, T. Kouda, F. Yoshinaga, and M. Shoda, "Production of bacterial cellulose by Acetobacter xylinumwith an air-lift reactor," *Biotechnology Techniques,* vol. 11, no. 11, pp. 829-832, 1997/11/01 1997, doi: 10.1023/A:1018433526709.
[116] Y. Chao, T. Ishida, Y. Sugano, and M. Shoda, "Bacterial cellulose production by Acetobacter xylinum in a 50-L internal-loop airlift reactor," *Biotechnology and Bioengineering,* vol. 68, no. 3, pp. 345-352, 2000, doi: https://doi.org/10.1002/(SICI)1097-0290(20000505)68:3<345::AID-BIT13>3.0.CO;2-M.
[117] H.-P. Cheng, P.-M. Wang, J.-W. Chen, and W.-T. Wu, "Cultivation of Acetobacter xylinum for bacterial cellulose production in a modified airlift reactor," *Biotechnology and Applied Biochemistry,* vol. 35, no. 2, pp. 125-132, 2002, doi: https://doi.org/10.1111/j.1470-8744.2002.tb01180.x.
[118] T. Ishida, M. Mitarai, Y. Sugano, and M. Shoda, "Role of water-soluble polysaccharides in bacterial cellulose production," *Biotechnology and Bioengineering,* vol. 83, no. 4, pp. 474-478, 2003, doi: https://doi.org/10.1002/bit.10690.
[119] K. Zuo, H.-P. Cheng, S.-C. Wu, and W.-T. Wu, "A hybrid model combining hydrodynamic and biological effects for production of bacterial cellulose with a pilot scale airlift reactor," *Biochemical Engineering Journal,* vol. 29, no. 1, pp. 81-90, 2006/04/01/ 2006, doi: https://doi.org/10.1016/j.bej.2005.02.020.
[120] L. Gardner, "Stability and design of stainless steel structures – Review and outlook," *Thin-Walled Structures,* vol. 141, pp. 208-216, 2019/08/01/ 2019, doi: https://doi.org/10.1016/j.tws.2019.04.019.

[121] L. Gardner, "The use of stainless steel in structures," *Progress in Structural Engineering and Materials,* vol. 7, no. 2, pp. 45-55, 2005, doi: https://doi.org/10.1002/pse.190.
[122] L. Gardner, "Aesthetics, economics and design of stainless steel structures," 2008.
[123] D. D. P. Systems. "Borosilicate Glass Equipment." https://www.ddpsinc.com/knowledge/what-are-the-benefits-of-glass-and-glass-lining (accessed.
[124] Y. Ramot, M. Haim-Zada, A. J. Domb, and A. Nyska, "Biocompatibility and safety of PLA and its copolymers," *Advanced Drug Delivery Reviews,* vol. 107, pp. 153-162, 2016/12/15/ 2016, doi: https://doi.org/10.1016/j.addr.2016.03.012.
[125] A. Barrett. "Advantages and Disadvantages of PLA." Bioplastics News. https://bioplasticsnews.com/2020/06/09/polylactic-acid-pla-dis-advantages/ (accessed.
[126] T. Lovaši, J. Pinc, and I. Voňavková, "Zinc-based Degradable Biomaterials–Limitations and Enhancements," *Manufacturing Technology,* vol. 19, no. 4, pp. 632-636, 2019.
[127] L. W. May and L. G. Seong, "A Narrative Review of Different Types and Processing Methods of Acrylic Denture Base Material," *Annals of Dentistry,* 2018.
[128] E. Pawar, "A review article on acrylic PMMA," *IOSR J. Mech. Civ. Eng,* vol. 13, no. 2, pp. 1-4, 2016.
[129] Steris Healthcare. "Everything About Autoclaves." https://www.steris.com/healthcare/knowledge-center/sterile-processing/everything-about-autoclaves (accessed.
[130] Medical Search. "Things to Consider When Buying Autoclaves & Sterilisers." https://www.medicalsearch.com.au/things-to-consider-when-buying-autoclaves-and-sterilisers/f/18247 (accessed.
[131] G. C. C. Mendes, T. R. S. Brandão, and C. L. M. Silva, "Ethylene oxide sterilization of medical devices: A review," *American Journal of Infection Control,* vol. 35, no. 9, pp. 574-581, 2007/11/01/ 2007, doi: https://doi.org/10.1016/j.ajic.2006.10.014.
[132] G. Kampf, "Efficacy of ethanol against viruses in hand disinfection," *Journal of Hospital Infection,* vol. 98, no. 4, pp. 331-338, 2018/04/01/ 2018, doi: https://doi.org/10.1016/j.jhin.2017.08.025.
[133] "PES (Polyethersulfone) Membranes." https://libraryguides.vu.edu.au/ieeereferencing/webbaseddocument (accessed February 25, 2022).
[134] C. H. Zierdt, "Adherence of bacteria, yeast, blood cells, and latex spheres to large-porosity membrane filters," *Applied and Environmental Microbiology,* vol. 38, no. 6, pp. 1166-1172, 1979, doi: doi:10.1128/aem.38.6.1166-1172.1979.
[135] A. Gössi *et al.*, "In-situ recovery of carboxylic acids from fermentation broths through membrane supported reactive extraction using membrane modules with improved stability," *Separation and Purification Technology,* vol. 241, p. 116694, 2020/06/15/ 2020, doi: https://doi.org/10.1016/j.seppur.2020.116694.
[136] S. Suresh, V. Srivastava, and I. Mishra, "Techniques for oxygen transfer measurement in bioreactors: a review," *Journal of Chemical Technology & Biotechnology,* vol. 84, no. 8, pp. 1091-1103, 2009, doi: https://doi.org/10.1002/jctb.2154.
[137] L. Cvitas and Z. Hocenski, "Automated Measurement System for Industrial Platinum Resistance Thermometer Manufacturing Industry," in *2007 IEEE International Symposium on Industrial Electronics*, 4-7 June 2007 2007, pp. 1377-1380, doi: 10.1109/ISIE.2007.4374801.
[138] A. Tong, "Improving the accuracy of temperature measurements," *Sensor Review,* vol. 21, no. 3, pp. 193-198, 2001, doi: 10.1108/02602280110398044.
[139] Apera Instruments. "Why Choose a pH Meter instead of a pH Test Strip." https://aperainst.com/blog/why-choose-a-ph-meter-instead-of-a-ph-test-strip/#:~:text=In%20summary%2C%20pH%20meters%20are,and%20accurate%20than%20test%

20strips.&text=When%20a%20test%20strip%20is,color%20comparison%20in%20different%20ways. (accessed.
[140] P. J. Carlson. "PH Meter Versus PH Paper." https://sciencing.com/ph-meter-versus-ph-paper-5840578.html (accessed.
[141] C. Meng, J. Hu, K. Gourlay, C. Yu, and J. N. Saddler, "Controllable synthesis uniform spherical bacterial cellulose and their potential applications," *Cellulose,* vol. 26, no. 15, pp. 8325-8336, 2019/10/01 2019, doi: 10.1007/s10570-019-02446-5.
[142] J. R. Davis, *Tensile testing*. ASM international, 2004.
[143] B. A. Wormer *et al.*, "The Green Operating Room: Simple Changes to Reduce Cost and Our Carbon Footprint," *The American Surgeon,* vol. 79, no. 7, pp. 666-671, 2013/07/01 2013, doi: 10.1177/000313481307900708.
[144] A. Vita, V. Castorani, M. Germani, and M. Marconi, "Comparative life cycle assessment and cost analysis of autoclave and pressure bag molding for producing CFRP components," *The International Journal of Advanced Manufacturing Technology,* vol. 105, no. 5, pp. 1967-1982, 2019/12/01 2019, doi: 10.1007/s00170-019-04384-9.
[145] P. Nayak, "Aluminum: Impacts and Disease," *Environmental Research,* vol. 89, no. 2, pp. 101-115, 2002/06/01/ 2002, doi: https://doi.org/10.1006/enrs.2002.4352.
[146] E. P. Centers. "Zinc Poisoning." https://www.environmentalpollutioncenters.org/zinc/#:~:text=Zinc%20can%20easily%20contaminate%20the,can%20cause%20various%20health%20problems (accessed.
[147] O. Adekomaya and T. Majozi, "Mitigating environmental impact of waste glass materials: review of the existing reclamation options and future outlook," *Environmental Science and Pollution Research,* vol. 28, no. 9, pp. 10488-10502, 2021/03/01 2021, doi: 10.1007/s11356-020-12263-0.
[148] P. Yadav, N. Ismail, M. Essalhi, M. Tysklind, D. Athanassiadis, and N. Tavajohi, "Assessment of the environmental impact of polymeric membrane production," *Journal of Membrane Science,* vol. 622, p. 118987, 2021/03/15/ 2021, doi: https://doi.org/10.1016/j.memsci.2020.118987.
[149] D. M. M. Yacout, M. A. Abd El-Kawi, and M. S. Hassouna, "Cradle to gate environmental impact assessment of acrylic fiber manufacturing," *The International Journal of Life Cycle Assessment,* vol. 21, no. 3, pp. 326-336, 2016/03/01 2016, doi: 10.1007/s11367-015-1023-3.
[150] A. Grec, F. Dumescu, and C. Maior, "Assessment of the environmental impacts generated by rubber processing activity," *Environmental engineering and management journal,* vol. 8, pp. 1533-1540, 11/01 2009, doi: 10.30638/eemj.2009.222.

Appendices

Appendix A: Constraints Test for Design Means

Table 53: Constraints Test for Developed Means

		Constraints			
Function	Means	Time	Money	Materials	Sterility
Pass (P) / Fail (F)					
1	Stainless Steel	P	P	P	P
	Glass	P	P	P	P
	3D Printed PLA	P	P	P	P
2	Arduino	P	P	P	P
	LabQuest mini	P	P	P	P
	LabQuest 2	P	F	P	P
	LabQuest 3	P	F	P	P
	Logger Pro	P	P	P	P
3	Autoclave	P	P	P	P
	Ethylene Oxide	P	P	P	P
	Gamma Irradiation	P	F	F	P
	Ethanol & PBS	P	P	P	P
4	Filter Membrane (PES)	P	P	P	P
	Teflon/Nylon	P	P	P	P
	Wire Mesh	P	P	P	P
5	Optical Dissolved Oxygen Probe	P	P	P	P
	Galvanic Oxygen Probe	P	P	P	P
	Stainless Steel Temperature Probe	P	P	P	P
	Thermometer	P	P	P	P
	pH strips	P	P	P	P
	pH Probe	P	P	P	P

Key Questions:

Time = Is using the material/item feasible within our 9-month time frame?

Money = Is the material/item affordable and able to be purchased within means of the budget?

Materials Availability = Is the material/item available for purchase?

Sterility = Is the material/item able to be sterilized and maintain sterility?

Appendix B: Bioreactor Standard Operating Procedure

Materials and Components:

- Glass Collection Vessel
- Glass Monitoring Vessel
- Two Side Port Caps for the Collection Vessel
- Four zinc 5-Inch Screws
- Six Acrylic Tiers:
    - 30 Inch-Diameter, 35 Inch-Diameter, 40 Inch-Diameter, 45 Inch-Diameter, 50 Inch-Diameter, 55 Inch-Diameter
- 40 Zinc Nuts
- Aluminum Showerhead acrylic topper for Collection Vessel
- Acrylic Top and Aluminum Outer Ring for the Monitoring Vessel
- Six PES Filter Membranes:
    - 30 mm-Diameter, 35 mm-Diameter, 40 mm-Diameter, 45 mm-Diameter, 50 mm-Diameter, 55 mm-Diameter
- Rubber Seal
- Optical DO Probe
- Temperature Probe
- Air Bubbler
- Breadboard Circuit and Two Peristaltic Pumps
- Vinyl Tubing (3mm inner diameter, 5mm outer diameter
    - Peristaltic tubing (2mm innter, 3 mm outer)

Sterilization of Bioreactor Components

Bioreactor glassware and all its components were sterilized using the necessary sterilization methods:

Autoclave:

- PES filter membranes
- HS Media
- Zinc alloy screws and nuts
- Aluminum showerhead
- Glass collection and monitoring vessels
- Monitoring aluminum outer ring
- Septum

EO:

- All tubing components including connectors
- Rubber seal
- Acrylic components
- Air valve

Collection Vessel Set-Up

Necessary Components:

- Glass Collection Vessel
- Four 5-Inch Screws
- Six Acrylic Tiers and Corresponding PES Filter membranes:

- 30 mm-Diameter, 35 mm-Diameter, 40 mm-Diameter, 45 mm-Diameter, 50 mm-Diameter, 55 mm-Diameter

- 40 Nuts
- Aluminum Showerhead and Acrylic Topper
- Rubber Seal

Method:

After sterilization techniques have been performed to sterilize the necessary components, this step in the set-up process must be completed in a biosafety cabinet to ensure the components of this vessel remain sterile.

1. Start by laying out each acrylic tier in decreasing order (55 mm-diameter to 25 mm-diameter) with their corresponding washers, for easy access.
2. Next place each PES filter membrane with their corresponding diameter in between the six acrylic tiers and their corresponding washers, so that your set up looks like Figure B1.

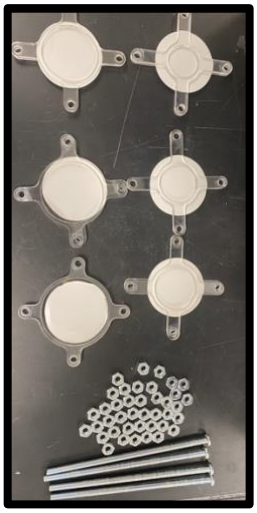


Figure B1: Component Preparation for Cap Assembly

3. Begin threading the tiers on the four 5-inch screws by first inserting the 55 mm-diameter tiers through the screws so that it is at the bottom of the screws and mirrors as illustrated in Figure B2.

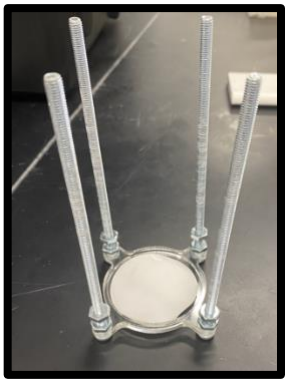


Figure B2: First Tier Threaded on Screws

4. Thread four nuts on each screw to secure the first tier in place.
5. Once the 55 mm-diameter acrylic tier is secured in place, thread four additional nuts to the bottom most black line.
6. Apply the 50 mm-diameter acrylic tier on top of the four screws and secure it in place with four more additional nuts. Your device should now look like Figure B3.

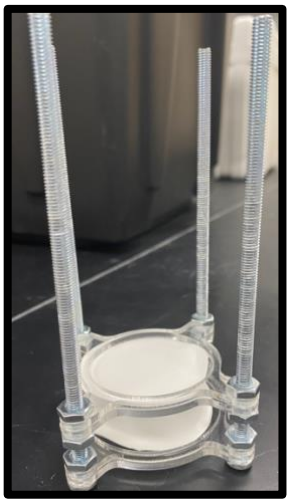


Figure B3: First Two Tiers Threaded on Screws

7. Repeat steps 5 and 6 except for threading four nuts to the bottom-most line that exceeds the previous line already threaded and applying the next acrylic tier in decreasing diameter until all the 40 nuts and the six tiers are fastened in place. Your final device should be like Figure B4.

Figure B4: Assembled Cap

8. Once all your tiers and nuts are in place, next thread the four 5-inch screws into the holes of the aluminum showerhead, ensuring it is tight and secure.
9. Before placing the device in the main glass vessel, apply the rubber seal over the tiers so that it is directly under the shower head.
10. Insert the finished device to the main glass collection vessel. The completed system should mirror Figure B5.

Figure B5: Completed System

Monitoring Vessel Set-Up

Materials and Components:

- Glass Monitoring Vessel
- Optical DO Probe
- Temperature Probe
- Vinyl Tubing
- Septum
- One- Way valve
- Air pump

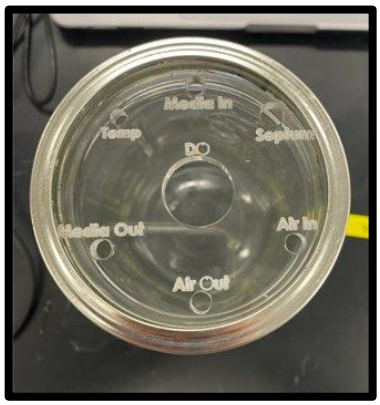


Figure B6: Monitoring System Top

Assembly of the monitoring vessel only requires in addition of media into the vessel. Once media is added, tubing can be added in the appropriate location ensuring that media is flowing such that it is taken from the monitoring vessel and added fed to the top of the collection vessel. The tubing should then be circulated from the bottom of the collection vessel back to the monitoring vessel. This requires two tube locations in the monitoring vessel (Media in and media out). Next, the air pump, DO probe, and temperature probe should be inserted in their appropriately sized holes in the acrylic lid. Finally, the one-way valve can be added to allow for the direction of

airflow out of the system to prevent air pressure build-up. And the septum can be added to its appropriate position, to add additional media, glucose or inoculate if necessary.

Arduino Breadboard Set-Up

Necessary Components:

- 3.3v to 5v driver
- 9v power supply adapter
- 2222A transistor
- 2x alligator clips/jumper cables
- U- Shape jumper wires
- Breadboard
- 10k ohm resistor

Method:

1. Align the board so that it is long way left to right and that the (+) is at the top and (-) is at the bottom.
2. Add 3.3v to 5V driver to the breadboard. Be sure to align up +/- allocation of the driver to match the orientation of the board. The driver power ports should face the rights and be flush with right side of the board. Ensure that all driver pins fit on the entire board, otherwise the back portion of the driver may hang off the edge. Ensure that the yellow voltage identifiers are placed on 3.3V to 5V to start. One or both yellow voltage pins can be changed to step up the voltage at any time.
3. Place potentiometer inline close to the driver along row J at least 5 columns left of the driver. The potentiometer wheel should face you.

a. EX: J31, J33, and J35

4. Attach a U-Shape wire from the + row of the board to behind the rightmost pin of the potentiometer.
    a. EX. (+) to G31
5. Place transistor appx 5 pegs to the left of the potentiometer in row F. the transistor has 3 pegs.
    a. EX. F43, F42, F41
    b. Align transistor so that the flat side faces toward you
6. Attach U shape wire behind the middlemost pin on the potentiometer to a peg in front of the middle transistor pin. The finalized circuit should look like Figure B7.
    a. EX. G33 to H42

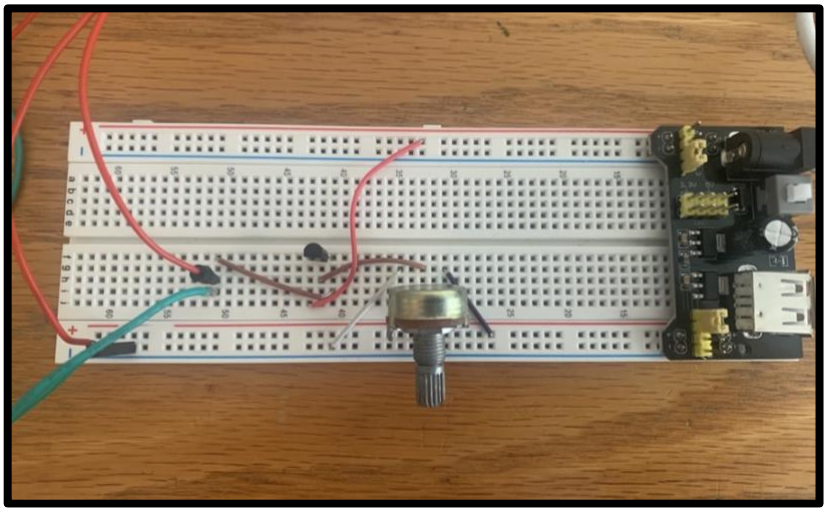


Figure B7: Finalized Circuit

7. Attach U shape wire behind the left most pin on the potentiometer to anywhere in the front most (-).
    a. EX. G35 to (-)
8. Attach U Shape wire in front of the left most pin on the transistor to the uppermost (+) row.
    a. EX. J43 to (+)33

9. Attach U Shape wire in front of the rightmost pin on the transistor to anywhere left in row F, G, or H
    a. EX. I41 to F51
10. Attach jumper wires to pumps
    a. Pump polarity is identified with a red dot near the attachment point. Red identifies (+)
11. Attach (+) pump jumper wire from each pump below the wire identified in step 9,
    a. EX. G51 and H51
12. Attach the other two pump jumper wires to front most (-)
13. Plug in the wall adapter and turn it on using the white button on the driver.

Post Set-Up – Starting a Cycle

Once the collection vessel and monitoring vessel are set up in a biosafety cabinet, the system is now a closed loop. This means that the two vessels, the tubing, and sensors can be carefully brought to the location of the monitoring equipment. To finalize the setup of the device, the pumps should bs should be added to the tubing sections. It is important to ensure that the polarity of the pumps is oriented so that the direction of the media flow is appropriate, if not, the polarity of the pumps can be switched by swapping the clips attached to the pump. Once the breadboard is plugged in, and the driver switched is put the on position, the pumps will run automatically, and the potentiometer can be rotated to slow the flow rate. It is recommended that the slowest flow rate is used to minimize agitation of the cellulose from the trickle bed; though pumps should be supplied with enough voltage so that they are both always running to ensure the equal flow of media. Pumps were run at driver 5V setting with 1K potentiometer turned about halfway. Finally, the DO and temperature probes should be plugged into the LabQuest mini and

laptop monitor. Next, open LoggerPro software on the computer, and change the time collection interval to the desired range. It is suggested to collect DO and temperature at least once per hour for the duration of the study. Once data collection intervals are set, press the green “collect” button at the top of the LoggerPro screen DO and temperature will automatically begin to record. It is recommended that laptop settings are changed so that it does not go into “sleep” mode so that the data collection is continuous.

Appendix C: Verification Testing Standard Operating Procedures

Durability Testing: Observational Study

Materials and Components:

- Bioreactor system set-up (see Bioreactor Standard Operating Procedure)

Methods:

1. Set up bioreactor (see Bioreactor Standard Operating Procedure).
2. Initiate cycle of bioreactor as described in the Set-Up Time Testing Verification Standard Operating Procedure.
3. Sterilize all components.
4. Take note of the condition of each component to ensure that they could be used for additional cultures including observations, comments, and potential reasoning, and planned adaptations.

User Friendly Testing: Set-Up Time of Bioreactor

Materials and Components:

- Glass Collection Vessel
- Two Caps
- Four zinc 5-Inch Screws
- Six Acrylic Tiers:
    - 30 Inch-Diameter, 35 Inch-Diameter, 40 Inch-Diameter, 45 Inch-Diameter, 50 Inch-Diameter, 55 Inch-Diameter
- 40 zinc Nuts
- Aluminum Showerhead and Acrylic Cap

- Six PES Filter Membranes:
    - 30 mm-Diameter, 35 mm-Diameter, 40 mm-Diameter, 45 mm-Diameter, 50 mm-Diameter, 55 mm-Diameter
- Rubber Seal
- Mason Jar
- Aluminum Cover
- Optical DO Probe
- pH meter
- Temperature Probe
- Air Bubbler
- Timer

Methods:

1. Separate all bioreactor components
2. Hit start on timer
3. Open logger pro on the laptop
4. Assemble the bioreactor as described in the Bioreactor Standard Operating Procedure
5. Set up the environmental monitoring system
    a. Plug the components (thermistor, Arduino, and DO) into the laptop.
    b. Place the DO probe and thermistor into the cap.
6. Set up the tubing
    a. Attach one end of one tube to the peristaltic pump and place the other in the main vessel.

b. Attach one end of a second tube to the peristaltic pump and place the other in the reservoir.

c. Repeat for the second pump.

7. Add 180 mL of HS media / Glucose to the reservoir.
8. Begin data collection.
9. Repeat this process three times each by three different people.

Sterility Testing: Coverslip Microscopic Analysis

Materials and Components:

- Bioreactor system set-up (see Bioreactor Standard Operating Procedure)
- Methylene Blue
- Glass coverslips
- Glass slides
- Nikon Eclipse E600 upright microscope

Methods:

1. Sterilize and set up the bioreactor (see Bioreactor Standard Operating Procedure).
2. Fill the vessel with 180 mL of media.
3. Set the bioreactor system was set to run for seven days.
4. On day seven, remove 50 μL of fluid from the monitoring and placed on a glass slide. Cover slide with glass coverslip (Complete this step in a biosafety cabinet).
5. Observe coverslip under a 10X or 20X microscope for bacterial presence.
6. Wash the coverslip with deionized water and reimage under the microscope to confirm that there are no bacteria present.

BC Washing Protocol

Materials and Components:

- BC
- Two beakers
- One strainer
- NaOH solution
- MilliQ Water
- Tweezers

Method:

1. Place produced BC in beaker
2. Submerge BC in a NaOH solution
3. Place the beaker in 60 °C for 3-hour duration
4. After the 3 hours has expired remove the beaker from the oven
5. Remove BC from NaOH solution utilizing a strainer and an additional beaker
6. Place BC back into the original beaker
7. At room temperature conduct a 15-minute rinse with MilliQ water
8. After time has expired remove BC from the MilliQ water with a strainer and beaker
9. Repeat the rinses three more times

Yield Testing: Gravimetrical Test

Lyophilize Method:

Materials and Components:

- BC
- 6-well plates

- Freezer
- Lyophilizer (lyo)
- parafilm

Freeze-Drying Method:

- After culture period place BC samples into a six well plate if they are not already in one
- Place the samples into the freezer until completely frozen
- Once completely frozen remove tops from the six well plate and place the samples into the lyo
- Leave samples in the lyo for two days or until water content is completely removed
- Once the two days are complete, remove the samples for the lyo and place the tops of the well plates back on
- Seal the well plates with parafilm until testing is commenced

Weighing and Recording Data Method:

Materials and Components:

- NewClassic Mettler Toledo MS104S scale
- Dried BC samples
- Tweezers
- Weight Boat

Method:

1. Make a similar table as shown below, adding as many rows as needed:

Sample/Cycle (#)	Dry Weight (g)	Glucose (g)	Calculated Yield (%)
1			
2			
3			

2. Zero your scale.
3. Apply your weight boat on the scale.
4. Zero the scale again – ensuring the value reads 0.00g or relatively close while the weigh boat is still on the scale.
5. Using the tweezers, take all of the components of a singular dried sample and place it onto the weigh boat.
6. Wait for the scale to read a constant value. Record this value under 'W_{dry}' for each sample.
7. Repeat steps 3 – 5 for all samples.
8. Once all of the ' W_{dry}' values have been record, recall the amount of carbon added to each culture and record this value in the column labeled 'C'.
9. Using the following formula, calculate the percent yield and record the resulting values under the column that reads 'Calculated Yield (%)': $Yield\ (\%) = \frac{W_{dry}}{C} \times 100$

Tensile Testing: Instron Test

Creating a Method on Bluehill Universal Version 4.25

Materials and Components:

- Bluehill Universal Version 4.25
- PC

Method:

1. Open the Bluehill Universal Software Version 4.25. Select the method module as shown in Figure C1.

Figure C1: Home Screen of Bluehill Universal Software

2. The next screen will appear as shown in Fig C2. Due to the fact that this test is a ring uniaxial tensile test, click the ‘tension method’ as shown in Fig. Be sure not to click the ‘Tension Creep/Relax method, as this method is different.

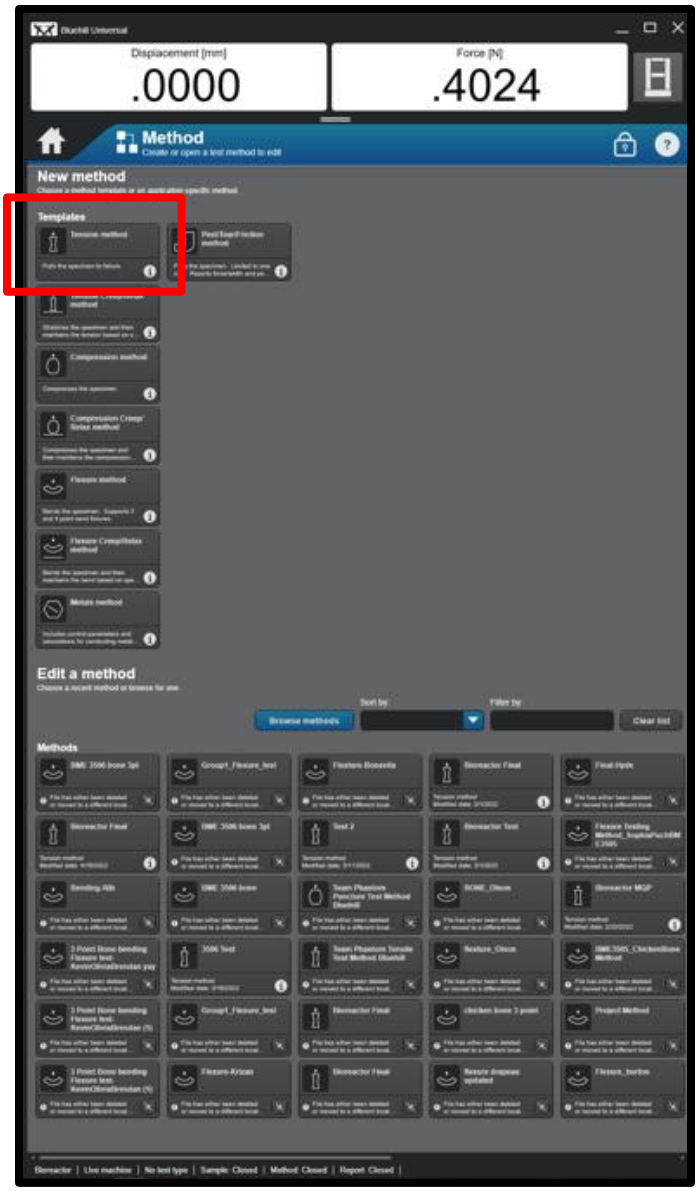


Figure C2: Creating a Method Screen

3. Under the ‘Method’ module, click the ‘Specimen’ module as shown below (Figure C3) First, change the geometry of the specimen shape from ‘Rectangular’ to ‘Tube Section’. We do this because the cellulose samples will be cut into rings and not rectangles. The resulting screen should look like the Figure C4.

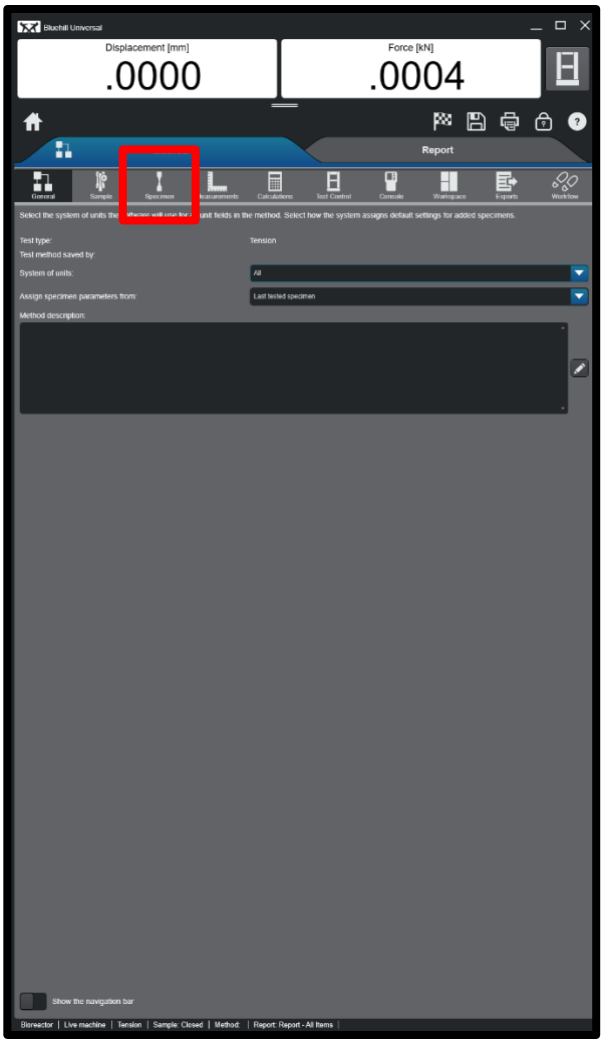


Figure C3: Creating a Method Screen

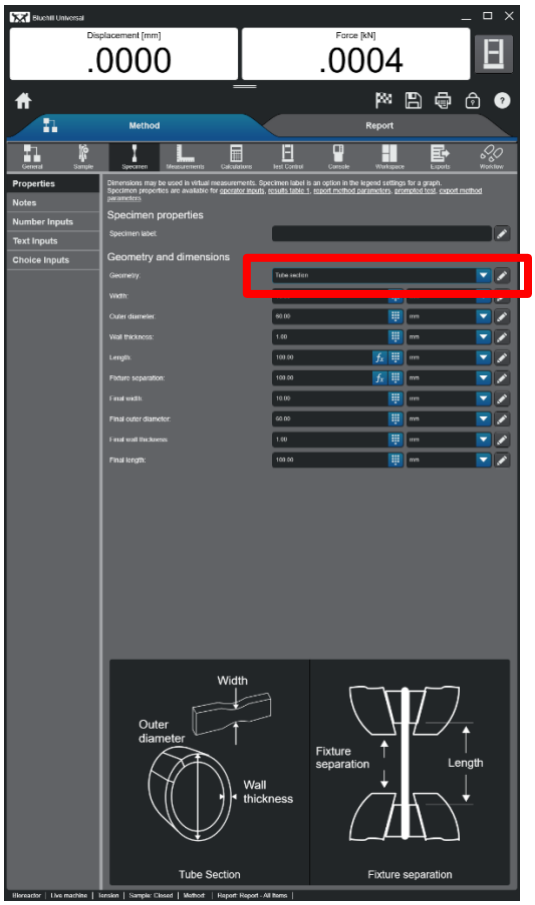


Figure C4: Changing Specimen Geometry

4. Next, click the ‘Measurements’ module and ensure that the Measurements available in the method include the following: Time, Displacement, Force, Tensile Strength (Displacement), Tensile Displacement, and Tenacity. Your screen should look like Figure C5.

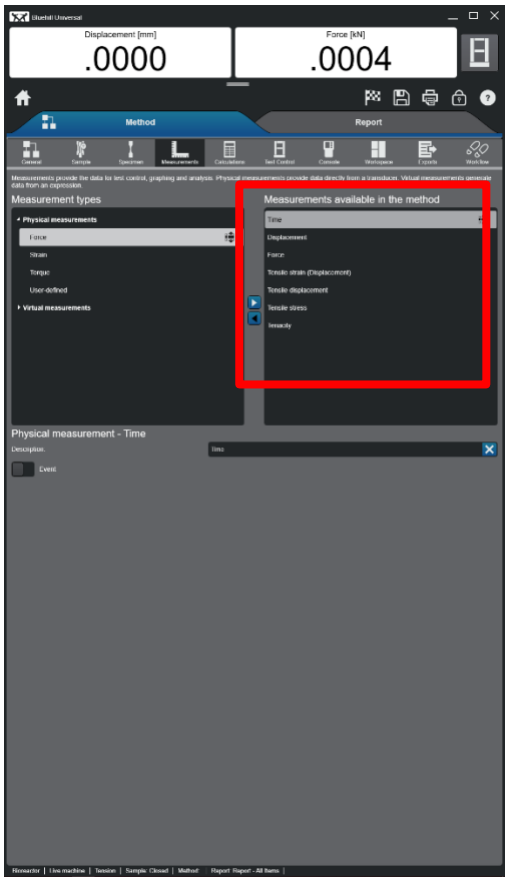


Figure C5: Selected Measurements

5. Next, click the ‘Calculations’ module. To add applicable calculations needed for the analysis of this experiment, click the calculation in the left ‘available calculations’ section and then click the right-facing arrow to pull that specific calculation of to the ‘selected calculations’ section. The following calculations should be selected for your analysis: Yield (Zero slope), and Modulus (Automatic Young’s) (Figure C6). The force at yield is the breaking point of the sample while the modulus is the stress over strain. Remove or add any additional calculations as needed.

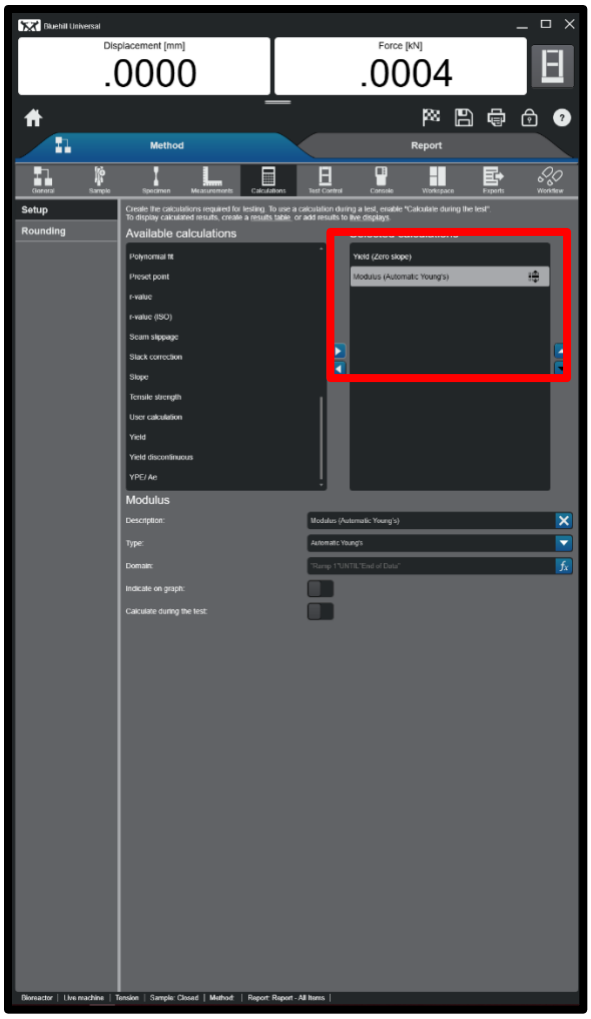


Figure C6: Selected Calculations

6. Next, click the ‘Test control’ module (Figure C7) First, under the ‘Start Test’ module on the left hand-side, ensure that the test is started ‘By the Start Button’. Second, go to the ‘Pre-Test’ module and unlock the ‘Preload’. Update the applicable ‘Preload’ parameters so that they are the same as in Fig. These parameters will ensure that your graph is starting at a base, thus a 5N preload will already be set and it gets to that preload before the start of experiment at the rate of 5 mm/min (C7). Third, click the ‘Test’ module on the left hand-side and change the rate of the displacement to that of existing literature which is ‘20 mm/min’ (Figure C8). Fourth, select the ‘End of Test’ module, and make sure that the measurement under the ‘Criteria’ is ‘Force’ and the ‘Sensitivity’ is set to ‘40%’. This

ensures that the test will end when your sample fails or breaks, where there will be an instantaneous drop of at least 40% of force, and then the test will end (Figure C9).

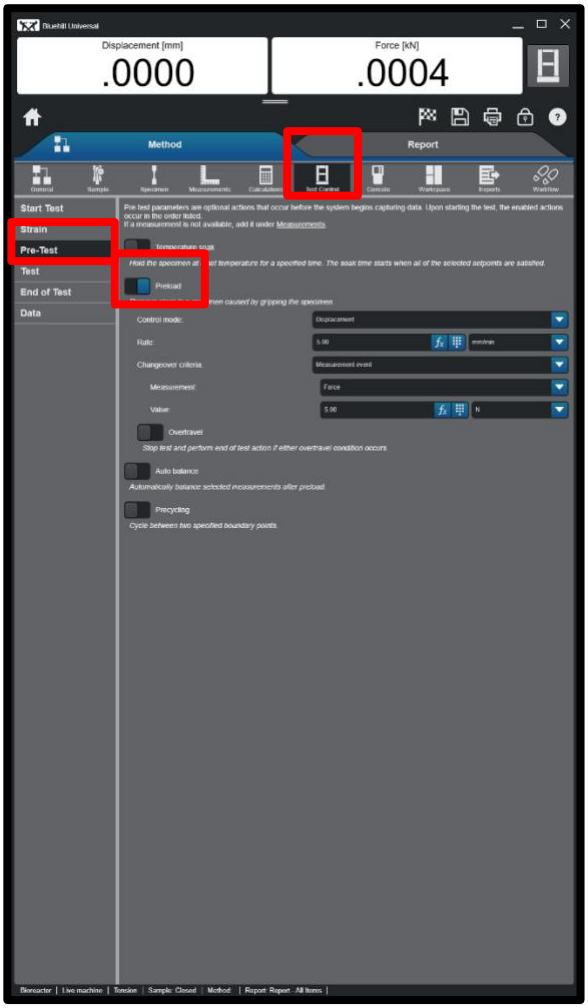


Figure C7: Test Control Module and Pre-test Parameters

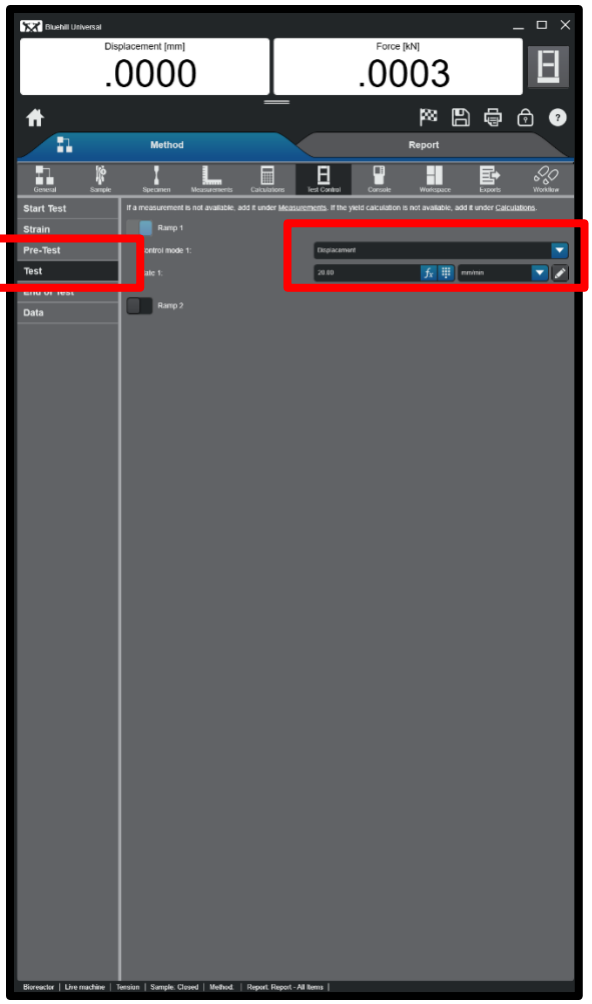


Figure C8: Rate of Displacement

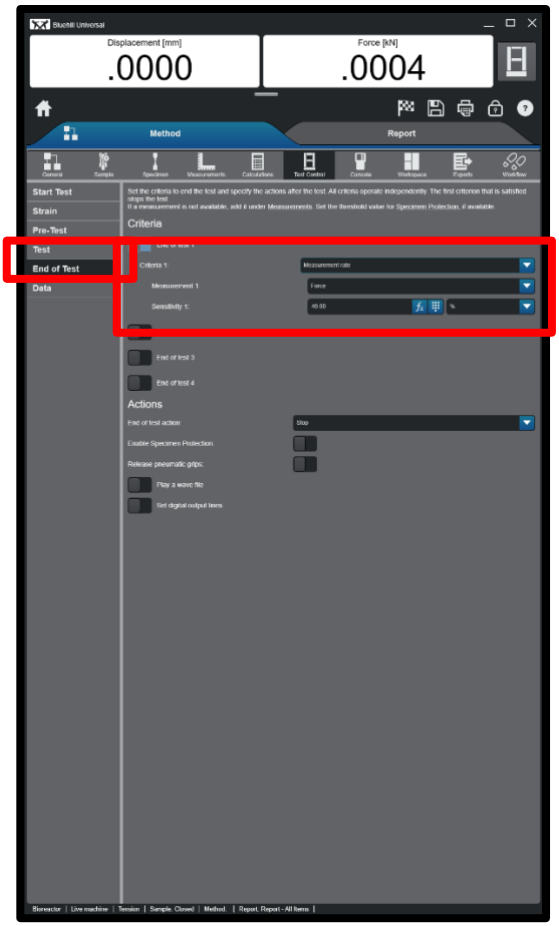


Figure C9: End of Test module changes

7. Next, click the ‘Workplace’ module (Figure C10). First, click on the ‘Results 1’ module and then pull over all of the calculated data needed. To pull over the ‘Modulus (Automatic Young’s), click the ‘Modulus (Automatic Young’s)’ in the ‘Available results’ section dropdown, then click ‘Modulus’ and pull it over to the ‘Selected results’ section by clicking the right-facing arrow (Figure C11). Ensure that the units for the Modulus are appropriate, changing them to either MPa or Pa (Figure C11). Next, bring over the ‘Tensile Stress’ and the ‘Force’ which is under the Yield (Zero Slope) dropdown (Figure C12). Similarly, ensure that these two results are appropriate (N for Force and MPa/Pa for Stress). Second, click ‘Graph 1’ in the left-hand-side module and make sure the ‘Multi-Specimen’ graph parameter is set for the ‘Graph Type’ to allow for multiple sample graphs to be displayed

on the same graph. Change the number of ‘Curves per graph’ as you see fit. It is standard to set this value to 10, however (Figure C13). Next, click the X-data module and make sure that the ‘displacement’ is the ‘measurement’ of the X-axis (Figure C14) and then click the Y-data module to make sure that ‘force’ is the ‘measurement’ on the Y-axis (Figure C15) both with appropriate units (mm and N, respectively). Third, click the ‘Graph 2’ module, and similar to the ‘Graph 1’ module, make sure the ‘Multi-Specimen’ graph parameter is set for the ‘Graph Type’ to allow for multiple sample graph to be displayed on the same graph and change the number of ‘Curves per graph’ as you see fit (10). Navigate to the ‘X-data’ module and change the ‘measurement’ of the X-axis to ‘Tensile strain (displacement)’ and ‘Units’ in ‘%’ (Figure C16). Next, change the Y-axis ‘measurements’ via the ‘Y-data’ tab, to ‘Tensile Stress’, and change the ‘units’ to ‘MPa’. The changes are made to this second graph so that two separate graphs of representatives can be showcased. Results should present a stress-strain curve and a force over displacement curve. Fourth, click the ‘Raw Data’ module and pull over the ‘Tensile strain (Displacement)’ and ‘Tensile stress’ from the ‘Available measurements’ section to the ‘Selected measurements’ section (Figure C17). Be sure to set the parameters for each of. The selected measurements, i.e. changing units, etc.

Figure C10: Workplace Module

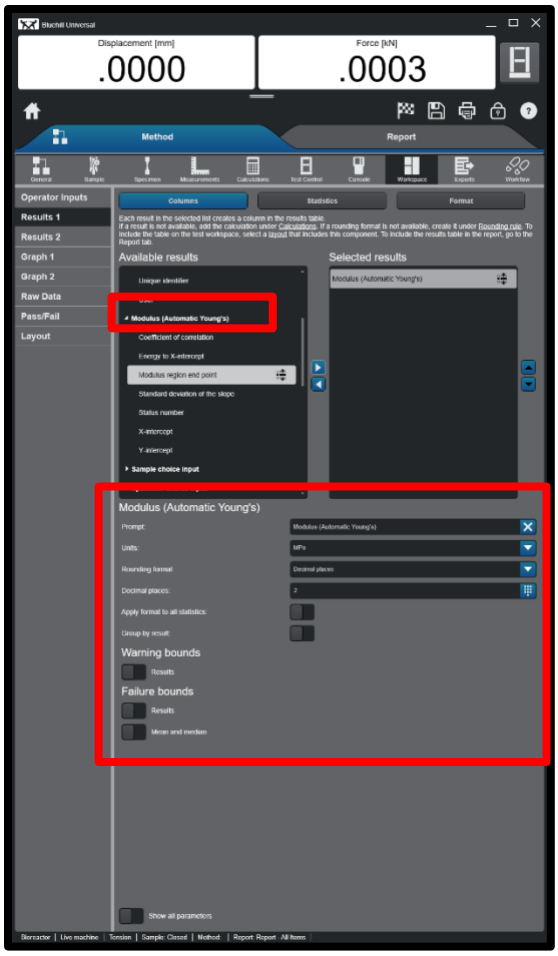


Figure C11: Modulus and Parameters

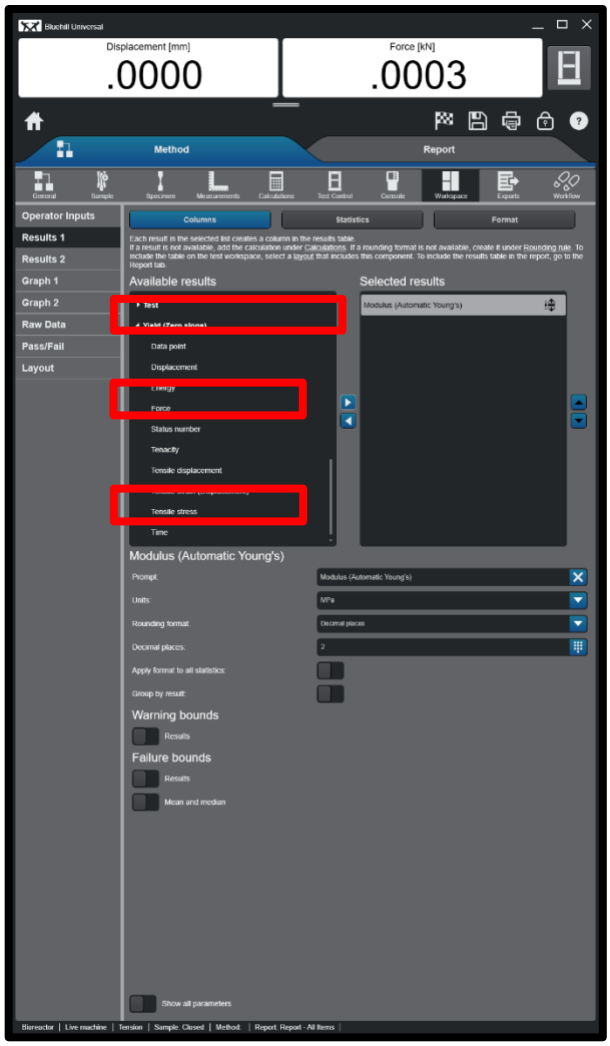


Figure C12: Yield (Zero Slope) and Parameters

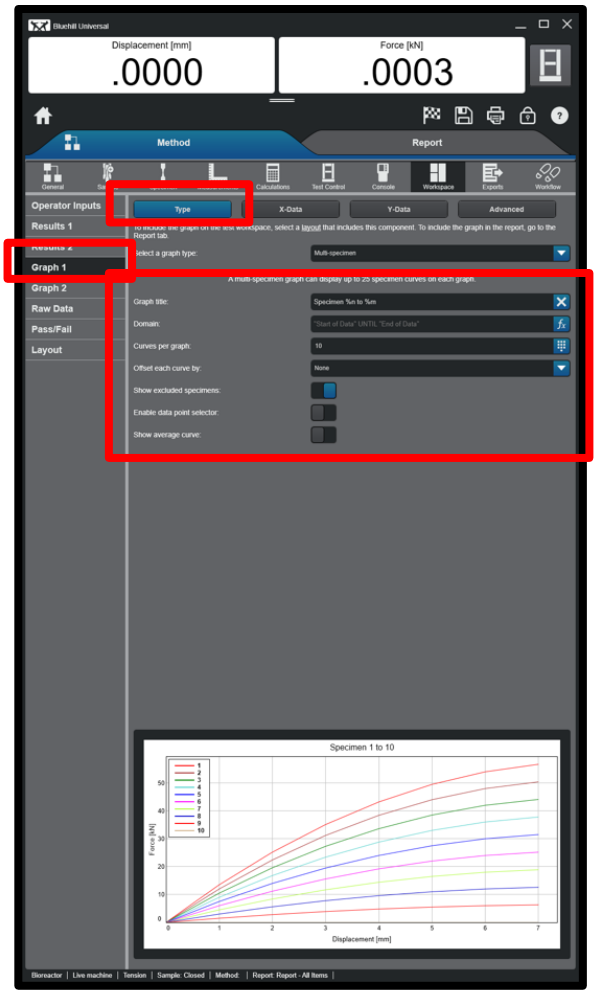


Figure C13: Graph 1 and Parameters

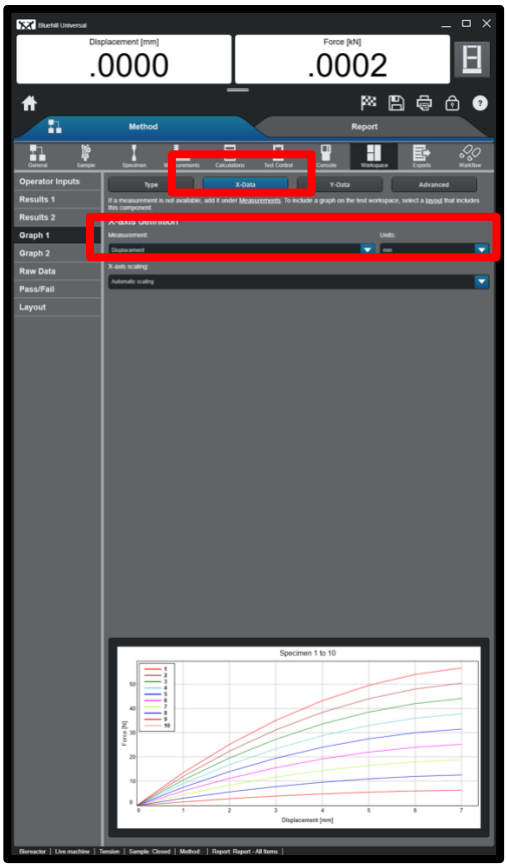


Figure C14: Graph 1 X-axis Parameters

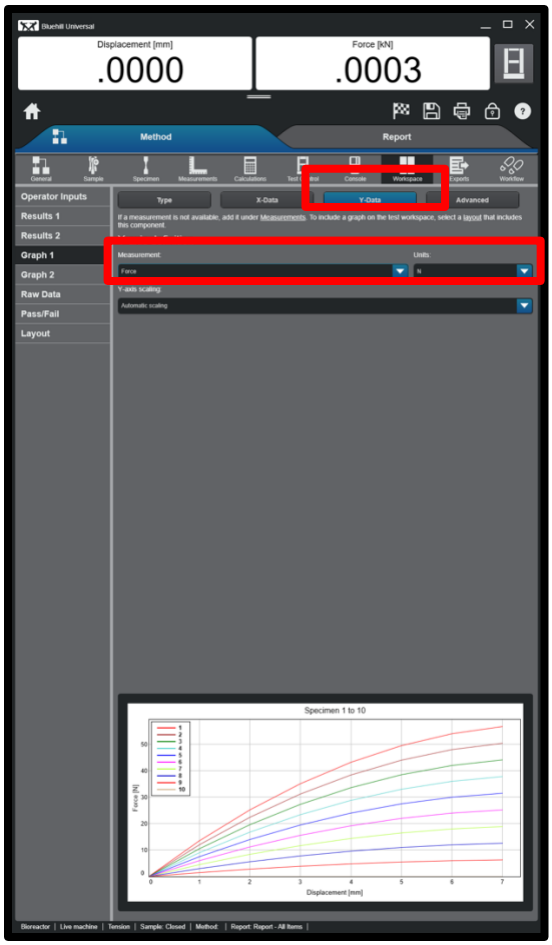


Figure C15: Graph 1, Y-axis Parameters

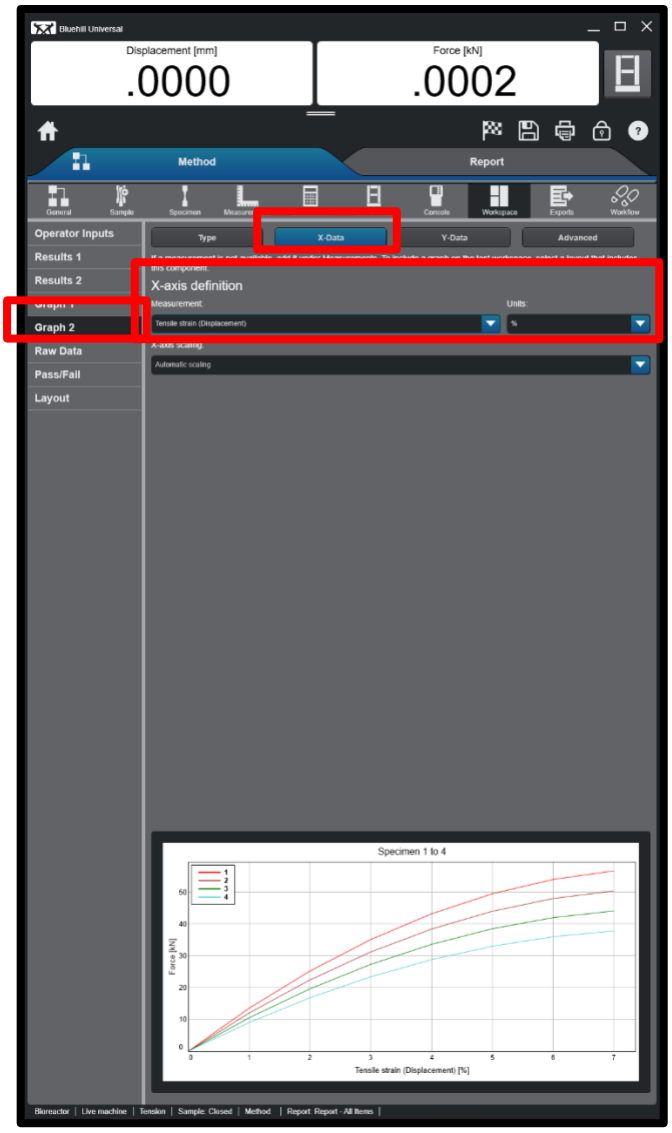


Figure C16: Graph 2, X-axis Parameters

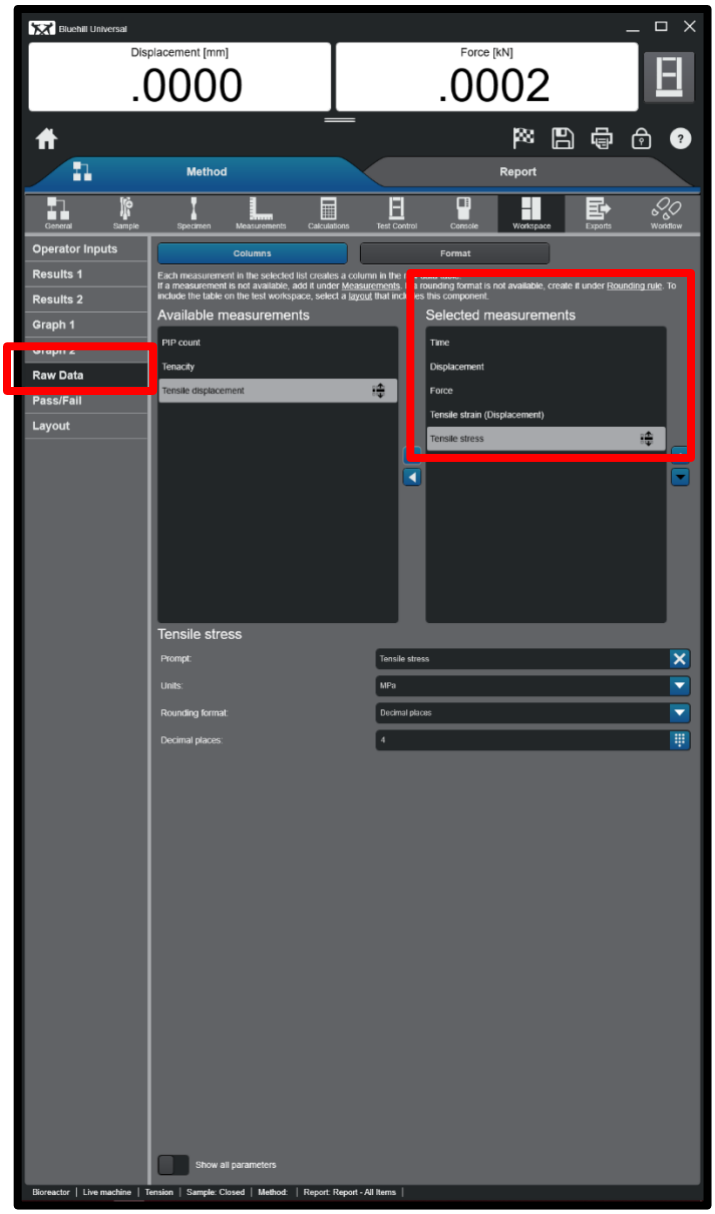


Figure C17: Raw Data, Selected Measurements

8. Go to the ‘Exports’ module. Click the ‘Exports 1’ module of the left-hand side. Change the ‘Export 1 frequency’ to ‘At finish’. Next, enable the ‘Create a file for each specimen’ and then disable the ‘Results table 2 results’ (Figure C18). This is done so that when you hit the finish button during testing, it will ask you where to save the data and then create a file for each specimen. Navigate to the ‘Export2’ module and changing the ‘Export 2 frequency’ to ‘At finish’ (Figure 19).

Figure C18: Exports, Export 1 Parameters

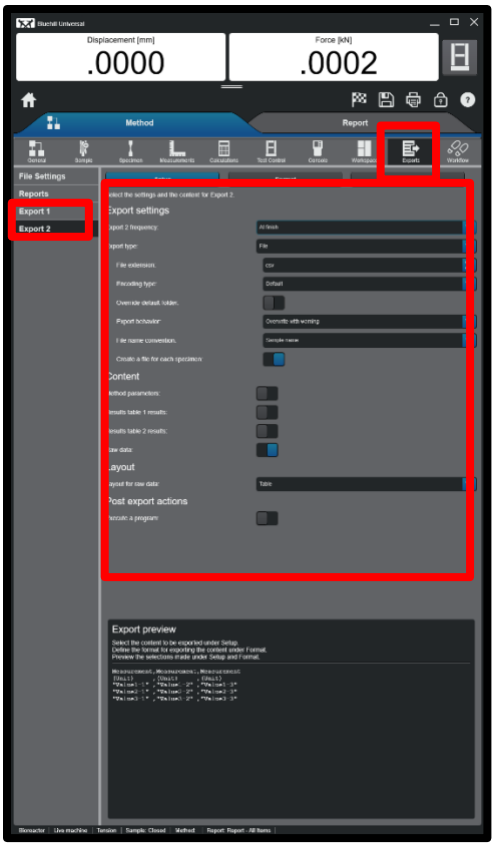


Figure C19: Exports, Export 2 Parameters

9. Lastly, click on the ‘Workflow’ module and then enable the ‘Run as a prompted test’ function. Next, disable the ‘show workspace after the test’, ‘prompt before calculating, and ‘show workplace after calculating’ functions (Figure 20). Finally, navigate to the ‘Before test’ tab on the flow diagram. In the ‘available parameters’ section, click the ‘specimen properties’ dropdown and pull over the ‘length’, ‘outer diameter’, ‘wall thickness’, and ‘width’ (Figure 21).

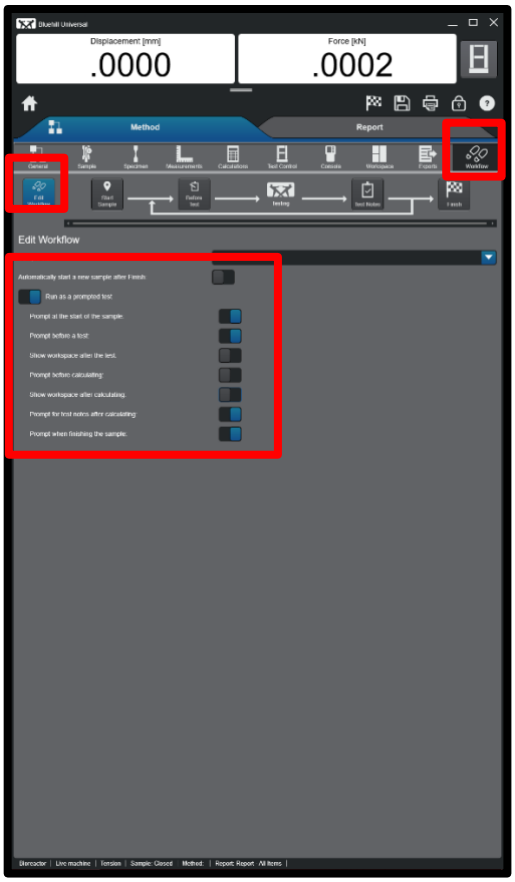
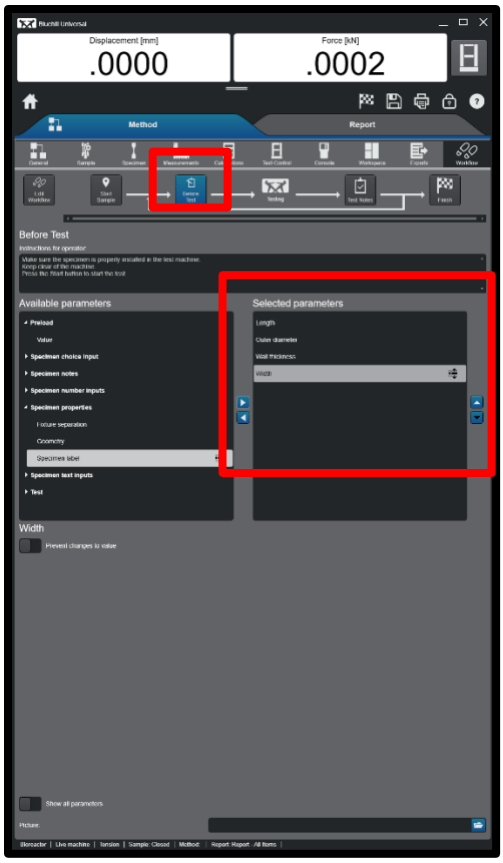


Figure C20: Editing Workflow Functions

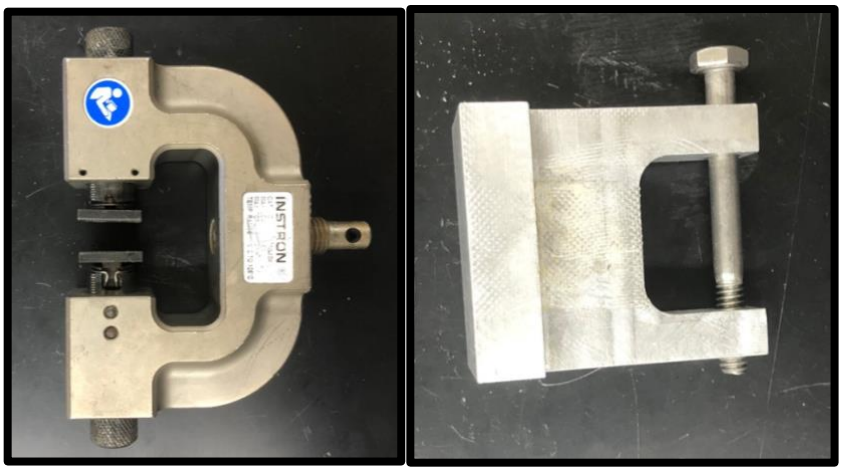


C21: Workflow, Before Test Parameters

Instron 5544 Machine Set Up

Materials and Components:

- Instron 5544
- Allen Wrench
- Pliers
- 2 sets of Grip and Fixtures (Specific to tension tests)



- 2 sets of Rivets (2 ring rivets and 2 rod rivets)

Instron Set-Up:

1. Set up the Instron by first turning the instron on.
2. Remove any components off the instron that are not needed for your test, using the pliers. The instron should look like Figure C22. before applying the tension based grips and fixtures.

Figure C22: Instron Without Added Components

3. Thread a ring rivet on the each of the Grips so that it looks like Figure C23.

Figure C23: Ring Rivet Threaded onto a Grip

4. Following Figures C24, C25, and C26, and place one grip into the upper connection and place rod rivet into the hole where the connection and grip align.

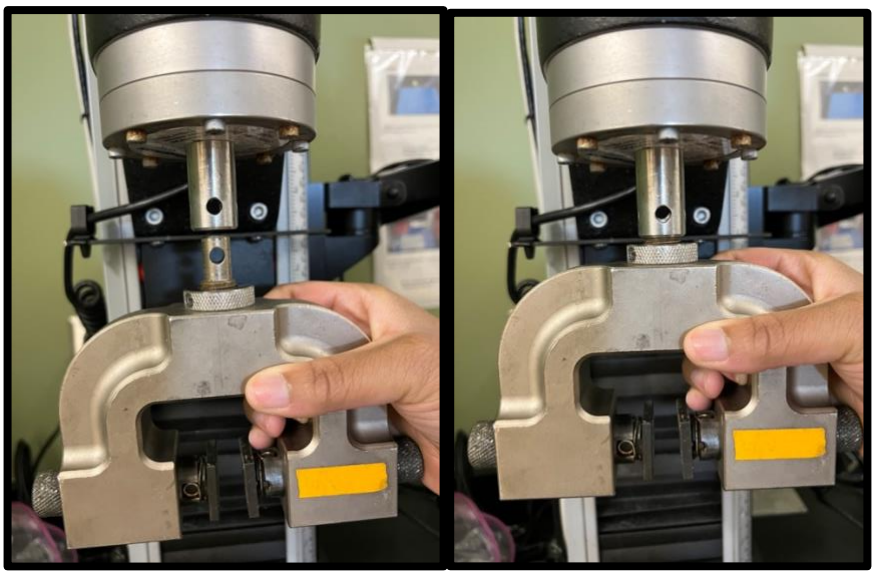


Figure C24: Inserting a Grip into the Upper Connection

Figure C25: Rod rivet inserted into the hole

5. As illustrated by Figure C26, with pliers, secure the ring rivet so the upper grip is secure.

Figure C26: Securing the ring rivet

6. Repeat steps 4-6 for the lower connection. The completed set up should look like Figure C27.

Figure C27: Set up without T-fixture

7. Take each of the tension fixtures and place them into the grip so that the fixture in the upper clamp is in the shape of a “T” and the fixture in the lower clamp is in the shape of an upside down “T”. Using the pliers, tighten the knobs on the grips to secure the fixtures in place as shown in Figure 28. Ensure that the two T fixtures are parallel so that when testing is conducted force is evenly applied to your sample. Your set up should like Figure C29 not Figure C30.

Figure C28: Tightening T-fixtures

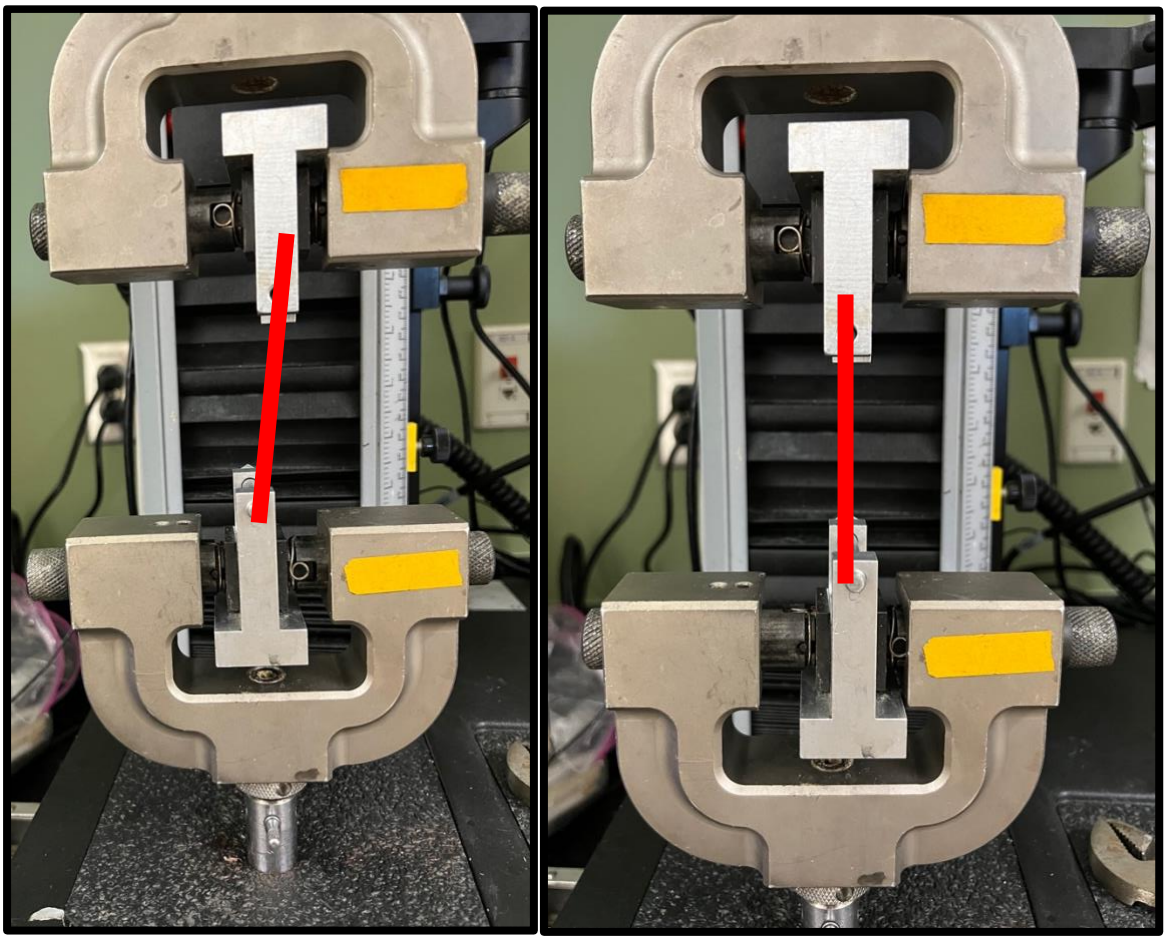


Figure C29: Your set up should not look like the image to the left, but rather the image to the right

8. Your final setup should look like Figure C30.

Figure C30: Completed Instron Set-Up

Conducting a Test

Materials and Components:

- Instron 5544
- Bluehill Universal 4.25
- Calipers
- Leather Hold Punch Set (Ranging from 10 mm to 40 mm hole punchers)
- Rubber Mallet
- Cutting Board
- Tweezers or Laboratory Forceps

Method:

Before beginning to test your BC samples, a method should be created on the Bluehill software that accurately reflects your desired testing strategy and results. For this experiment, a tension test method was created and enabled exports of raw data, graphs showcasing force vs displacement, and resulting values of stress, force, and Young's modulus. An in-depth method protocol can be found above. To ensure your method is working effectively, materials such as

elastic rubber bands should be tested on the Instron before testing BC samples. Once it has been confirmed that your method is working effectively, then BC samples should be tested to make sure the Instron is within range of your test materials. Troubleshoot any problems that may occur during these testing phases. Once confirmed that your Bluehill protocol works effectively and that you are receiving data that looks correct, you may begin testing your control and bioreactor samples.

Preparation of BC Samples

1. Collect all viable samples (those appearing visually uniform throughout) and purify them by rinsing them with 0.1M NaOH and letting them sit in a NaOH bath at 80°C for approximately four hours to remove bacterial cells possibly attached to the BC pellicles. After four hours have passed, rinse the samples with 0.1M of distilled water and let them sit in a distilled water bath until ready for use.
2. Before testing, collect the purified and washed samples and place one on a cutting board.
3. Using a paper towel, slightly dry the sample so that it is still damp but not saturated with distilled water. This will make it easier to visually see the sample when cutting and easier to handle the sample after cutting.
4. Using a leather hole puncher of your desired size, place the leather hole punch in the center of the BC sample.

    * It is recommended that you do not use a hole larger than 5 mm from the diameter of the BC sample. For example, if your BC sample is 55 mm in diameter, it recommended not to use a hole larger than 50 mm in diameter. This is to ensure that the rings are not too thin or fragile and will not break upon simple handling.

5. Using the rubber mallet, hammer the hole puncher through the BC sample to ensure a clean-cut ring (Figure C31).

Figure C31: BC Ring Preparation

6. Remove the inner circle of the ring using tweezers or forceps.

7. Repeat steps 2 –6 until all samples are in the shape of a ring, as illustrated in Figure C32.

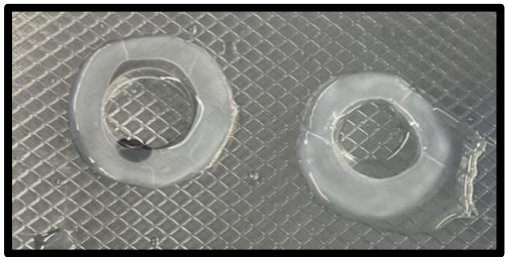


Figure C32: BC Rings

Testing Method:

1. Turn on the Instron machine using the button on the back right side.
2. After logging into the PC connected to the Instron, open the Bluehill Universal Program v. 4.25, Click the ‘Test’ module (Figure C33).

Figure C33: Home Screen Bluehill Universal

3. Under ‘New Sample’, open your saved method by clicking ‘Browse Methods’. Locate where you have saved your previously made testing method and open the file (Figure C34).

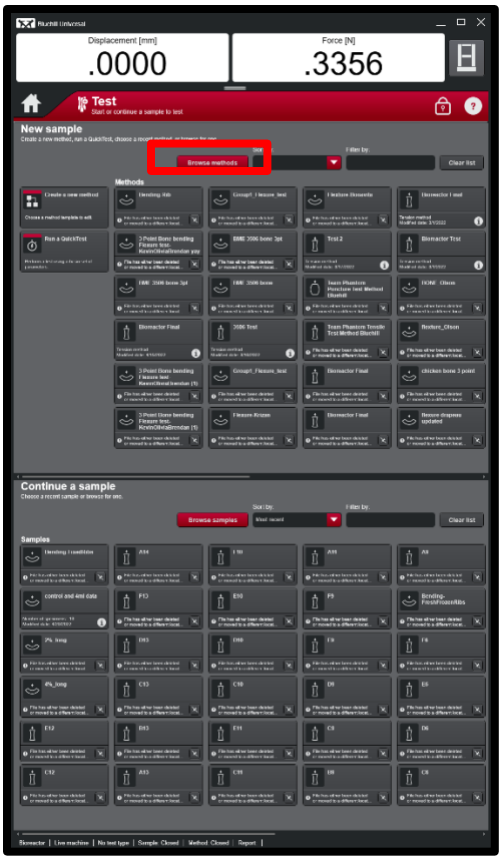


Figure C34: Browse Screen for Create Methods

4. The following (Figure C35) will be displayed once you open your testing method. Set the appropriate crosshead travel limits to reduce the risk of bodily injury and damage to the specimen or system. Do this by twisting the black knob counterclockwise and moving the crosshead to appropriate height of 25 inches or or more. Click OK once finished.

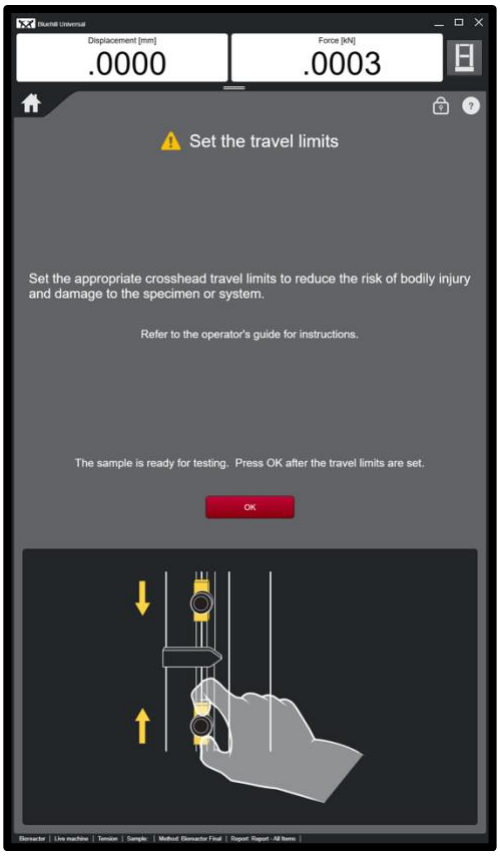


Figure C35: Setting the Crosshead

5. The following screen will then appear (Figure C36). Click the method module to ensure that proper method is being used and that all of your testing parameters are correct (ensuring they are the same as those listed in the Bluehill program SOP). Once it has been confirmed that the proper method and parameters is being and are set, go back to the 'Test' module and select 'Before Test' (Figure C37).

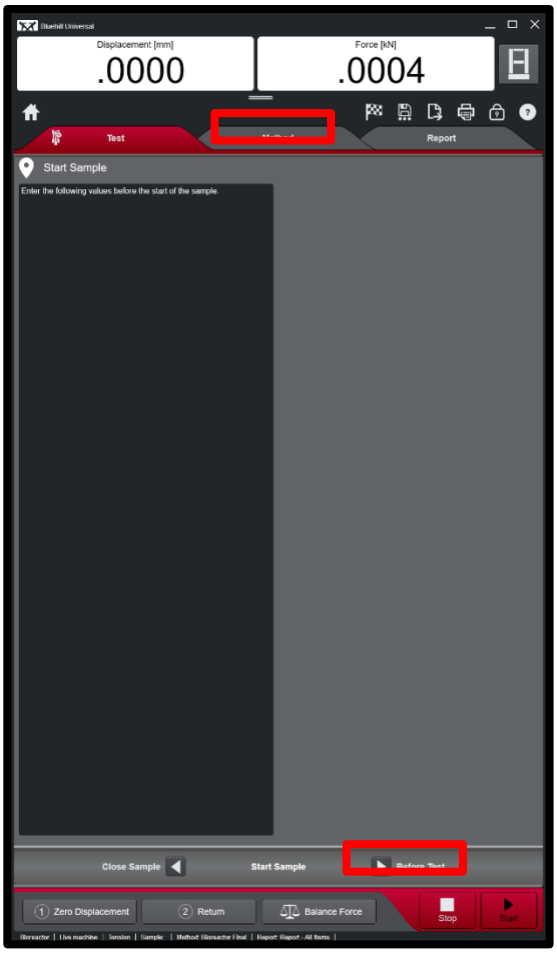


Figure C37: Before test button Bluehill

6. The following screen will appear (Figure C38). Before applying your specimen to the machine, calibrate the machine (zero the force applied on the machine) by clicking the icon to the top right of Figure C38.

Figure C38: Calibrate Button and Before Test Parameters Screen

7. The following pop-up screen will appear. Click the third icon from the left, titled ‘Force Transducer Settings’ as shown in Figure C39.

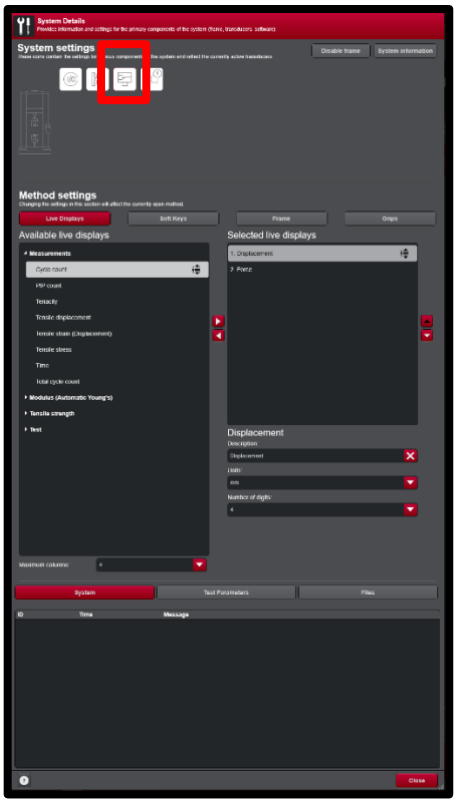


Figure C39: Force Transducer Settings

8. The following pop-up screen will appear (Figure C40). Click the ‘Calibrate’ button and wait for the system to be calibrated. Once finished, close out of this pop-up screen and the previous one.

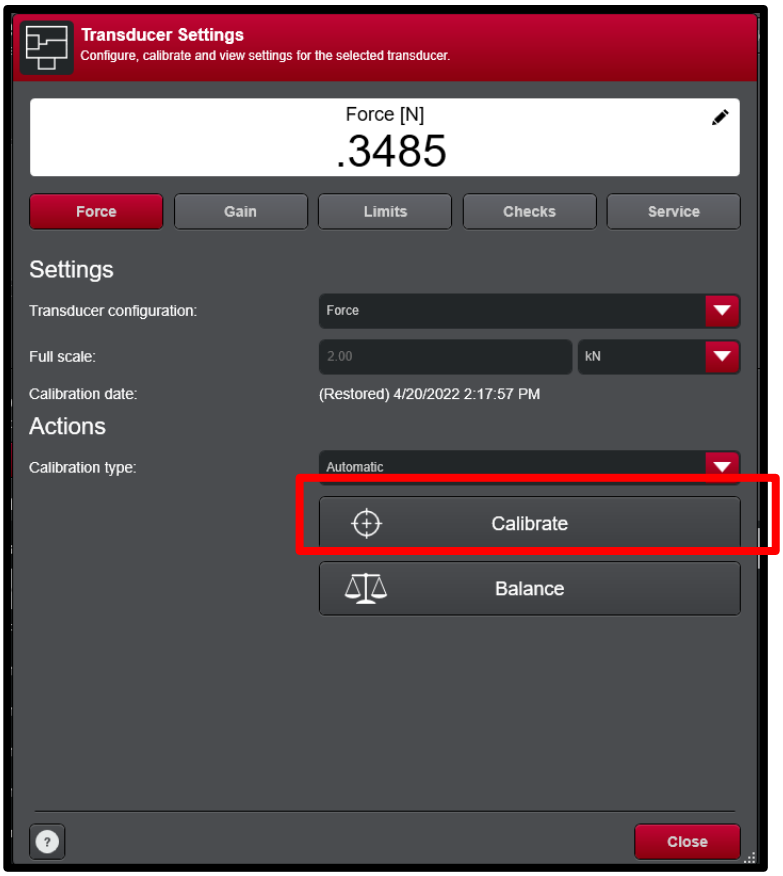


Figure C40: Calibrate Button

9. Next, apply the specimen to set up Instron machine so that it looks similar to Figure C41.

   Jog up and down the upper lever as needed to ensure the specimen is taut on the device.

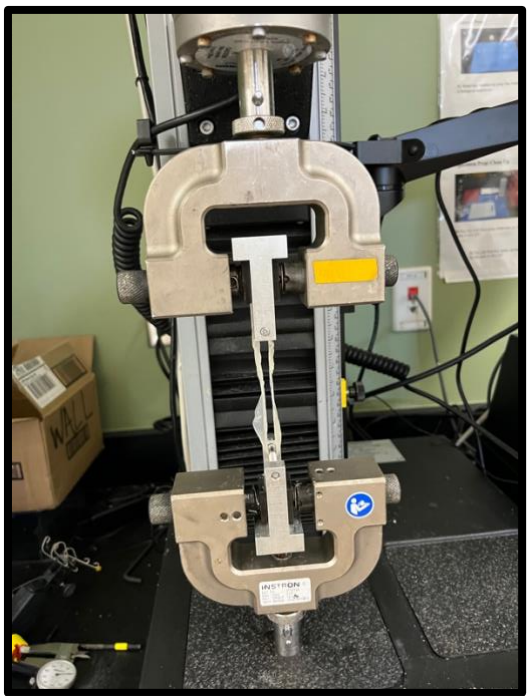


Figure C41: Specimen on Instron

10. Using calipers, measure the outer diameter and the wall thickness in mm of the sample while on the Instron machine, and input those values on this screen. Be sure to ‘Zero Displacement’, ‘Return’, and ‘Balance Force’ by clicking the following buttons on the bottom of the screen. Start the test by clicking ‘Start’ (Figure C42).

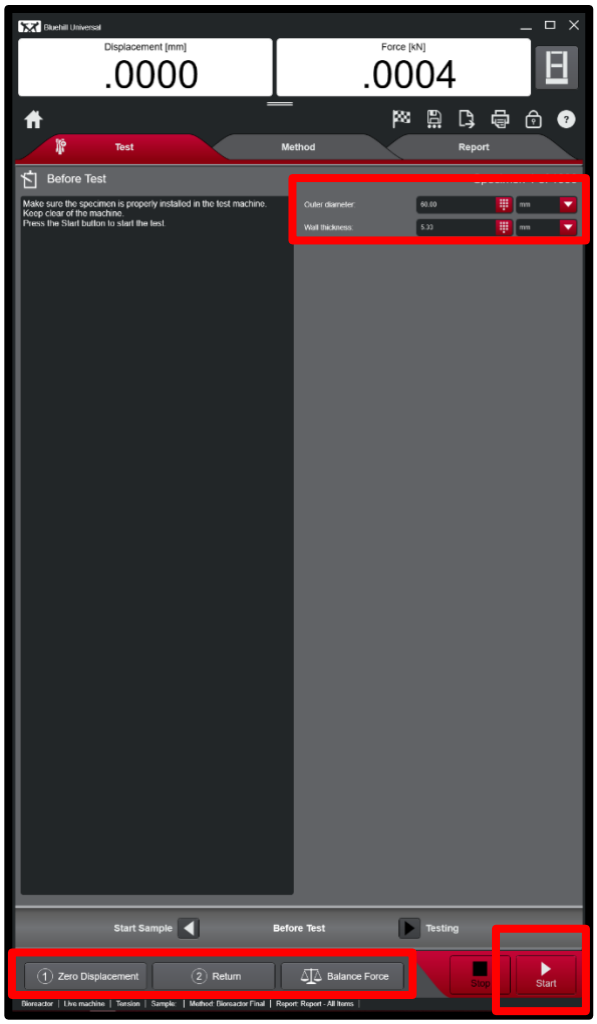


Figure C42: Pretest buttons in Bluehill Software

11. Once the sample has failed (when it breaks), click the stop button (Figure C43).

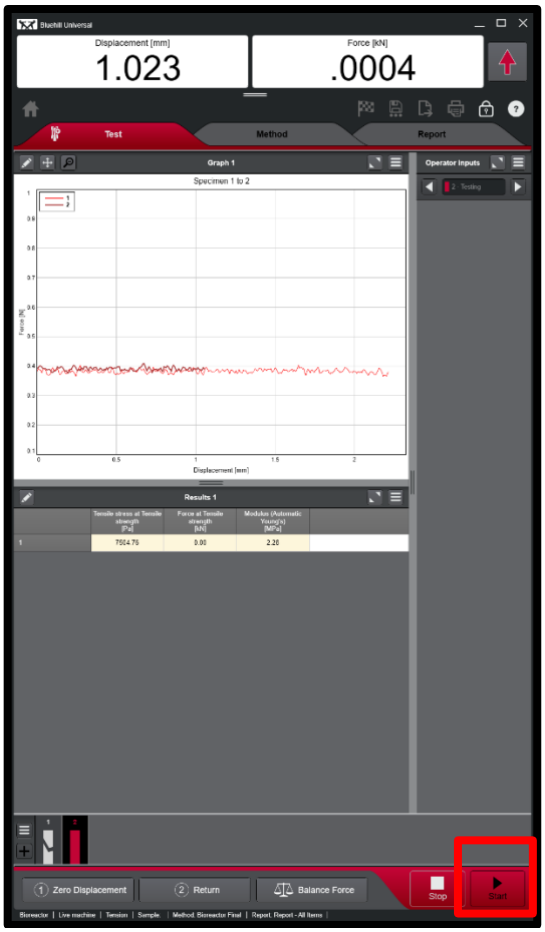


Figure C43: Stop Button

12. Save the sample by clicking the ‘save’ button. Click the ‘save as’ button and name the file and put it in a folder where it can be easily accessed for analysis later. Then click the export button as shown in Fig. and export both cvs files. These files will automatically save in the same folder that you saved the raw data. If you are testing more than one sample, you can save them all under the same test. Up to 1000 samples tested under one test. Begin the next test by clicking the ‘before test button again’. Repeat steps 9 – 12 for each sample.

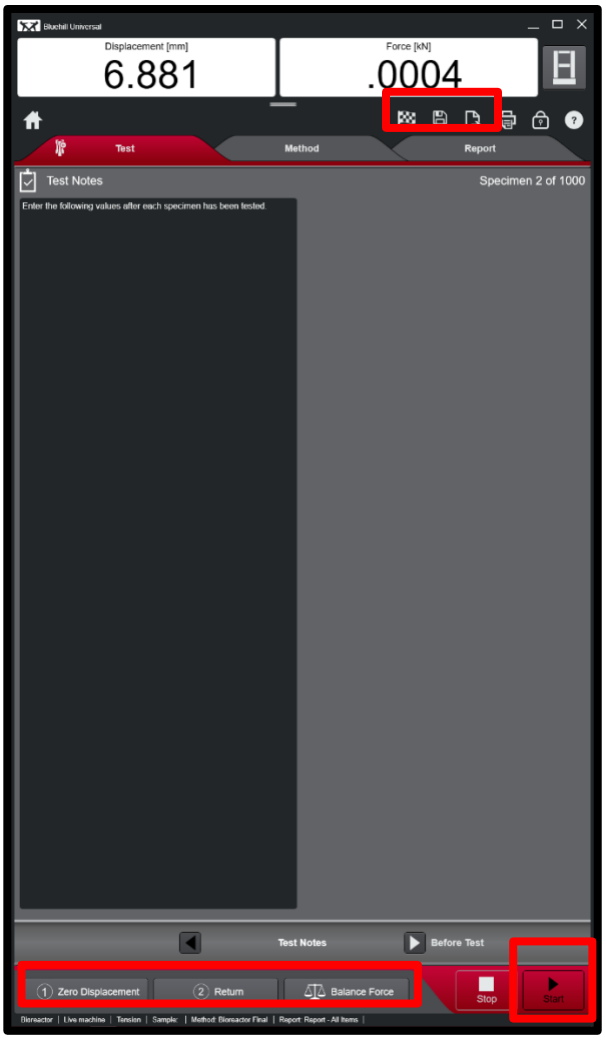


Figure C44: Saving Buttons and Starting a new test

13. When you are done testing all of your samples, click the finish button as shown in Figure C44. Save your final pdf graph in the folder where you are saving all of the other tests.
14. Before closing out of the program, be sure to save your method incase any changes were made.
15. Be sure to send your data to yourself either via email or save it on a flash drive, so that you can further analyze your data later.

Uniformity Testing: ImageJ Test

Materials and Components:

- Bacterial Cellulose Samples
- Cutting Board
- Black /dark colored platform
- ImageJ software

Method:

Preparation of BC Samples

Collect all viable samples (those appearing visually uniform throughout) and purify them by rinsing them with 0.1M NaOH and letting them sit in a NaOH bath at 80°C for approximately four hours to remove bacterial cells possibly attached to the BC pellicles. After four hours has passed, rinse the samples with 0.1M of distilled water and let them sit in a distilled water bath until ready for use.

Data Collection Procedure

1. Take a sample of BC and place on dark background.
2. Take picture of sample in lighting that minimizes reflection.
3. Open picture in ImageJ.
4. Click “oval” selection as seen in Figure C45 and adjust to outline BC.

Figure C45: Oval selection tool in ImageJ

5. Click Edit ->clear outside.
6. Click image ->type->32-bit

7. Click Analyze->set measurements and ensure that the measurements shown in Figure C46 are selected.

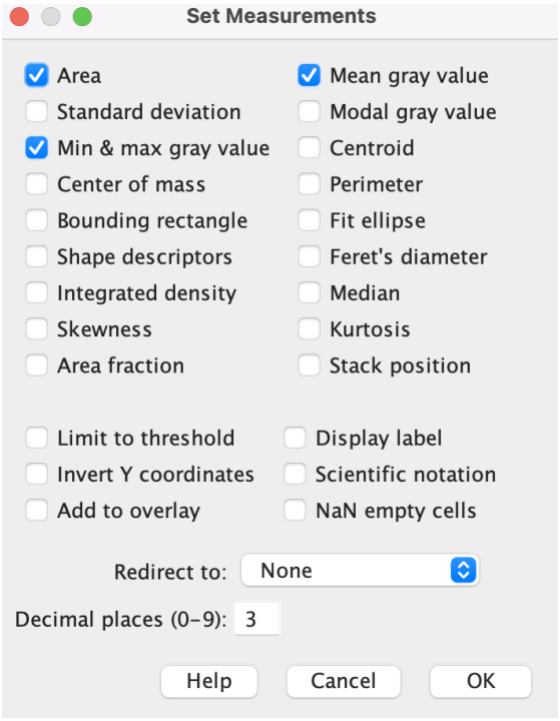
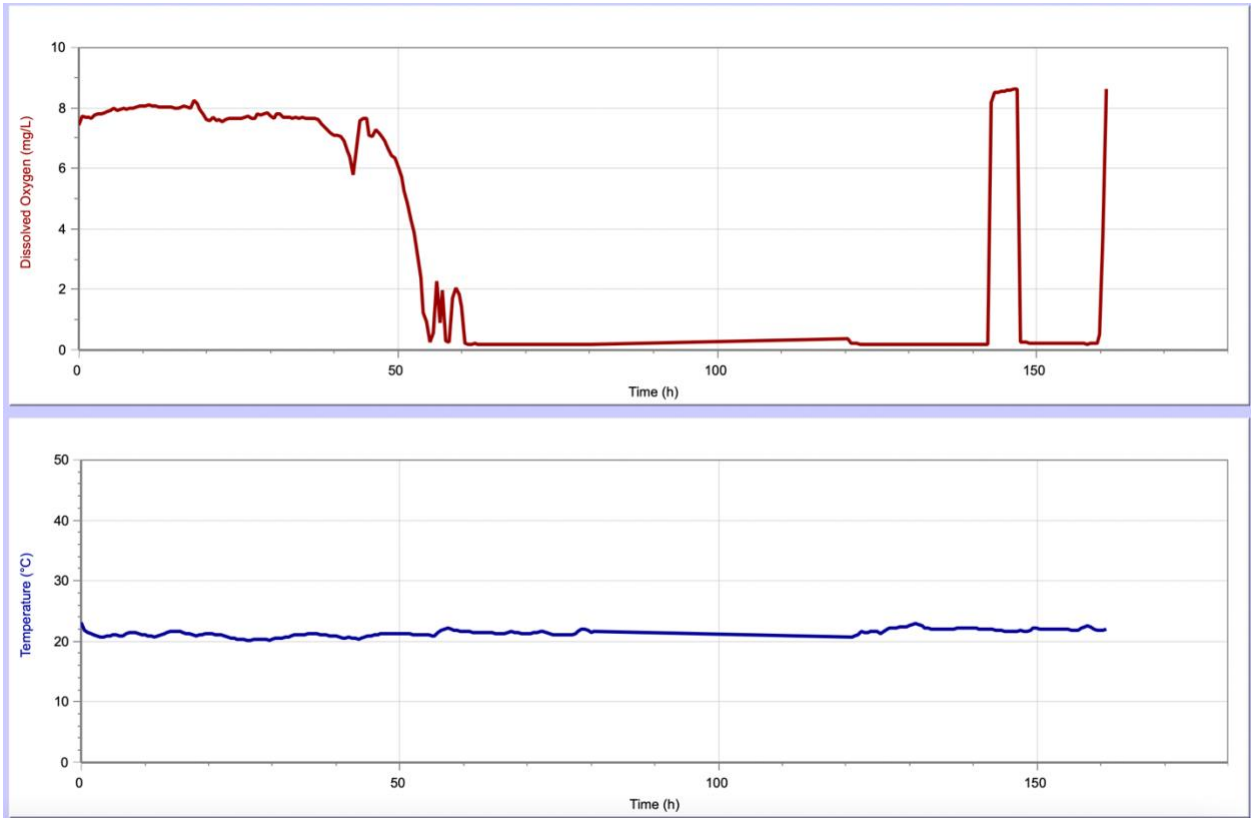


Figure C46: Measurement selection ImageJ

8. Click Analyze-> measure.
9. Document the resulting measurements in an Excel spreadsheet for further analysis.

Appendix D: LoggerPro Data Collection



Latest: Time (h)	Latest: Dissolved Oxygen (mg/L)	Latest: Temperature (¬∞C)
0	7.419521857	23.16729707
0.5	7.720286524	21.90018432
1	7.68027499	21.4151963
1.5	7.672476132	21.21186234
2	7.650096799	20.99370164
2.5	7.765723351	20.82077774
3	7.788441765	20.72253004
3.5	7.810821097	20.71812981
4	7.830148703	20.78852233

4.5	7.872872883	20.86328879
5	7.922039599	20.97612241
5.5	7.973918961	21.02299724
6	7.916275226	20.8999295
6.5	7.952556871	20.94389019
7	7.980361496	21.16356291
7.5	7.951539629	21.39033742
8	7.973918961	21.46636836
8.5	7.980022416	21.37863823
9	8.005453475	21.25283578
9.5	8.044786848	21.13135765
10	8.048516737	21.01127946
10.5	8.066148938	20.90872235
11	8.077338605	20.83397173
11.5	8.070217908	20.78412349
12	8.0668271	20.86622027
12.5	8.034614424	21.07425529
13	8.006131637	21.25429899
13.5	8.027832808	21.40788513
14	8.011896011	21.53360689
14.5	8.01155693	21.59790506
15	7.967137345	21.6636477

15.5	7.996298294	21.54529869
16	8.005453475	21.35523816
16.5	8.039361555	21.22649654
17	8.005792556	21.16063535
17.5	7.996637375	21.05375348
18	8.248913488	20.93656402
18.5	8.13057429	20.99370164
19	7.947470659	21.09329101
19.5	7.802683158	21.16649044
20	7.59109674	21.21917956
20.5	7.560240388	21.16356291
21	7.665694516	21.01860315
21.5	7.580246155	21.01860315
22	7.591435821	20.97319241
22.5	7.549389802	20.83397173
23	7.603981811	20.69466039
23.5	7.630769194	20.589015
24	7.646705991	20.49359364
24.5	7.646705991	20.41134796
25	7.635855406	20.33788529
25.5	7.659591062	20.26586489
26	7.680953152	20.22028701

26.5	7.702993404	20.19528804
27	7.646705991	20.23205009
27.5	7.635516325	20.39225032
28	7.813533744	20.38490458
28.5	7.763010705	20.30996221
29	7.808447532	20.23057974
29.5	7.841338369	20.13645389
30	7.725372736	20.24381244
30.5	7.646705991	20.44072533
31	7.790137169	20.47009835
31.5	7.791154411	20.4612869
32	7.689430172	20.62570358
32.5	7.665694516	20.78412349
33	7.673154294	20.8999295
33.5	7.659930142	20.99370164
34	7.682648556	21.06254019
34.5	7.645688749	21.1093969
35	7.665694516	21.15185243
35.5	7.635516325	21.21186234
36	7.65450485	21.27770901
36.5	7.651792203	21.25868855
37	7.659930142	21.18405481

37.5	7.596522033	21.11086102
38	7.46326328	21.0376436
38.5	7.345941324	20.97905238
39	7.243538923	20.93949452
39.5	7.143510087	20.90872235
40	7.104176715	20.8310398
40.5	7.077050251	20.64037717
41	7.03398699	20.44219409
41.5	6.891912135	20.46569267
42	6.549440529	20.61102893
42.5	6.39244612	20.59048267
43	5.780066199	20.50093535
43.5	6.668457889	20.33788529
44	7.588045013	20.55965938
44.5	7.630769194	20.77972455
45	7.651792203	20.87648016
45.5	7.104176715	20.90286049
46	7.050262868	20.9775874
46.5	7.264901013	21.15038858
47	7.165211258	21.30404251
47.5	7.017711111	21.31574534
48	6.885808681	21.30696827

48.5	6.646417638	21.26746742
49	6.399905898	21.24698288
49.5	6.323612718	21.25283578
50	6.053365322	21.2338133
50.5	5.693939676	21.21039887
51	5.244657619	21.22503316
51.5	4.838438823	21.2045449
52	4.325070495	21.15624393
52.5	3.894437882	21.13721349
53	3.116586532	21.15038858
53.5	2.389936382	21.10793278
54	1.228584649	21.07571963
54.5	0.937653325	21.04496641
55	0.25983081	20.92044559
55.5	0.549744892	20.93363349
56	2.271597184	21.44443879
56.5	0.873906135	21.81844234
57	1.98100494	22.08109829
57.5	0.276445769	22.26189831
58	0.25983081	22.07818124
58.5	1.708383978	21.89288693
59	2.062384331	21.76588078

59.5	1.84062549	21.68994004
60	1.406941149	21.63443083
60.5	0.203882478	21.58329328
61	0.197779024	21.54529869
61.5	0.199135347	21.51460655
62	0.217106629	21.48098699
62.5	0.190997408	21.43566641
63	0.190658327	21.42396942
63.5	0.190997408	21.39764912
64	0.190658327	21.38302549
64.5	0.191336489	21.38010066
65	0.190658327	21.29087613
65.5	0.189980166	21.21039887
66	0.190658327	21.17088166
66.5	0.190997408	21.16209913
67	0.199813509	21.4868342
67.5	0.185572115	21.60374954
68	0.190997408	21.45905872
68.5	0.185233034	21.38156308
69	0.190319246	21.32159655
69.5	0.190997408	21.2806351
70	0.190658327	21.28209813

70.5	0.188623842	21.3318358
71	0.190997408	21.42250725
71.5	0.185572115	21.52776079
72	0.185572115	21.59498277
72.5	0.185572115	21.5862157
73	0.185911196	21.39472447
73.5	0.190658327	21.22210638
74	0.190319246	21.11232513
74.5	0.197779024	21.08450549
75	0.199135347	21.06400461
75.5	0.190658327	21.05082449
76	0.198796266	21.04350187
76.5	0.190658327	21.04496641
77	0.190319246	21.02739125
77.5	0.187267519	21.28941315
78	0.184215792	21.69286124
78.5	0.177095095	21.94104612
79	0.176756014	22.07526416
79.5	0.182859469	21.77318162
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125.9141667	0.193370974	21.57160325
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129.9141667	0.184554873	22.55328572
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131.4141667	0.178451418	22.82695465

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143.9141667	8.520178127	21.76003997
144.4141667	8.536793086	21.72791316
144.9141667	8.549678156	21.70892729
145.4141667	8.577143701	21.6767942
145.9141667	8.576126458	21.68117622
146.4141667	8.601557518	21.67241211
146.9141667	8.622580528	21.68117622
147.4141667	0.271359557	21.71769017
147.9141667	0.24694574	21.70746678
148.4141667	0.242537689	21.66218693
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149.9141667	0.213376741	22.1510986
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150.9141667	0.213715821	22.00962203
151.4141667	0.212698579	21.97752477
151.9141667	0.212698579	21.96585212
152.4141667	0.213715821	21.96731123
152.9141667	0.213715821	21.95855647
153.4141667	0.212698579	21.94396459

153.9141667	0.212698579	21.93666836
154.4141667	0.213715821	21.92353467
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157.4141667	0.205577882	22.37557156
157.9141667	0.20049167	22.54600427
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158.9141667	0.205577882	22.00962203
159.4141667	0.207612367	21.74397706
159.9141667	0.522618428	21.73229432
160.4141667	3.82967345	21.88996792
160.9141667	8.610373619	22.02712809