Cellular Encapsulation in Edible Bioreactors

A Major Qualifying Project Report:

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By:

_____	_____
Gabriella Cerbo	Liam Cox
_____	_____
Jillian Doyle	Luca Ialongo

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_____
Professor Eric M. Young
Chemical Engineering Advisor

Abstract

This project was completed under the sponsorship of Incredible Foods. Edible alginate membranes can be used to encapsulate foods. This has utility in the food packaging industry for plastic waste reduction and extending shelf life. We explored a new use for encapsidated foods - an edible bioreactor for cultured probiotics. We confirmed the feasibility of yogurt production in alginate spheres quantifying growth progression. We also showed that probiotic yeasts can survive in yogurt culture, demonstrating that designer probiotics can be cultured while encapsidated. This study builds the foundation for a unique biomanufacturing modality where cultured products can be grown after encapsidation.

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Table of Contents

Table of Figures

Table of Tables

Introduction

The food industry is massively growing and changing. It has produced around 20,000 new food and beverage products each year over the past decade (Sinclair, W. & Zeballos E., 2021). However, the rate of product failure can reach up to levels of up to 90% in some years (Kansas State University, 2019). It is imperative for companies to take risks and explore new potential ideas to keep up and expand this innovative industry through both making new foods, and sometimes foods that make themselves.

Our project sponsor, Incredible Foods, is a small company based out of Boston founded in 2012. They specialize in creating nutritional snacks, called “foodberries,” through the use of their plant based technologies. Their patented foodberries consist of an encapsulation which simulates different foods utilizing some of the basic components of fruit skins and other similar natural casings such as chitin and alginate. These casings contain a secondary food element with a complimentary flavor to make their snack such as their hummus poppers which is hummus encapsulated within a roasted red pepper casing.

Incredible Foods require further research into the possibility of creating small scale bioreactors within the foodberries. Their current lines of production require creating their products with pre-existing foods and the encapsulations they create. It would be beneficial to their production, and products shelf life, if the components required for a foodberry could be put within the bioreactor encapsulation and their products create themselves. For example, the yogurt berries Incredible Foods made would be able to culture themselves, and the yogurt culture could be utilized for many batches saving resources and time.

The goal of our team is to bring a new level of depth to Incredible Foods’s understanding of small scale bioreactors within their products. Our team's goal is to test different aspects of fermentation cultures within the encapsulations such as product output, reactant consumption, and cell counts to provide Incredible Foods with an analysis of the feasibility of their bioreactor endeavors, and where to focus their efforts.

Background

Determining ways in which food production companies can reduce the accumulation of waste in the environment is a challenge that is prevalent in history and still a modern problem today. At Incredible Foods, creating new and innovative ways to help minimize litter by developing edible food packaging is the foundational purpose of their company. By incorporating an edible food packaging material, the food product can benefit from an extended shelf life and added nutrients while also reducing packaging waste (Incredible Foods, 2020). The end goal of these materials is to increase the health benefits and sustainability of food products without compensating the flavor and texture of the original ingredients. As this is a growing field in food science, there is much to be studied and researched about the limits of biodegradable food packaging technologies.

Benefits of Edible Food Packaging

Food encapsulation methods are a relatively new process but exhibit viable benefits for future applications. Perhaps the most important benefit is prolonging the shelf life of the food product in the same way a plastic or paper packaging material would - especially for probiotic foods (Timilsena, Y. , Haque, M. and Adhikari, B, 2020). Due to the semipermeable membrane of the packaging material, the layer will prevent aromas, ingredients, or moisture from escaping the capsules while also allowing necessary gasses produced and consumed in food respiration to undergo mass transport freely through the membrane (oxygen, carbon dioxide, etc.) (Petkoska, A. T. et. al., 2021). For this same reason, the edible layer provides ideal conditions and potential to host a probiotic food product that incorporates additional active cultures that can provide nutritional benefits such as enhancing growth development, strengthening the immune system, and increasing production of important nutrients such as vitamins and minerals (Goldin, B. R., 2019).

As for determining the extended shelf life of the food products, shelf life depends on four main attributes; formulation (the selection of raw ingredients for the packaging), processing (subjecting the raw, formulated ingredients to unfavorable conditions to test the limits of the material), packaging (examining the microenvironment within the raw ingredients), and storage conditions (analyzing whether the product was stored properly to favor the preservation of the raw materials) (Galic, K., Kurek, M., & Scetar, M., 2011). If one is only considering the packaging material for the food product, the shelf life is impacted in both the packaging and processing attributes. Therefore, choosing a durable and sustainable packaging material that is also biodegradable will result in a prolonged shelf life for the food product as compared to the food being open to the same storage conditions. There are also processes of making edible films and capsules that do not involve any extreme changes in the thermal conditions of the system. For probiotic foods especially, being able to create films without heating or cooling allows for the food to be undisturbed throughout the packaging process.

Lastly, the development of edible food encapsulation technologies eliminates the possibility of food packaging litter that negatively impacts the ecosystem. Similarly, the aforementioned increased shelf life also suggests the possibility of reducing food waste in homes, as foods will not expire as quickly (Dikes-Hoffman, L. S., et. al., 2018). When food production was first industrialized and mass manufactured in the 1980's, food waste levels rapidly began to increase (Guillard, V., et. al., 2018). Today, nearly 40% of U.S. landfills are composed of food waste and their associated packaging, costing the country about $165 billion in unused, purchased food every year (Barrett, A., 2018). In response to this, plastic and paper food packaging was introduced to increase the shelf life of foods, but this only slightly reduced waste levels and only added more materials to the landfills. Introducing an edible packaging material where traditional plastic and paper packaging is used will not completely eliminate food waste in U.S. landfills, but it does suggest a way to reduce plastic and paper packaging waste in the environment.

Description and History of Food Encapsulation Processes

There are several ways to encapsulate food products in edible films, and biodegradable food packaging technologies have been used in countless food production facilities since the first biodegradable plastic was introduced by Japanese scientists in 1975 (Nedovic, V. A. & Zuidam, N. J., 2010). Food encapsulation can be defined as the process of entrapping a food substance within another substance, in this case an edible, biodegradable material (Nedovic, V. et. al., 2011). Polysaccharides are the most common encapsulation material, but proteins and lipids are also frequently used (Nedovic, V. A. & Zuidam, N. J., 2010).The encapsulate, or the core and membrane complex, has multiple functions, the most important being prolonging shelf life by stabilizing ingredients and increasing the bioavailability of active probiotic ingredients. Specifically for probiotics, the encapsulate can act as an edible bioreactor where the membrane acts as the reactor that is semipermeable to the environment, and the core contains the proliferating live culture.

The three most common and simplest ways to encapsulate food products are injection, extrusion, and emulsification (Nedovic, V. A. & Zuidam, N. J., 2010). Injection and extrusion both have the same first two steps. The first step is to prepare the membrane coating in liquid form. The second is to disperse the solid, active food ingredients into the coating, ensuring that the entirety of the substance is coated in the liquid. The third step is an extrusion step; for injection, this is exhibited through filtration, and for extrusion, encapsulates are separated through a twin-screw extrusion machine. Lastly, the encapsulates are left to cool and set. A simpler and similar extrusion process involves immersing the food ingredients in a liquid membrane bath that gels when in contact with a specific agent, then "dropping" the submerged food ingredients into the gel-activating agent. Emulsification is a two-step process, the first being to choose whether the food substance and emulsifiers should be dissolved in a water or oil phase. The second step is suspending the core mixture in the opposite phase to create the membrane. Lastly, the

encapsulates are again left to set and create a protective film. If the membrane solution is already in liquid form, no extreme heating is required, which further simplifies the process, and other than a two-screw extrusion machine, no advanced machinery or materials are required. There is another widely-used process of encapsulation known as spray-drying which involves heating the food product-membrane mixture and atomizing the mixture in the heaters to dehydrate and develop a film at the food surface. All aforementioned processes take less than an hour to complete, often only taking several minutes.

Benefits of Vitamin Incorporated Yogurt

A popular addition to food products in recent years is incorporating various vitamins and nutrients into commonly consumed foods in order to increase their nutritional value. These vitamin enriched foods also have the potential to prevent various illnesses and diseases; for example, the consumption of vitamin E fortified products decreases the chances of vascular disease (Widhalm, H. K., et al., 2011). Another prominent example can be found in a study where adolescents and young adults consumed 300g of vitamin supplemented yogurt every day for two months. Researchers found that at the end of the study, when compared to the control group, the supplemented participants had both lower total cholesterol and higher vitamin A readings (Widhalm, H. K., et al., 2011). Another study looked at enriching dairy products to aid menopausal women and found that consistently eating yogurt enriched with calcium and vitamins D, K, and C significantly increased the bone mass in the experimental group (Morato-Martinez, M., et al., 2020). This study showed that vitamin enriched food products can decrease the risk of osteoporosis without the standard pharmacological treatment; this conclusion can then be the inspiration to nutritionally alter foods in other ways to aid in the prevention of other diseases.

Another common addition to yogurt products is probiotic bacteria and yeast. Probiotics increase the amount of good bacteria in the gut microbiome, which is generally why people eat probiotic products. One study conducted looked at patients with inflammatory bowel disease who consumed yogurt cultured with *Lactobacillus rhamnosus* for thirty days. When compared to a control group, it was shown that the probiotic enriched yogurt had significant anti-inflammatory effects on IBD patients (Lorea Baroja, M., et al., 2007). Another group of individuals that can benefit from the effects of probiotic yogurt are type 2 diabetic patients. Probiotic yogurt is especially good at reducing oxidative stress, which is the main driving force of type 2 diabetes. In a study where the experimental group consumed 300g of probiotic yogurt a day for six weeks, it was shown that the total antioxidant status increases significantly compared to the control group (Ejtahed, H. S., et al., 2012).

Project Scope

This MQP project will focus on two main phenomena: yogurt bacterial culturing in a self-contained edible bioreactor and the fermentation of probiotic yeasts in yogurt. The overall goal in the study of these two focus areas is to predict and test the feasibility of probiotic yogurt production inside a self-contained edible bioreactor. This project will comprise several experimental processes.

The study of yogurt's incubation inside alginate-chitin matrix shells necessitated the creation of a mesophilic yogurt. Doing so was a simple process, only requiring the team to pasteurize store-bought milk in the lab and then add the culture bacteria. This mixture was then poured into spherical ice molds and frozen to enable enclosure into the shells. Alginate and crosslinking baths were prepared per instructions provided by Incredible Foods. To enclose the prepared spheres in the alginate crosslinking matrix, the frozen yogurt culture spheres were first treated in liquid nitrogen to rapidly decrease their temperature. Next, the spheres were coated in the alginate bath, which partially froze onto the yogurt core. The spheres were then rolled along two metal chopsticks to allow excess alginate to drip off. Finally, the alginate-covered spheres were placed into the crosslinking bath, which sat on a stir plate to ensure uniform coverage of the spheres for optimal crosslinking.

Throughout the duration of this project, the team will evaluate the effectiveness of the yogurt production and modification by observing quantitative properties such as pH, cell count, water content, and viscosity, as well as the qualitative properties including appearance, taste, smell, and texture. In doing so, the team will be able to judge the efficacy of probiotic yogurt production within alginate-chitin matrix spheres.

Methodology

In order to determine and quantify the growth during incubation of the encapsulated yogurt spheres over the course of multiple trials, several steps of preparatory work and a concrete incubation procedure were required. Most of the procedures below were adapted from protocols developed by Incredible Foods, Inc. The purpose of the methods described below were to replicate the same materials used in the yogurt edible bioreactor study conducted by Incredible Foods, Inc., and then modify the data measurements and analytics to better quantify and describe how the growth progression of the enrobed yogurt cultures compares across each trial. Additionally, we aimed to study how the encapsulated yogurt cellular proliferation rate is represented by different parameter measurements. These procedures are detailed below in a step-by-step format to explain the specific steps taken to achieve the aforementioned objectives.

Alginate Bath

The sodium alginate bath is the first coating step for the yogurt cores in the enrobing process. It is important that the alginate ions are fully incorporated into the water as a homogeneous solution so the coating process is successful. Because of this, heat and an immersion blender is necessary to ensure a well-mixed system. The purpose of the alginate bath is to provide the initial coating of alginate onto a material to be enrobed, before immersion into a calcium-rich bath that diffuses through the alginate layer, causing crosslinking to occur and the alginate layer to coagulate.

Materials:

- Distilled water
- Sodium alginate
- Scale
- Hot plate
- Thermometer
- 1 L beaker
- Weigh boat
- Scoop
- Immersion blender
- 500 mL Schott bottle (for storage)

Procedure (taken from Incredible Foods Protocol (2021)):

1. Measure 294.0 g of distilled water into a beaker. Heat up water on a hot plate to 80 ℃.
2. Measure 6.0 g of sodium alginate.
3. Once the water reaches 80 ℃, gradually add sodium alginate to water and mix with an immersion blender to avoid clumping.

    a. Keep the sodium alginate solution at a constant temperature to avoid boiling. If the total mass of the solution after immersion is less than 300 g, q.s. with distilled water to 300 g.
4. Allow the solution to cool to around 60 ℃, occasionally swirling the solution.
5. Store solution in 4 ℃ to 8 ℃ refrigerator for future use.

Cross-Linking Bath

The spheres were transferred into a proprietary crosslinking bath containing calcium ions, which sat on a stir plate to ensure uniform crosslinking of the alginate coating. This bath is the second step of the enrobing process. After the core is submerged in a sodium alginate bath and fully coated, it is transported to the cross-linking bath. The purpose of the cross-linking bath in the enrobing process is to diffuse the calcium through the alginate layer and cause cross-linking interactions to occur, leading to coagulation and completing the enrobing process. Similar to the alginate bath, the cross-linking bath also requires a heat source and immersion blender to ensure the solution is homogeneous and well-mixed.

Yogurt Core

In order for the yogurt core to be enrobed, it must first be frozen. Additionally, the yogurt core must also incubate at room temperature for easier sampling. Therefore, a mesophilic yogurt starter culture is required for incubation. A core diameter of 2.85 cm was initially used, then switched to a core diameter of 1.5 cm to increase membrane strength. The two cultures that were used in experimentation were Filmjölk and Viili strains. The mixture of the starter culture and whole milk must be done gently to reduce bubble formation that may interfere with a homogeneous freezing process or stunt the yogurt incubation. Additionally, the yogurt cores should be placed into the freezer as soon as the mixture is incorporated to limit any initial bacterial growth. The purpose of developing the yogurt cores is to prepare a solid core for the encapsulation process.

Materials:

- 4 freeze-dried packets of a mesophilic yogurt starter culture (Filmjölk or Viili)
- 4 cups pasteurized whole milk
- 1 L beaker
- Whisk
- 2 38-sphere ice trays

Procedure (taken from Bacillus Bulgaricus Viili Yogurt instructions (2022)):

1. Pour 4 cups of pasteurized whole milk into the 1 L beaker.
2. Add 4 freeze-dried packets of Filmjölk yogurt starter culture and mix completely with a whisk. Avoid frothing.

3. Pour the yogurt-milk mixture evenly into 2 38-sphere ice trays, close the ice trays carefully but completely, and store the filled ice trays in the freezer overnight or until the cores are frozen.

Tomato-Milk Agar Petri Dishes

Scientists in the 1920's discovered through experimenting with different petri dish media that the *lactobacillus acidophilus* bacteria culture found in yogurt grow best in a medium with milk and tomato juice because milk provides a carbon and nitrogen source and tomato juice provides a mineral source (Synthetic Biology, 2017). The purpose of using petri dishes for the experiment is to directly measure the increase in growth of the yogurt cultures over time by counting colony forming units (CFUs). The serial dilution factor for the yogurt samples was determined through research and preliminary trials. Based on research, most yogurt cultures have around one billion CFUs per mL of yogurt (Nyanzi, R., Jooste, P. J., & Buys, E. M., 2021). So, if 0.5 mL of yogurt were added to each petri dish, a dilution factor of 10^6 to 10^7 may be necessary depending on the growth rate of each specific yogurt culture.

Materials:

- Canned tomatoes (14.5 oz)
- Skim milk
- Agar
- Distilled water
- 1 L beaker
- Stir plate
- Stir bar
- Graduated cylinder
- Weigh boat
- Scoop
- Scale
- 9 cm Petri dishes
- Autoclave
- Thermometer
- 2 L Schott bottle

Procedure (taken from Synthetic Biology website (2017)):

1. Separate the tomato juice from the canned tomatoes into a beaker using a coffee filter.
2. Measure 100 mL of tomato juice.
3. Measure 100 mL of skim milk.

4. Measure 23 g of agar.
5. Mix the tomato juice, skim milk, and agar using a stir bar and stir plate.
6. Add distilled water to q.s. to 1 L, and stir until fully homogeneous.
7. Autoclave the solution per the autoclave instructions.
8. Allow the solution to cool to 60 ℃ and apportion into the empty petri dishes, filling about halfway (fills about 50).
9. Store filled petri dishes in 4 ℃ to 8 ℃ refrigerator for future use.

Yogurt Core Encapsulation

The procedure of encapsulating the frozen yogurt cores using the alginate and cross-linking baths are the most significant in relation to the data collection and analysis for the project. The purpose of this procedure is to create the edible bioreactors for the individual yogurt spheres. By placing the yogurt cores in a liquid nitrogen bath, the alginate will freeze evenly onto the core and ensure an equally distributed alginate shell as well as allowing the yogurt cores to stay frozen for longer. An even coating is required because if there are any weak points in the coating the yogurt may leak or the cross-linking reaction may not occur properly. Similarly, the crosslinking bath should be well-mixed to ensure that the cross-linking reaction occurs at all points of the sphere.

Materials:

- Prepared alginate and crosslinking baths, chilled
- Stir plate
- Stir bar
- 1 L beakers
- Prepared frozen yogurt spheres
- Chopsticks
- Spoon
- Paper towel
- Tupperware container

Procedure (taken from Incredible Foods Protocol (2021)):

1. Remove the chilled alginate and crosslinking baths from the refrigerator.
2. Stir the crosslinking bath on a stir plate.
3. When ready, remove the frozen yogurt spheres from the freezer and place in a plastic bowl.
4. Fill the plastic bowl with a liquid nitrogen bath to super freeze the yogurt spheres.
5. One at a time, drop a frozen sphere into the alginate bath and ensure the core is fully coated. Remove the coated sphere from the alginate bath and transfer the coated sphere into the crosslinking bath and allow the core to thaw completely to ensure the coating is fully set.

6. Remove the finished spheres from the crosslinking bath as they set, and roll gently on a paper towel to remove excess liquid.
7. Store spheres in a tupperware container for future use.

Yogurt Incubation

The yogurt incubation procedure takes place immediately after the encapsulation process. Since the yogurt cultures are mesophilic, incubation can take place at relatively lower temperatures than thermophilic cultures, but the incubation process takes longer. The purpose of the incubation step is to allow the *lactobacillus* bacteria to cultivate in the milk, allowing the consistency of the mixture to grow more viscous over time and to convert the milk into yogurt. The incubation procedure was adapted to the instructions listed on the freeze-dried yogurt packages for each specific culture. It is important that bacteria exposure to light is minimized, as the *lactobacillus* growth is negatively impacted and can be stunted by light exposure.

Materials:

- Recently-encapsulated yogurt spheres
- Tupperware

Procedure:

1. Immediately after encapsulating and thawing the frozen yogurt cores, place all spheres in a closed tupperware container for storage.
2. Store at room temperature until desired yogurt consistency is reached (about 16 hours).

Viscometer

The viscometer is an instrument that measures dynamic viscosity of a given sample. The purpose of this procedure is to measure the viscosity of the yogurt samples over the course of the yogurt incubation period. In order to have consistent steps in our measurements, the following procedure was saved into the viscometer software so the examination would run for the same amount of time and the rotating piece would consistently rotate at the same speed each run. It was important to keep the rotational speed constant because yogurt is a non-Newtonian fluid and varies in viscosity when faced with different stresses. Trials were repeated three times and an average of the three trials was recorded for each sample measured. The viscosity values indirectly represent the growth rate of the yogurt, because as viscosity increases, the amount of bacteria in the yogurt increases.

Materials:

- Anton Parr ViscoQC 300 viscometer
- 2 mL yogurt sample, taken from an incubated, prepared, encapsulated sphere

Procedure (taken from Anton Parr ViscoQC 300 instruction guide):

1. Fill 2 mL of a sample into the sample cup, and dip the measuring bob into the sample.
2. Place and rotate the cup into the machine adaptor until it locks into place, and ensure the measuring bob is fully connected to the measuring head.
3. Set the measuring parameters and select "Start."
4. When the progress bar turns green and displays "Finished," record the measured dynamic viscosity value (mPa*s).
5. Repeat steps 1-4 twice and record the average value.

Using all the procedures described above, the collected data allowed for the quantification and representation of the growth and life span of the *lactobacillus* cultures in the yogurt. Samples of the encapsulated yogurt were taken roughly every 1-2 hours during the incubation period, and once a day following incubation until the data values remained relatively stagnant (usually 1-2 days). Measuring the dynamic viscosity, sugar concentration (using a brix refractometer), and pH (using paper pH strips), were all ways of indirectly measuring the growth progression of the yogurt cultures, while counting CFUs using a petri dish was a direct way of quantifying growth. Measuring the weight % loss of the sphere over time is a measurement of the barrier properties of the membrane. The previous work done with the encapsulated yogurt samples by Incredible Foods aimed to study the feasibility of using the alginate layer as a semipermeable bioreactor, capable of culturing the yogurt. The data acquired by that study were taken at timepoints of 1, 12, 24, and 48 hours. The main objective of these procedures is to extend the study to examine how well the alginate layer functions as a bioreactor by creating a graph of data points versus time to both prove that successful fermentation has occurred and to analyze the results of the graph to explain how cellular proliferation is represented by the trendlines. Additionally, these trials were completed several times under the same conditions to test the robustness and repeatability of the efficacy of the alginate layer and analyze how this may have impacted the proliferation process of the yogurt as well.

Results and Discussion

After the completion of our experimental trials, the resulting data was analyzed through the use of graphs and comparing the specific project data to both literature values commonly observed in yogurt and the values obtained by Incredible Foods in their similar experimentation. The first three trials were completed with the objective of demonstrating repeatability and robustness in the yogurt culturing process and were conducted per the instructions on the yogurt starter package. One trial was completed as a control, with the encapsulated core consisting of milk absent of yogurt starter cultures. Lastly, two trials were completed by changing the temperature conditions for the incubation period at a temperature higher and lower than ambient temperature. For all trials, data collection was conducted at specific time points throughout the incubation period to measure pH, weight %, CFUs, dynamic viscosity, and sugar concentration. Each of these parameters either directly or indirectly measure the growth rate of the yogurt culture during incubation except for weight % which is a measure of the moisture permeability of the membrane . The condition of the alginate spheres were also observed and recorded over time. The purpose of this data collection is to represent the capability of the alginate capsule to serve as an edible bioreactor through analyzing the various growth curves of the yogurt cores, examining the effect of varying environmental conditions on the function of the capsules, and overall observing how the capsule layer withstands the incubation process over time.

Colony Forming Units (CFUs)

One parameter that was measured and collected during experimentation was the number of Colony Forming Units, or CFUs. Approximately every eight hours, a yogurt sample was first diluted by a factor of 1,000,000 (DF was selected due to a known range of CFUs/mL of yogurt as $5.5 - 65 * 10^7$), then a 10 µL sample was placed onto a prepared tomato-milk agar petri dish (Erhard, F., Jaros, D. & Kneifel, W., 1993). After 2-3 days, the number of CFUs was counted by hand and recorded to create a growth plot over time. The purpose of measuring CFUs over time was to create a cell growth curve for the yogurt colony during incubation conditions. Collecting CFU values over time was important towards creating a growth curve because it is the only direct way of analyzing the yogurt culture growth. Measuring pH and viscosity were all indirect methods of measuring cell growth and, while still viable and accurate data, is not as reliable as measuring CFUs to create a growth curve and approximate a growth rate. The number of CFUs versus time have been plotted for all trials and are transposed into one graph below in **Figure 1**.

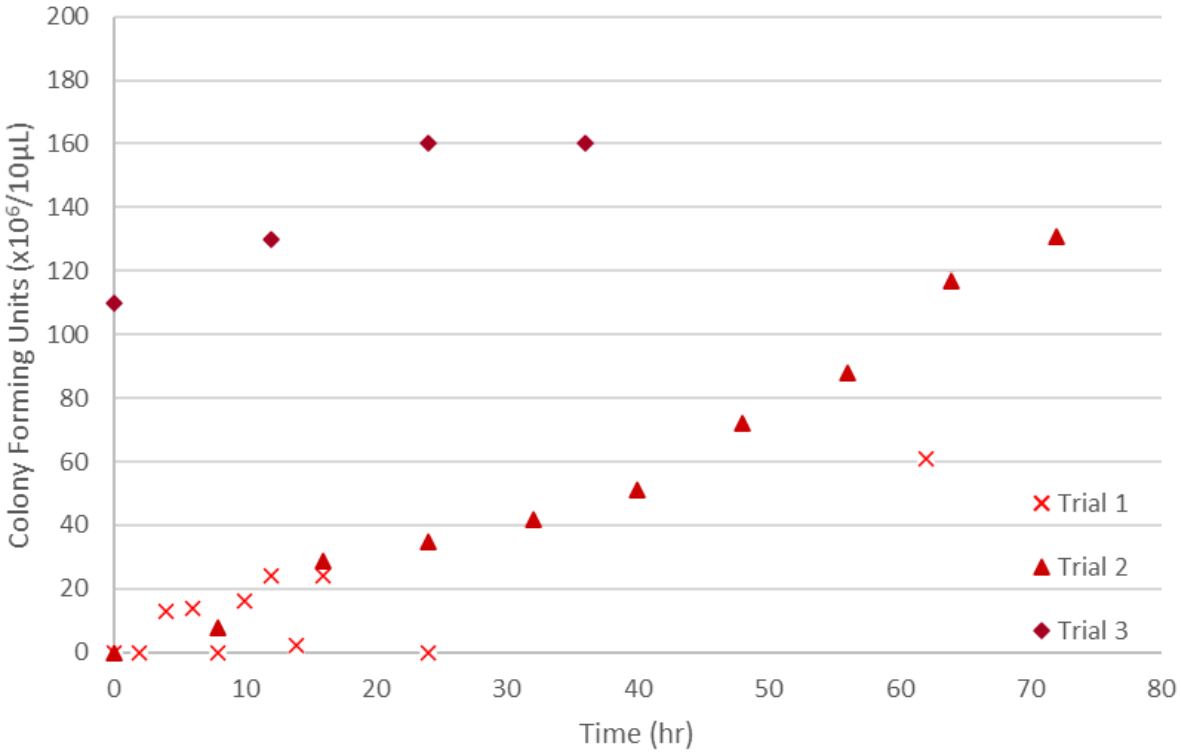

Figure 1: *Colony Forming Units vs time for all trials*

As can be seen by all trials, the CFU count is zero at the initiation of incubation (t=0). Then, values accelerate between 0 and 8 hours, then follow a linear trend throughout the 72 hour incubation period. This trend is commonly observed in microbial growth curves, which is composed primarily of a lag phase, log (or exponential) phase, stationary phase, and death phase. The lag phase is the period before cell growth begins, the log phase is where exponential growth occurs due to the abundance of resources available for growth, the stationary phase is where exponential growth slows and stagnates, and the death phase is where microbes die due to lack of nutrients (Muller, C. & Straube, T., 2016). These phases are shown in **Figure 2** below.

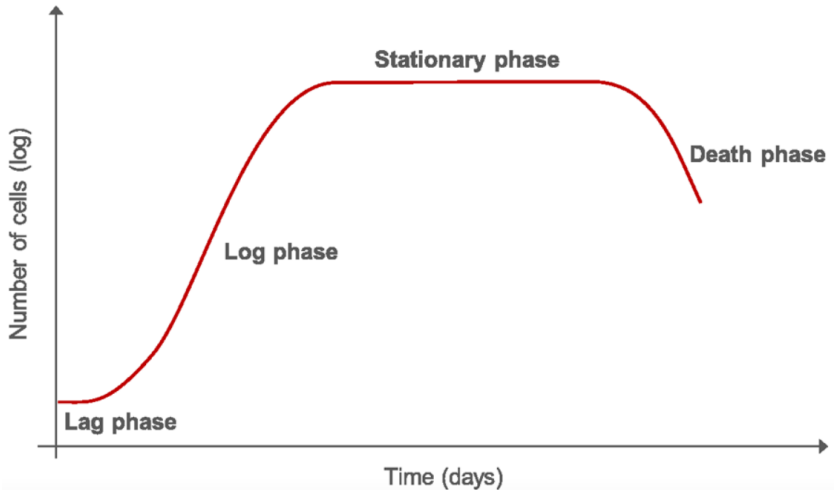

Figure 2: *Microbial growth curve phases*

By assuming that the log phase follows a linear trend, the cell growth rate during the log phase was determined by calculating the slope of the graph for each trial. The growth rate of each trial should be comparable, as all trials were performed under similar environmental conditions with the same yogurt starter culture. These values are in **Table 1** below.

Trial	**Growth Rate** (Day 1) (CFUs/μL/hr)
1	1.00
2	1.46
3	0.67
Average (CFUs/hr)	1.04
Variance (σ^2)	0.16

Table 1: *Cell growth rate during the log phase for each trial*

The purpose of comparing the growth rates for each trial was to demonstrate that the alginate spheres exhibit repeatability in functioning as a bioreactor for yogurt incubation. As shown by the relatively low value of variance among all trials, it can be said that the alginate spheres can function with high reproducibility and robustness as edible

bioreactors for yogurt cultures, as growth has occurred at a comparable rate for each of the trials that were performed.

It should be noted that the stationary and death phases were not observed during the 72 hour sample collection period. This was most likely due to the fact that more time is required for these phases to be reached, and a future recommendation for repeating these trials would be to continue CFU data collection past the log phase of cell growth. Analyzing CFUs over an extended period of time could also suggest an estimated shelf life for the yogurt during the incubation phase as well as determining how long the yogurt culture can be exposed to ambient conditions until there are no slim to no probiotic benefits upon consumption.

Sugar Concentration

One way to indirectly measure the growth of a cell culture is by creating a substrate consumption curve. In the case of the *lactobacillus* bacterial culture, the bacteria consumes lactose available in the whole milk in which the yogurt starter is mixed. To measure the sugar (mostly lactose) concentration in the yogurt spheres, a Brix refractometer was used. By placing a droplet of sample on the screen and placing the sample under a light source, the refractometer instantly measures the amount that light is refracted off the sample which indicates the sugar concentration. Due to a Brix meter measuring dissolved solids, the value on the Brix meter should theoretically never read zero for yogurt due to the presence of proteins and other compounds that can refract light. Generally speaking, a substrate consumption curve tends to follow the opposite trend of its corresponding microbe growth curve (Markov, S. M., 2011). This is demonstrated in the example graph below in **Figure 3.**

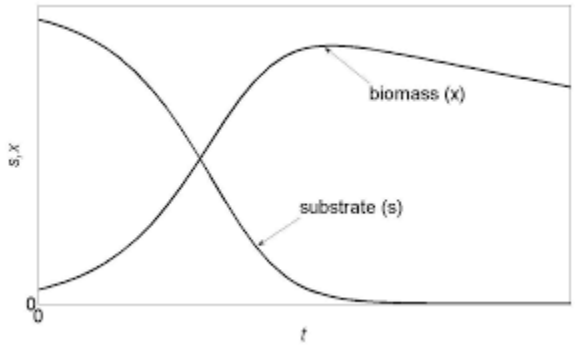

Figure 3: *Substrate consumption curve compared to a microbe (biomass) growth curve*

As seen in the figure, as biomass grows exponentially, substrate concentration falls exponentially, and at about the same rate as growth is exhibited. Then, as the stationary phase and death phase of growth is reached, the substrate concentration also plateaus at a concentration of zero, which indicates the substrate concentration has been completely depleted by the biomass.

By using the Brix refractometer and measuring the sugar concentration of the yogurt sample approximately every eight hours, we were able to construct a substrate consumption curve. The results of each trial were then superimposed onto a graph to see if each trial were comparable and to study the reasons for possible outliers. The resulting graph is shown below in **Figure 4**.

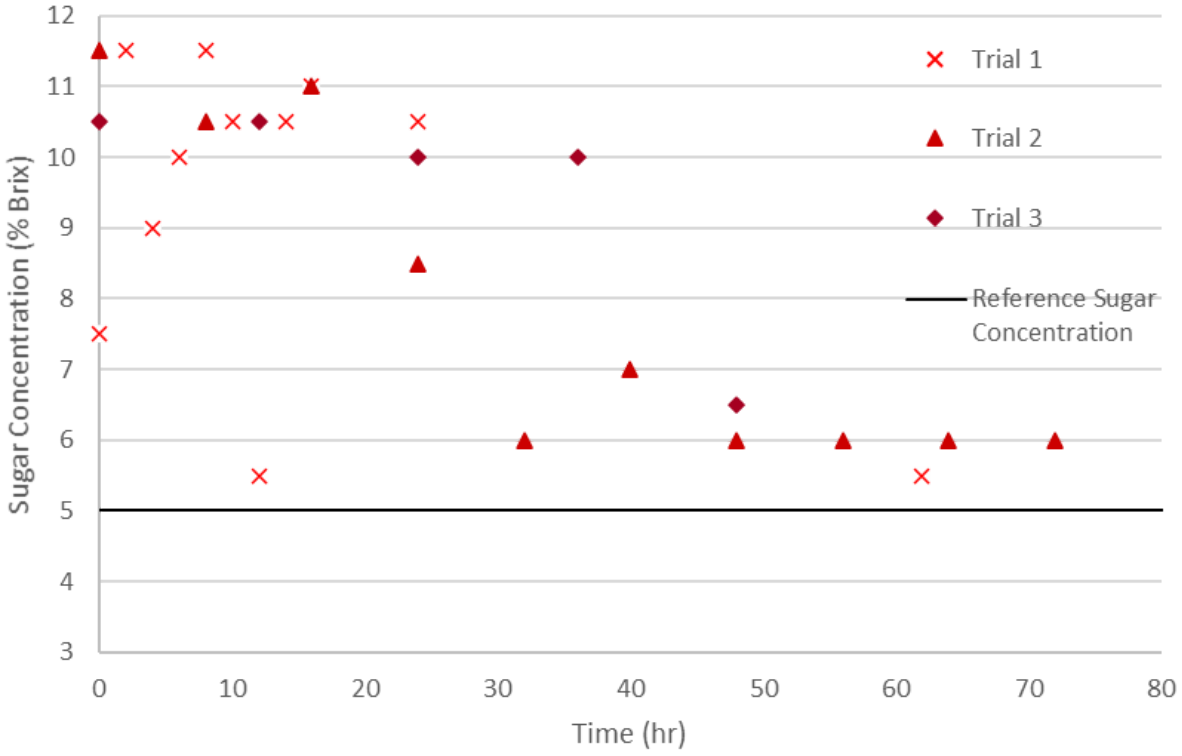

Figure 4: *Sugar concentration vs time for all trials*

As can be seen by the trials, the sugar concentration reading over time tends to vary and ultimately causes the data to fluctuate. However, when looking at the overall trend in the data, the values of sugar concentration do decrease over the course of the yogurt incubation. This suggests that substrate consumption by the yogurt culture is occurring since the supply is depleting. Additionally, near the tail end of the graph, it is observed that the sugar concentration plateaus at the lower value. Although this value is not zero, this does represent the point in cell growth where cells have stopped consuming the substrate at such an exponential rate. This phase in cell growth corresponds to the stationary phase.

In regards to explaining the variation in data points between each measurement point, there are a few reasons that may explain this. First, the sugar concentration in the yogurt is entirely sourced from the lactose in the whole milk used to create the yogurt. The Brix refractometer can read sugar concentrations from 0 to 32% by volume. As can be seen in our results curve, sugar concentration does not reach higher than about 13%, and only

drops to about 6%. This only allowed for a small margin of error in sugar concentration measurements, and the refractometer reading was often blurry and difficult to record. So, a future recommendation would be to introduce a small amount of lactose to increase the starting amount of sugar and allow for a wider range of sugar concentrations to be recorded. Second, the blurriness of the refractometer reading may have caused the uncertainty in measurement to be greater than expected by calculation. More time could have been dedicated to properly calibrating the instrument, or attempting to increase the light intensity that is exposed to the sample may also cause the reading to display a more distinct line. Lastly, the Brix refractometer may not have been the most accurate instrument to measure the sugar concentration in the yogurt. Although a Brix refractometer can provide accurate measurements under certain conditions (homogeneous solution, clear light source), the instrument is more commonly used to estimate an overall sugar concentration (as opposed to a focus on lactose or glucose), which could lead to inaccuracies in measurements. In the future, a lactose assay kit in combination with a spectrometer or colorimeter may provide more reliable data.

Weight

Another parameter which was measured during the experiment was the weight of a sphere. To measure this parameter, one sphere was set aside at the beginning of the test. This sphere was never burst during the test, and instead was weighed at every time interval which data was taken. Originally, this measuring of the weight was intended to measure yogurt production and the corresponding protein conversion. During the trials, however, it became apparent that the decreasing weight of the sphere was a much better measurement of moisture loss than of protein conversion. Nevertheless, it remains a valuable tool in understanding the robustness and permeability of the membranes.

Due to the properties which a semipermeable membrane exhibits, the alginate spheres will naturally release moisture throughout the growth of the yogurt culture core. Incredible Foods, when conducting a similar experiment, also measured the weight loss of the spheres over time, so the results of our experimentation were compared to the sponsor's values and trends. The data obtained from all trials were superimposed into the graph in **Figure 5** below.

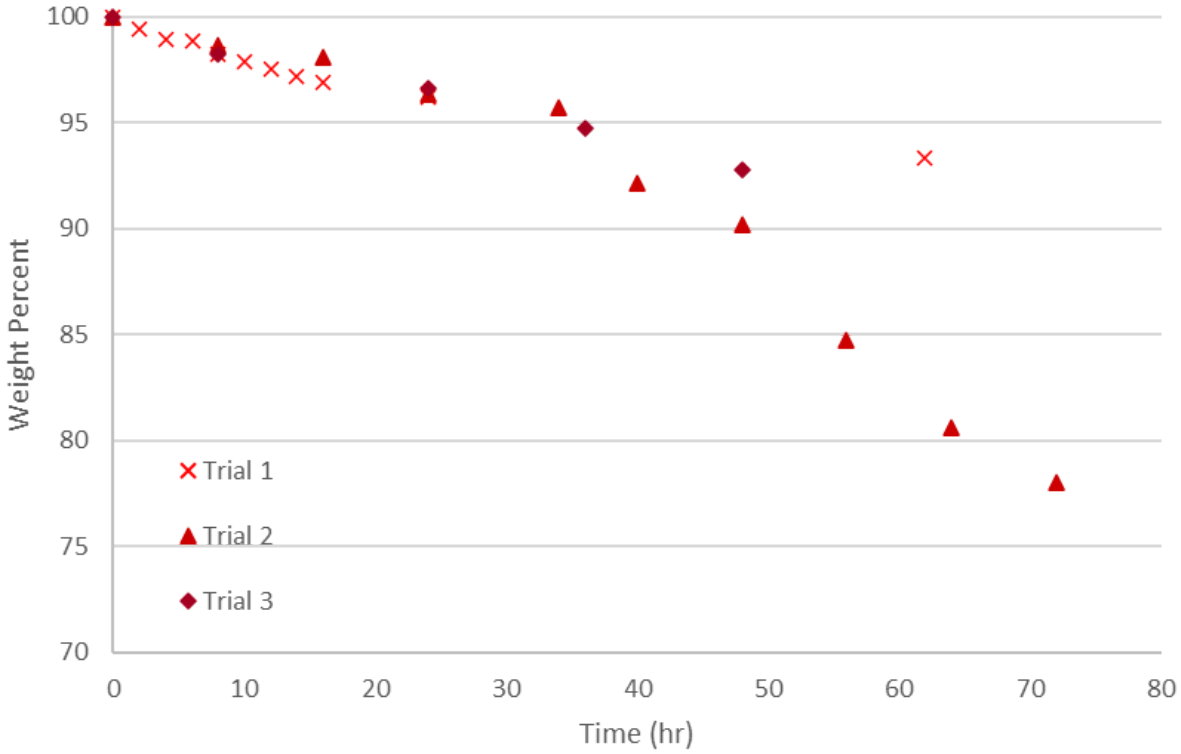

Figure 5: *Weight loss vs time for all trials*

As can be seen by the trendlines, the weight loss measured over time for all trials proceeded at a steady, linear rate. Since the initial weight of each sphere differed among trials, comparing the slopes of each weight loss rate can suggest whether the trials demonstrated robustness and repeatability. In accordance with the work completed by Incredible Foods (2021), an enrobed sample was measured once a day to obtain the percentage of weight retained by the sphere. Below is a table of the weight for the weight measurement obtained by Incredible Foods in **Table 2** as compared to the average weight for all trials.

Time (hr)	**Weight %** (Incredible Foods)	**Weight %** (Average)
0	100	100
24	98.2	96.4
48	97.4	91.5
72	97.2	78.05

Table 2: *Weight loss rate for each trial*

As shown above, the weight loss rates for each trial were generally similar and comparable and the low variance represents a minimal deviation in data, although it should be noted that the weight % dropped faster in the average trials, most likely due to a thinner membrane and possible leakage. This suggests that the membranes for each trial were of similar physical quality and that moisture was diffusing at constant similar rates. However, although the quality of each membrane may have been similar, they were observed to be very frail and thin especially for the first two trials which consisted of larger spheres (12 mL cores as opposed to 2 mL cores used later in experimentation). Due to this, the membrane may have ruptured slightly and caused the sphere to leak. Although no yogurt leak was initially observed, the possibility of a miniscule leak cannot be neglected. In any sense, it is clear that moisture (most likely water) was being removed from the spheres at a steady rate during cell proliferation, however there is no proof that this is a result of cell growth and is more likely due to the physical and material properties of the membrane.

pH

The pH of yogurt extricated from a sphere was also measured during the experiment. Each time a sphere was burst, standard paper pH strips were used to determine the pH of the yogurt released. The pH of the developing yogurt was measured to demonstrate the acidifying of the mixture as bacterial metabolism of the milk's sugars progressed. There have been several studies on the change in pH over time for lactic acid fermentation. One study from the Journal of Dairy Science explains how the change in pH over time of a homemade yogurt culture follows a sigmoidal trend, very similar to the Gompertz model, which describes growth as being slowest at the start and end of a life cycle (Soukoulis, C. et. al., 2007). One example of the Gompertz model is observed from the lag phase to the stationary phase of the biomass growth curve. The expected pH trend for yogurt incubation is depicted below in **Figure 6**.

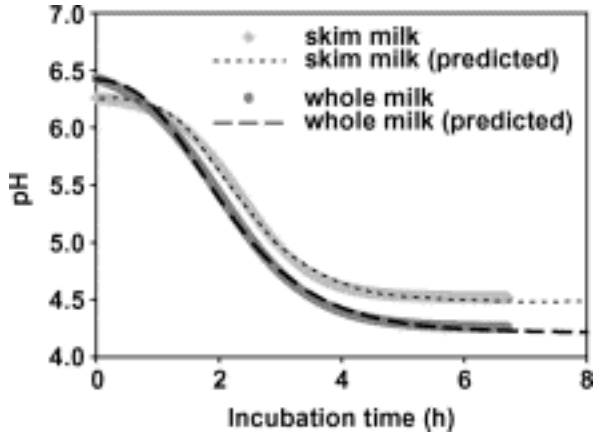

Figure 6: *pH over time in skim milk and whole milk yogurt, compared to a Gompertz model fit*

The pH in each of the trials was recorded every eight hours over the span of at least 62 hours. A superimposed graph of the measured pH over time for all trials was developed for comparison and is depicted in ***Figure* 7** below.

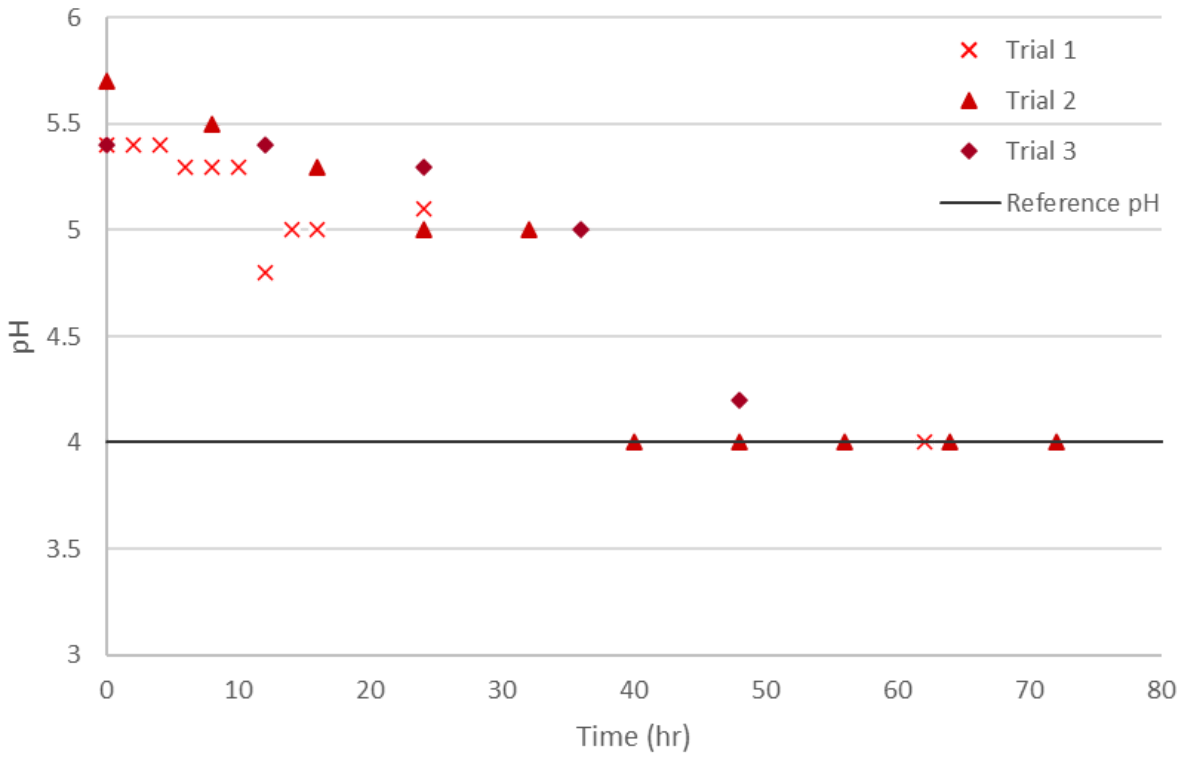

***Figure* 7:** *pH vs time for all trials*

According to the results, there is a pH drop across all trials. This suggests that lactic acid fermentation has occurred because the production of lactic acid as a byproduct of fermentation has a low pH, which causes the yogurt to become more acidic with time. It is also evident that the pH drops fastest during the middle of incubation. Although the Gompertz model fit may not be the most accurate fit for the data collected, the general theory that the bacterial growth is slowest during the start and end of incubation is supported by the results. One reason for the discrepancies in data is that the pH test strips measured in increments of 0.4 or 0.5, and the general range of pH recorded was from 5.7 to 4.0. Due to the concern that a pH probe may interfere or interact with the yogurt growth, the measurements were carried through with pH test strips, which are less accurate and less reliable, especially as the yogurt becomes more viscous and difficult to dissolve onto the paper surface. In the future, the use of a pH probe may be more beneficial for the study as long as careful consideration is made with regards to the bacteria-probe interactions.

Additionally, Incredible Foods recorded measurements of the yogurt pH in the enrobed cores when they completed a similar experiment (2021). They used these values to verify that the bacteria culture had successfully developed into yogurt. These values for the first two days of experimentation are compared with the average of all our trials in **Table 3** below. It should be noted that different yogurt starter cultures and slightly different methods were used between the experiments completed at Worcester Polytechnic Institute and at Incredible Foods and should be taken into consideration when comparing these values.

Time (hr)	**pH** (Incredible Foods)	**pH** (Average)
0	6.37	5.5
24	4.59	5.13
48	4.38	4.07

Table 3: *pH comparison between trials and results gathered from Incredible Foods*

As can be seen from the data above, similar pH drops are observed between the start and end of incubation. This further suggests that our trials had successfully reached yogurt development.

Dynamic Viscosity

Another parameter measured during the experiment was the dynamic viscosity of the yogurt. This was measured using a viscometer, the operation of which entailed filling a well with yogurt and inserting a small weight, which the machine then spun to measure the resistance. Yogurt thickens as bacteria produces lactic acid, causing the pH to drop and ultimately leading to protein denaturation and gel formation. This measurement was undertaken to illustrate the corresponding increase in viscosity. Therefore, measuring the viscosity over time is an indirect way of measuring the growth of the yogurt culture. The results for these viscosity measurements for all trials superimposed are shown below in **Figure 8**.

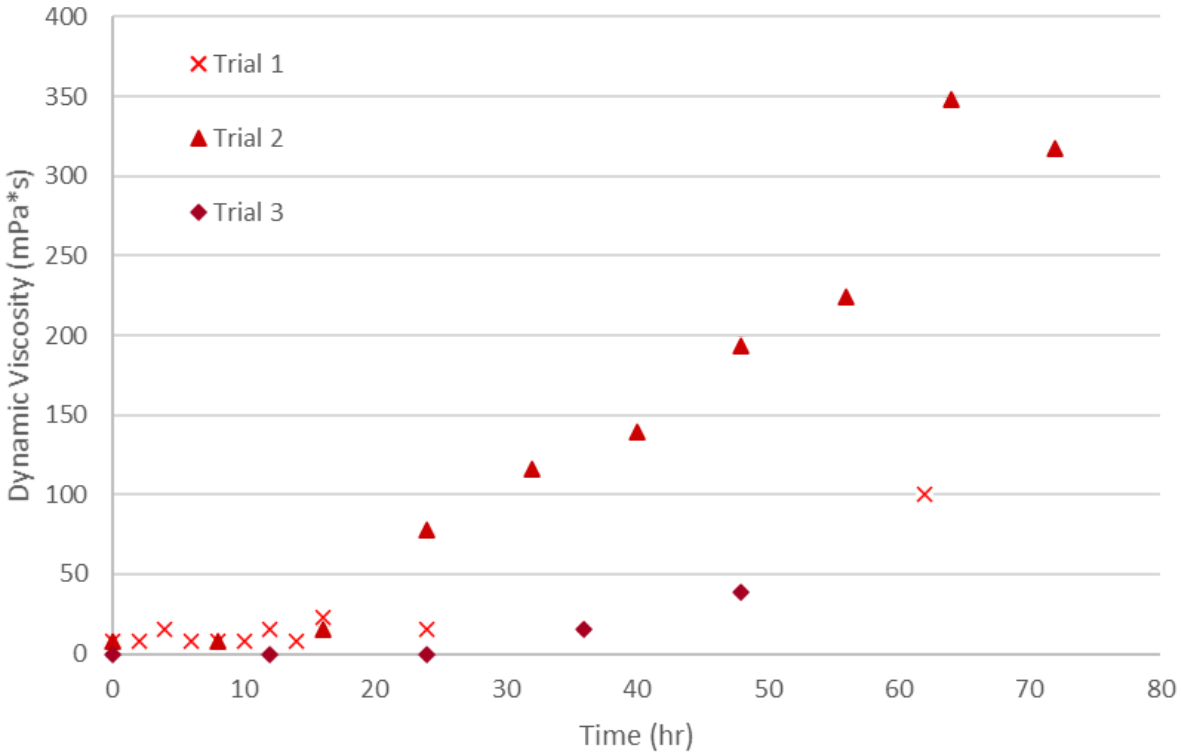

Figure 8: *Dynamic viscosity vs time for all trials*

One of the first observations that was made was that the dynamic viscosity of the yogurt for generally the first 24 hours of incubation consistently sat at about 7.7 mPa*s. The consistency of the yogurt during this time period was still very similar to milk, indicating that the material was not very viscous. The particular viscometer that was used to record measurements was only calibrated to read viscosities at a specific range, and it may be possible that the 7.7 mPa*s value is the lowest possible value that the machine can measure (the known dynamic viscosity of whole milk is between 2 and 5 mPa*s). However, once the yogurt became thick enough to generate higher readings on the viscometer, the data began to develop a positive trend on the graph. This suggests that cellular proliferation had taken place.

However, there was some variation in dynamic viscosity among trials most likely due to how the sample was prepared in the viscometer. First, there was very little space between the rotating weight and the sample well, and this may have caused air bubbles to become trapped along the sides of the well, impacting the viscosity measurement. Additionally, the sample may not have been mixed properly before being placed into the sample well, and this may have caused an uneven consistency to be in contact with the rotating weight, hindering the measurement. Due to these two operational challenges, the viscometer reading would slowly drop over the course of the measurement. It is unclear whether this drop was a common occurrence, and this may cause the data collected to be

less reliable. Albeit, the overall general trend is that viscosity increases in the yogurt samples over time, which proposes that the bacteria cultures are successfully growing in the yogurt.

Yeast Culture Preliminary Results

Lastly, a preliminary study was conducted to assess the possibility of incorporating a probiotic yeast into the yogurt mixture for added nutritional benefits. For this experiment, the yeast culture *D. hansenii* (or Candida Famata) was grown on agar dishes and frozen until immediately prior to use. Candida Famata functions to produce riboflavin (or vitamin B) which is a necessary component for cell growth and repair. Yeast was mixed in a 1:1 ratio by mass with the yogurt bacteria starter and the yogurt mixture was prepared using the same original method. Prior to beginning the yogurt-yeast culturing process, a small sample of the yeast culture was plated on a petri dish to examine the shape, color, and opacity of the CFUs. In order to tell if the yeast had grown successfully, the yeast cultures would have to be recognizable on the petri dish amongst the bacteria cultures. So, although pH, weight loss percent, and sugar concentration were measured as well just to ensure that general growth was still exhibited, observing differences in the CFUs was the important factor in determining that the yeast culture specifically was able to grow in the yogurt environment. Samples were taken once a day for four days. **Figure 9** below shows what the yeast CFUs look like, while **Figure 10** shows the CFUs for each day of sampling.

Figure 9: *Image of yeast petri dish*

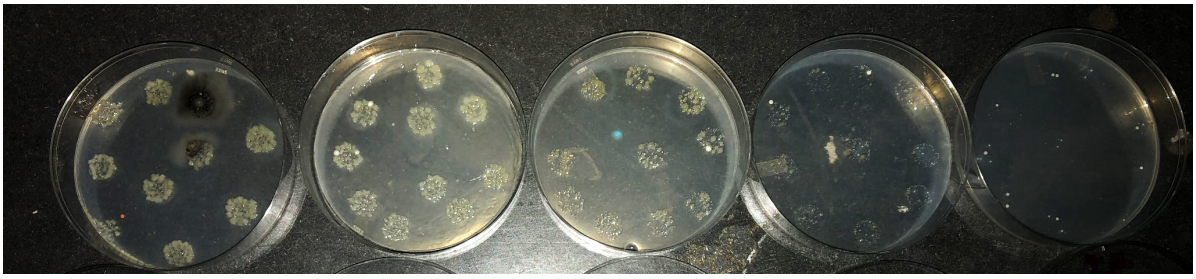

Figure 10: *Image of all yeast-incorporated yogurt petri dishes taken daily (left to right is day 1 to day 5)*

As can be seen by the two figures above, the yeast CFUs are very similar to the bacteria CFUs observed from previous trials, so it is difficult to tell if yeast cultures actually proliferated. There are small differences in the opacity of the cultures, where some appeared to have a matte finish and others had a more shiny finish, and it could be said that the bacteria cultures were shiny while the yeast cultures were matte (*lactobacillus* and *D. hansenii* are known to exhibit these features). However, all of the colonies appeared far too similar to draw an accurate and supported conclusion.

There are some subsequent observations and measurements that were made that suggested general growth was still evident in both cultures and that the growth rate may have increased for this trial as compared to the trials without yeast. **Table 4** below shows the conditions of the yeast-yogurt spheres at the beginning and end of the trial.

Time (hr)	**pH**	**Sugar Concentration** (mass %)	**Weight** (mass %)	**CFUs** (per µL yogurt)
0	5.1	11	100	63
96	4	5	91.01	82

Table 4*: Start and end measurements for yeast growth in the yogurt core*

From the table, the yeast-yogurt mixture has experienced a pH, sugar concentration, and weight drop, which was also observed during the pure yogurt trials. This suggests that growth was occurring in the spheres. Additionally, the number of CFUs counted on the petri dishes with yeast were higher than the petri dishes of pure yogurt. This may resemble a faster growth rate due to the presence of more starter cultures in the cores with the addition of the yeast.

One way to better differentiate the two colonies would be to dye one culture with a carbon-based dye, so one culture would be differently colored. It was very difficult to

deduce which CFUs belonged to which culture, and although there were a few small observations made during experimentation like an increased growth rate, an accurate and supported conclusion cannot be drawn as to whether the yeast can reliably and repeatedly grow in the yogurt culture.

Conclusions and Recommendations

Overall, it can be concluded by the data obtained from experimentation that the alginate layer around a food berry has proved to be a robust method for encapsulating food products as well as functioning as an edible bioreactor. Not only did the five measured parameters suggest that the yogurt cultured successfully, but the repeatability in data measurements also demonstrates that the membrane is able to protect and reliably house the food core when prepared properly. The results from this project is important for Incredible Foods because there was successful microbial growth observed and recorded across all trials.

This study could be improved by taking parameter measurements more often and over a longer period of time to better solidify and represent the microbial growth curve and substrate consumption curves. Recording more data points on graphs can lead to more reliable data and more accurate trend conclusions. We also suggest that the yeast preliminary study be carried forth due to the nutritional benefits that could come from successfully incorporating and growing a probiotic yeast within a yogurt culture. Lastly, an important parameter that should also be considered is the taste quality of the yogurt. Conducting a taste test will help decide when the yogurt has expired and if it needs to be fortified with flavors and added sweeteners to better suit the consumer.

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