Engineering Kombucha: Characterization of *K. xylinus*

Major Qualifying Project

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DISCLAIMER

Expected completion of this project was impeded by the unprecedented COVID-19 outbreak. As a result of the major societal changes due to this virus outbreak, necessary resources for the completion of project goals, such as laboratory access, became unavailable. Many of the experiments we planned for late March and April 2020 were unable to be performed. The data collected prior to the outbreak as well as expected data for the unexecuted experiments are presented in this report and identified accordingly.

ABSTRACT

Komagataeibacter xylinus is a strain of bacteria found in kombucha that produces cellulose, a versatile biomaterial. However, the study of *K. xylinus* is limited by a lack of metabolic modeling of cellulose biosynthesis and a lack of genetic tools for engineering cellulose biosynthesis with synthetic biology. Specifically, there is no method for high-throughput transformation *K. xylinus*. Therefore, this project sought to develop a scalable chemical transformation method for *K. xylinus* by adapting existing protocols for similar bacteria and to perform carbon balances for cellulose biosynthesis by *K. xylinus* in different media. Due to time constraints neither objective was fully accomplished, but the study collected significant research on *K. xylinus*, contributing to the characterization of the bacteria and future efforts to achieve a scalable transformation method.

INTRODUCTION

Section I: Bacterial Cellulose and Kombucha

Cellulose is a tough, fibrous organic polymer with a degree of polymerization ranging from several hundred to over ten thousand subunits of glucose. It is the most abundant polymer on earth [1]. Bacterial cellulose (BC), also known as microbial cellulose, is a biodegradable, natural cellulose that is synthesized by bacteria. BC is highly pure and has superior water retention properties to plant cellulose [2]. Plant cellulose expresses water retention values of 60%, compared to 1000% for BC [2]. The high water retention of bacterial cellulose allows BC to be highly-crystalline yet smooth and moldable [2].

BC has potential applications in a variety of areas, such as paper products, electronics, acoustics, optics, and biomedical devices. In the biomedical field, BC's main advantages are that it is highly biocompatible and that the human body does not naturally degrade it [3]. Given that BC is not naturally degraded by the body, this characteristic can be tuned as needed for different applications by adding active sites throughout the polymer. Moreover, different compounds can be added to add specific chemical reaction sites that can give different functionalities to the polymer. Some examples of BC applications have been wound-healing devices for severely damaged skin and as a small-diameter blood vessel replacement [3].

In order to take full advantage of the potential applications, it is necessary to find affordable and scalable sources of BC. One source of BC is kombucha tea, which is a probiotic beverage made by fermenting sugared black tea with a community of fungal and bacterial organisms. This symbiotic culture of bacteria and yeast, also known as SCOBY, is composed of acetic acid bacteria and osmophilic yeast species [4]. The SCOBY can be recognized as a floating cellulose network which appears like surface mold on the undisturbed, unshaken medium. The exact microbial composition of kombucha varies based on the specific brewing conditions and the source of inoculum for the tea fermentation. The yeasts ferment glucose and fructose to ethanol, which is then oxidized by acetic acid bacteria to acetic acid. This is the main metabolic path of kombucha fermentation, and acetic acid is a dominant organic compound in the beverage [4].

Komagataeibacter xylinus is a strain of bacteria found in kombucha, which has been found to be an effective producer of bacterial cellulose. *K. xylinus* can produce BC from various carbon sources including glucose, fructose, and glycerol [5]. In one study, an extensive matrix of different growth conditions including media, incubation time, inoculum volume, surface area and media volume were investigated in order to maximize the yield of bacterial cellulose produced by *K. xylinus* [6]. The yield of cellulose was maximized through experimentation, which involved reactor surface area design and media optimization. The results from said study concluded that mannitol is the carbon source that yields in the highest bacterial cellulose production in *K. xylinus* [6].

Even though *K. xylinus* is the most studied cellulose-producing microorganism, it is not a model organism (like *E. coli*, for instance). The limitations of working with non-model organisms is that many aspects of their physical characteristics, metabolism, and internal processes are unknown. Several genomic studies in *K. xylinus* [7] and other related cellulose-producing species [7, 8] have been performed to try to understand their characteristics and metabolism better. These types of studies are very important because they give synthetic biologists and genetic engineers more information about how the cell works, and therefore, how to modify it for our best advantage. This highlights the need for metabolic modeling of the organism to understand the production of bacterial cellulose and the factors affecting it. Once the organisms' characteristics and processes are defined, possibilities start opening in the field of cell and protein engineering. Genetic engineering can be used to make BC production more efficient, or to add chemical features to the cellulose polymer, as discussed previously. In order to be able to introduce any of these interesting genetic modifications in *K. xylinus*, specific transformation protocols need to be developed first. Therefore, the main goals of this project are: (1) to develop a high-throughput transformation protocol for *K. xylinus*; and (2) to develop a black-box metabolic model of cellulose production in *K. xylinus*.

Section II: Transformation of *K. xylinus*

The interdisciplinary field of synthetic biology has led to vast advancements in biochemical fields such as pharmaceuticals, agriculture and environmental sustainability. While the conventional biochemical manufacturing industry has streamlined processes for developing useful compounds, synthetic biology offers a platform for developing more dynamic and unique compounds that would be impossible to manufacture using traditional techniques. Synthetic biology pulls together a myriad of subjects, such as fundamental biological research, genetic engineering, chemical engineering and even electrical engineering, to alter natural or create new biochemical structures and processes. These "designer molecules" produced through synthetic biology are "responsive and multifunctional" in their environments [9]. As a result, the molecules "can be tailored for particular functions," which have led to breakthroughs in biological research.

A tool at the forefront of synthetic biology is genetic engineering. Through genetic engineering, synthetic biologists can utilize and alter the mechanisms within cells to produce desired products. Bacterial cells are extremely useful in genetic engineering because of their relatively simple DNA structure and ease of growth. Because bacteria contain all of their genetic information within their cytoplasm, plasmids containing new DNA can be added directly into the cell through the cell's membrane. This process is known as transformation.

Transformation plays a foundational role in synthetic biology and genetic engineering. Since DNA is likened to the "software" of cells, modifying a cell's DNA programs the cell to behave differently. For example, at a very basic level a single operon may be added to a

bacterium to make it produce a particular enzyme. However, genetic engineering in industry is often much more complex. Typically, rather than coding for one enzyme, the aim is to comprehensively modify the metabolic network to increase product formation. These many genetic engineering changes require reliable and scalable genetic transformation methods. With the ability to transform DNA into a bacteria that codes for specific functions leads to synthetic control of the natural metabolic network [10].

There are several methods for transforming cells with varying tradeoffs, such as efficiency and effectiveness that depend on the organism. Moreover, each transformation method has not yet been applied to all organisms. Currently, the only transformation method being used for *K. xylinus* and other cellulose-producing bacteria is via electroporation [7, 11-13]. This method basically consists of exposing the mixture of cells and external DNA to a brief electric shock that opens the cell walls temporarily, allowing the DNA to enter the cytoplasm. The cells must be made chemically competent previous to the electroporation, by exposing them to a buffer that provides the necessary salts to accomplish the opening of the cell wall. This process is typically done one at a time and is difficult to scale.

One of the biggest challenges in synthetic biology currently is the ability to translate lab bench work and results into industry. Problems like scalability, toxicity, and difficulties in product separations are some of the main limitations for implementing synthetic biology advances in industry [9]. Therefore, it is important to take into account issues like scalability when designing cell factories for a product of interest. This poses a potential problem with the use of electroporation as the main method for the transformation of cellulose-producing bacteria, like *K. xylinus*. The problem with electroporation is that it is a low-throughput method, which can only transform a small volume of cells at the one time. When handling large volumes of cells or many different samples, it becomes a very tedious and time-consuming process. Other common transformation methods, like heat-shock (also known as chemical transformation), allow for larger volumes of cells to be transformed at the same time. The goal of this project is to develop a high-throughput transformation method for *K. xylinus* and other similar cellulose-producing bacterial species.

Given that *K. xylinus* is not a model organism, there are no heat-shock protocols adapted for this specific bacterial strain; however, there are many available protocols that have been optimized to transform *E. coli*, a more well known organism [14]. Other bacterial strains, like *Salmonella typhimurium* (also an acetobacter gram-negative species), have been successfully transformed through heat-shock [15]. We will be using these protocols as starting points for developing a chemical transformation method for *K. xylinus*. Other interesting methods that have been developed for bacteria include the use of micro-polymers that either pierce cells to introduce the plasmid DNA [16], or use a capsule-like structure that merges with the cell wall and introduces the DNA into the cell [17]. These methods might be harder to scale-up, but they are good options if the chemical transformation proves unsuccessful in *K. xylinus*.

Section III: Metabolic Modeling of *K. xylinus*

Metabolic modeling is a representation of the pathways within an organism and a mathematical balance of the overall substrate and product flow rates[18]. Over recent years, metabolic models have developed into much more refined and specific representations of organisms, which delineate each reaction pathway within the organism. During the developmental years of metabolic modeling, primarily from 1995 to 2010, scientists focused on quantifying only the overall input and outputs of organisms, rather than the internal reaction mechanics - a process known as black-box modeling. The black-box model calculations give a "quantitative analysis of metabolic system components [...] and outputs," but not an explanation as to "how a metabolite concentration or a metabolic flux emerges from the interactions in the underlying networks" [19]. The information from these black-box models gave scientists a way to characterize and generically understand different strains of bacteria, ultimately serving as a foundation for the higher-level studies at the present.

The goal of developing this black-box metabolic model is to describe the *K. xylinus* isolate in terms of growth and cellulose yields in different conditions. Therefore, studying the underlying reaction networks within the organism is unnecessary and out of the scope of this project. Instead, this project will quantify only the inputs and outputs of *K. xylinus*, using the black-box model approach. This model will calculate the specific uptake rate for each substrate, the specific rate of formation for each product and the overall cell biomass growth [19]. Figure 1 shows the generic schematic for a black-box model.

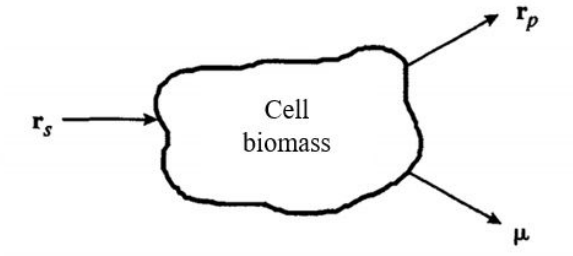

Figure 1: Diagram of the black-box model, where r_s is the rate of substrate uptake, r_p is the rate of product formation and μ is the accumulation of biomass [18].

In order to gain a more basic understanding of *K. xylinus*, our research project will develop a metabolic model for the bacteria's cellulose-production process. For this project, the before and after masses of different sugar and sugar alcohol feeds and the corresponding cellulose and cell masses will be measured. These measurements should give insight into optimal sugar and sugar alcohol feed ratios as well as cellulose yield for *K. xylinus* [20]. The components measured in this project will be D-mannitol, L-arabitol, acetic acid, ethanol, xylose, and glucose.

METHODOLOGY

Section I: Transformation of *K. xylinus*

Antibiotic Resistance Protocol

1. Inoculate 5mL of HS media with cellulase. Let the culture grow in shaker at 200 rpm and 30°C until they reach an OD of 0.1 (about 2-3 days).
2. Measure OD of cells after culturing for a couple of days.
3. Preparing the 96-well plate:
    a. We will be using a multi-channel micropipette to get serial dilutions of the antibiotic from 1- to 16-fold dilution.
    b. Pipette 200 µL of media with cells plus 2 µL of antibiotic for the main runs, and kanamycin for the positive controls.
        i. Antibiotics used were: Kanamycin (positive control), Spectinomycin, Nourseothricin, Chloramphenicol, Hygromycin B, and Ampicillin.
4. All wells should have a final volume of 100 µL. To set up the plate, start by loading 100 µL of cells and media to all wells except for the 100 µg/mL column. Then use the channel micropipette to pour 200 µL of media with cells and antibiotic at the 100 µg/mL concentration. Then set the micropipette to 100 µL and transfer half of the contents of the wells in the highest concentration column to the one right next to it, thus diluting it 2-fold. Mix well and then transfer 100 µL into the next column. Continue like this until you reach the lowest concentration, discarding the remaining 100 µL. DO NOT mix anything on the last column (concentration of 0 µg/mL).
5. The negative control will be one well with no antibiotic, and the positive control will be using kanamycin (which we know works for this bacteria).
6. Use the plate reader to measure the OD at the start of the experiment, record the data.
7. Let the cultures grow for 7 days.
8. Measure the OD at the end of the experiment, record the data.

Transformation Protocols

Before testing different methods for the transformation of *K. xylinus* it was first important to successfully transform *E. coli*. *E. coli* was chosen as the control for this experiment because it has several reports of successful chemical transformation. It is also a gram-negative bacteria, as is *K. xylinus*. A basic chemical transformation method for *E. coli* was used which was found on bio-protocol.

E. coli Transformation Protocol

Materials:

1. Strains of E. coli
2. DMSO: Oxidation products of DMSO, presumably dimethyl sulfone and dimethyl sulfide, are inhibitors of transformation
3. PIPES
4. Deionized H2O
5. Yeast Extract
6. Tryptone
7. KCl
8. NaCl
9. NaOH
10. MgCl2
11. MgSO4
12. Antibiotic
13. (MnCl2)4 H2O
14. (CaCl2)2 H2O
15. Glucose
16. Liquid Nitrogen
17. Ethanol
18. Sugar
19. Inoue Transformation Buffer
20. SOB Medium
21. SOC Medium
22. Luria-Bertani (LB) Medium

Equipment:

1. Centrifuges and Rotors
2. Milli-Q filtration system
3. Polypropylene 2059 tubes (17x 100mm), chilled in ice
4. Shaking Incubator (18C)
5. Water Bath (42C)
6. Nalgene Filter
7. Disposable pre rinsed Nalgene filter
8. 250-mL flask
9. Sorvall GSA rotor
10. Vacuum Aspirator
11. Bent glass rod
12. Bunsen burner

13. .22um filter

Preparation of cells:

1. Prepare Inoue transformation buffer (chilled to 0°C before use). Organic contaminants in the H_2O used to prepare transformation buffers can reduce the efficiency of transformation of competent bacteria. H_2O obtained directly from a well-serviced Milli-Q filtration system usually gives good results. If problems should arise, treat the deionized H_2O with activated charcoal before use.
    a. Prepare 0.5 M PIPES (pH 6.7). Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H_2O to bring the final volume to 100 ml. Sterilize the solution by filtration through a disposable pre-rinsed Nalgene filter. Divide into aliquots and store frozen at -20 °C
    b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H2O.
    c. Sterilize Inoue transformation buffer by filtration through a pre-rinsed 0.45-mm Nalgene filter. Divide into aliquots and store at -20 °C.
2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 h at 37°C. Transfer the colony into 10 ml of SOB medium in a 250 ml flask. Incubate the culture for 6-8 hours at 37 °C with vigorous shaking (250-300 rpm).
3. At about 6 o'clock in the evening, measure the OD of the starting culture. Then use it to inoculate three 250 mL flasks, each containing 100 ml of SOB media. The first flask receives 4 ml of starter culture, the second receives 1.6 ml, and the third receives 0.8 ml. Incubate all three flasks overnight at 20 °C with moderate shaking.
4. The following morning, read the OD600 of all three cultures. Continue to monitor the OD every 45 min.
5. When the OD600 of one of the cultures reaches 0.55, transfer the culture vessel to an ice-water bath for 10 min. Discard the two other cultures.
6. The ambient temperature of most laboratories rises during the day and falls during the night. The number of degrees and the timing of the drop from peak to trough varies depending on the time of year, the number of people working in the laboratory at night, and so on. Because of this variability, it is difficult to predict the rate at which cultures will grow on any given night. Using three different inocula increases the chances that one of the cultures will be at the correct density after an overnight incubation.
7. Harvest the cells by centrifugation at 2,500 x g (3,900 rpm in a Sorvall GSA rotor) for 10 min at 4 °C.
8. **[Do this step in the Biosafety cabinet]** Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 min. Use a vacuum aspirator to remove

any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck.

9. Resuspend the cells gently in 80 ml of ice-cold Inoue transformation buffer. The cells are best suspended by swirling rather than pipetting or vortexing.
10. Harvest the cells by centrifugation at 2,500 x g (3,900 rpm in a Sorvall GSA rotor) for 10 min at 4 °C.
11. **[Biosafety cabinet]** Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 min.
12. Use a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube or trapped in its neck.

Freezing of competent cells (skip if necessary):

1. Resuspend the cells gently in 20 ml of ice-cold Inoue transformation buffer.
2. Add 1.5 ml of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 min.
3. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes.
4. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the tubes at -70 °C until needed. Freezing in liquid nitrogen enhances transformation efficiency by ~5-fold. For most cloning purposes, 50 ml aliquots of the competent-cell suspension will be more than adequate. However, when large numbers of transformed colonies are required (e.g., when constructing cDNA libraries), larger aliquots may be necessary.
5. When needed, remove a tube of competent cells from the -70 °C freezer. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice bath. Store the cells on ice for 10 min. 6. Use a chilled, sterile pipette tip to transfer the competent cells to chilled, sterile 17 x 100-mm polypropylene tubes. Store the cells on ice. Glass tubes should not be used since they lower the efficiency of transformation by ~10-fold.

Transformation:

1. Include all of the appropriate positive and negative controls.
2. Add the transforming DNA (up to 25 ng per 50 ml of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 min.

3. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. Do not shake the tubes. Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
5. Add 800 ml of SOC medium to each tube. Warm the cultures to 37 °C in a water bath, and then transfer the tubes to a shaking incubator set at 37 °C. Incubate the cultures for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.
6. Transfer the appropriate volume (up to 200 ml per 90 mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and Kanamycin. When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 sec at room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml of SOB medium by tapping the sides of the tube. **IMPORTANT** Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to RT, spread the transformed cells gently over the surface of the agar plate. When selecting for resistance to Kanamycin, transformed cells should be plated at low density (<104 colonies per 90 mm plate), and the plates should not be incubated for more than 20 h at 37°C. The enzyme b-lactamase is secreted into the medium from kanamycin-resistant transformants and can rapidly inactivate the antibiotic in regions surrounding the colonies. Thus, plating cells at high density or incubating them for long periods of time results in the appearance of kanamycin-sensitive satellite colonies. This problem is ameliorated, but not completely eliminated, by using carbenicillin rather than kanamycin in selective media and increasing the concentration of antibiotic from 60 mg/ml to 100 mg/ml. The number of kanamycin-resistant colonies does not increase in linear proportion to the number of cells applied to the plate, perhaps because of growth-inhibiting substances released from the cells killed by the antibiotic.
7. Store the plates at RT until the liquid has been absorbed (about 5 minutes).
8. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12-16h.

After successfully transforming *E. coli*, the aforementioned protocol was adapted for *K. xylinus*. The protocol was first minimally changed by scaling down the amounts used, and making modifications for the appropriate culturing and growth of *K. xylinus*. The media used was changed from LB to HS, which is the media in which *K. xylinus* is commonly grown. The

incubation temperatures were changed from 37°C to 30°C, which is the incubation temperature used for *K. xylinus* at the Young Lab (since cells were observed to grow better at a lower temperature). The inoculation and growth times were also modified to adjust for the slower growth of *K. xylinus* as compared to *E. coli*.

After trying the minimally modified transformation protocol, further modifications were made in an effort to successfully transform *K. xylinus*. Four iterations of the adapted protocol were created with the following variables changed respectively: heat shock temperature, heat shock length, post-heat shock recovery time and growth media.

Temperature gradient: The *E. coli* protocol heat shock temperature was 42°C. Compared to *E. coli*, which incubates at a temperature of 37°C, *K. xylinus* grows best under the relatively milder incubation conditions of 30°C. Because of this temperature variation in growing conditions, we hypothesized that *K. xylinus* is more sensitive to higher temperatures. Therefore, for the first iteration of a modified transformation protocol, a temperature gradient from 30°C to 42°C was used for the heat shock of *K. xylinus.* Two sets of eight samples of competent *K. xylinus* cells and transforming DNA in PCR tubes were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at temperatures of 30.2°C, 30.8°C, 32.3°C, 34.5°C, 37.5°C, 39.8°C, 41.3°C and 41.9°C for 30 seconds. After a 45 minute recovery period, the first set of eight heat shocked samples were plated on plates with HS only (control), the second set was plated on plates with HS and kanamycin (for selection of transformed colonies). The plates were incubated at 30°C for three to four days. Optimal heat shock temperature was chosen as the highest temperature at which cells were viable on the plates, as this suggests that the temperature is sufficiently high enough to transmit the DNA across the membrane without permanently denaturing the cell membrane.

Heat shock time: After determining an acceptable heat shock temperature, heat shock time was studied. The *E. coli* protocol heat shocks the cells for 30 seconds. For this study, three sets of two *K. xylinus* cells and transforming DNA samples in PCR tubes were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at 42°C for 15, 30 and 60 seconds respectively. After a 45 minute recovery period, a sample from each heat-shock set was plated on either HS only plates or HS and kanamycin plates. The plates were incubated at 30°C for three to four days and then examined for growth.

Recovery time: After determining acceptable heat shock temperatures and times, post-heat shock recovery time was varied. Because it takes a few hours for *K. xylinus* to express the kanamycin resistance gene, it was hypothesized that short recovery periods prevented detectability of this transformation indicator on the plates, and killing the cells before they were able to become antibiotic resistant. Therefore, three recovery periods were studied: 45 minutes, 4 hours and 24 hours. Three sets of two *K. xylinus* cells and transforming DNA samples in PCR tubes were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at 42°C for 30 seconds. After a 45 minute recovery period, the first set of two heat shocked samples were plated on a plate with HS only and on plate with HS and kanamycin respectively. Likewise, after 4

hours and 24 hours, the remaining sets of samples were plated on the HS only and HS and kanamycin plates.

Growth media: The final modification to the transformation protocol was modifying the *K. xylinus* growth media by adding Mg^{2+} cations. According to an *E. coli* transformation study by Hanahan, "addition of Mg^{2+} cations to the LB medium during growth [...] gave 60% more transformants" [21]. This is thought to occur because the cations neutralize the negative ions on the cell membrane, which normally repulse the negatively charged DNA [21]. HS media with 20 mM of Mg^{2+} was used for the initial growth of *K. xylinus.* The transformation protocol adapted for *K. xylinus* was then carried out using a heat shock temperature of 42°C for 30 seconds, with a recovery time of 45 minutes.

Salmonella typhimurium Transformation Protocol

In addition to the *E. coli* transformation protocol and testing out different modifications, we also tried using a transformation protocol used for *Salmonella typhimurium* LT2 [15]. The transformation protocol for this bacteria was selected because salmonella shares multiple characteristics with *K. xylinus*. Salmonella is gram-negative and produces cellulose and acetic acid during fermentation, which are also all properties of *K. xylinus*. There are a few other bacteria that also share these properties, but Salmonella had more research done on successful chemical transformations. The same method for the preparation of cells was used as the *E. coli* transformation protocol, only the transformation steps were adjusted.

Procedure:

1. Grow 10 mL *K. xylinus* at 30°C in HS media with shaking to an OD of 0.55.
2. Centrifuge cells at 2500 rpm for 10 min at 4°C and resuspend them in 0.4 mL cold (4°C) 0.1 M $CaCl_2$.
3. Dispense 0.2 mL of competent cells and mix with plasmid DNA (10-100 ng).
4. Chill the mixture for 20 min at 4°C.
5. Heat-shock the mixture of cells and DNA for 30 seconds in a thermocycler set at 42°C.
6. Add 0.8 mL HS media and incubate the mixture for 30 min.
7. Plate 0.1 mL cells on L-agar plates containing HS+Kanamycin.
8. Incubate at 30°C overnight and monitor for cell growth.

Section II: Metabolic Modeling of *K. xylinus*

Due to constraints resulting from the COVID-19 global pandemic, we were not able to perform the experiments relating to this section of the project. However, we did develop an experimental plan to get the data we needed for this project goal.

To develop a black-box metabolic model, we needed to grow and ferment the cells for a period of time, and then measure the concentrations of the main substrates and products related to cellulose production. The variable we were studying was the carbon source, i.e. the type of sugar being fed to the cells. The data outputs we were hoping to collect were (1) the cellulose mass produced after the fermentation period, (2) the substrate concentration over time, and (3) the by-products concentration over time. The by-products of cellulose production in *K. xylinus* are ethanol and acetate, given that it is an acetic acid bacteria. The cellulose was to be measured by weighing at the end of the experiment. However, for the substrates and by-products that are normally dissolved in the media, we needed to measure in another way. Enzymatic kits were going to be used for this purpose. The sugars that we were going to use as our varying carbon sources are: glucose, xylose, L-arabitol, and D-mannitol. Glucose and xylose were selected because they are inexpensive and commonly-used sugars for cell culturing and fermentation. L-arabitol and D-mannitol, on the other hand, were selected because during other studies from project collaborators, they were shown to yield in cellulose with interesting morphology and translucency.

Before performing the fermentation experiment, it was necessary to create standard curves for the different enzymatic kits. To do this, we needed to create solutions of the different sugars, ethanol, and acetate at a range of concentrations, and then run the corresponding enzymatic assay. Then, the absorbances for each of the concentrations were to be measured, and a plot of concentration vs absorbance would be constructed to use as reference in the subsequent experiments where the concentrations of the sugars and by-products were unknown.

For the fermentation experiment, we were to set-up a large cell culture in HS media with no antibiotics and no cellulase (since we wanted to measure the cellulose produced). One large flask was set up for each of the carbon sources studied. From those stock cultures, about 25% of the volume was to be used as samples for the different enzymatic kits. The plan was to take three samples for each of the components to be measured (sugar, ethanol, and acetic acid) at each timepoint, distributed at time 0h, 24h, 48h, 96h, and so on. The samples were to be frozen immediately after taking them from the stock culture, and when all samples were obtained, the corresponding enzymatic assays were to be performed. The data obtained would allow us to plot a graph of concentration over time for the sugar, ethanol, and acetic acid. Moreover, we would be able to calculate the yield of cellulose for each of the sugars tested.

RESULTS

Section I: Transformation of *K. xylinus*

Antibiotic Resistance Experiment

Before conducting any transformation experiments, it was necessary to make sure that the antibiotic chosen for selection, kanamycin, was effective in killing wild-type *K. xylinus*. Moreover, we wanted to test the effectiveness of other antibiotics in killing the bacteria of interest, so we included spectinomycin, nourseothricin, chloramphenicol, hygromycin B, and ampicillin to the experiment. The cells were placed in a 96-well plate containing the six antibiotics at four different concentrations each: 0, 50, 100, and 200 mg/mL. The cells were then grown with shaking for 7 days, with optical density measured at time 0 and at the end of the experiment. There were three replicates for each of the data points, which allowed us to create a bar graph showing the results.

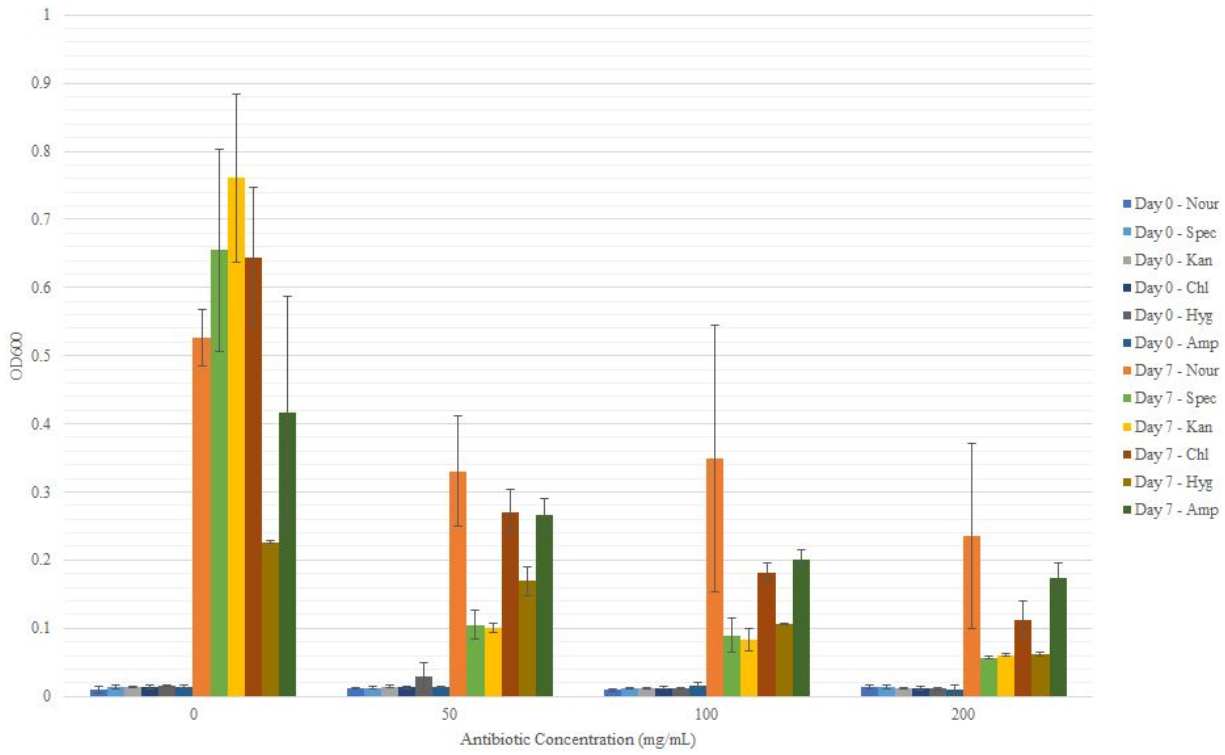

Figure 2: OD600 measurement for *K. xylinus* cultures in 96-well plate at time zero and after 7 days. Cells were exposed to six different antibiotics: Kanamycin, Spectinomycin, Nourseothricin, Chloramphenicol, Hygromycin B, and Ampicillin. Error bars represent standard deviation calculated for each sample.

We can see that, as expected, kanamycin was one of the most effective antibiotics, reducing the growth of *K. xylinus* significantly as compared to the untreated sample (on the left),

even at the lowest concentration tested, of 50 mg/mL. Spectinomycin also proved to be as effective as kanamycin in stopping *K. xylinus* growth. Nourseothricin was the least effective in halting or slowing growth, so we would not recommend working with that antibiotic, since very high concentrations need to be used to get an effect on cell growth. Chloramphenicol and ampicillin were relatively effective in halting *K. xylinus* growth, but were not as effective as kanamycin and spectinomycin. Finally, the hygromycin B set of samples showed no significant growth in all of the cases (including the no antibiotic one), which could indicate some problem with the experiment setup. Sometimes the plate has some sections that are more prone to condensation, which can make the results less accurate. Overall, based on these results we would recommend that, when working with *K. xylinus*, the antibiotics of choice for selection should be kanamycin or spectinomycin.

Heat Shock Transformations

The first goal of this project was to develop a high-throughput transformation method for *K. xylinus*. Given that the currently used transformation protocol (electroporation) was not suitable for scale-up, we decided to try to adapt a chemical transformation, also known as heat shock, protocol for use in *K. xylinus*. The first step followed was to make small adaptations to an *E. coli* heat shock protocol [14], to accomodate for the timing and conditions that were optimum for *K. xylinus*. This was the starting point for our experiments, and we also performed a control transformation using the original protocol on *E. coli*. Figure 3 below shows the successfully transformed *E. coli* cells in an agar plate, with the incorporated plasmid evidenced both by the antibiotic resistance and the red color of the colonies provided by the RFP gene.

Figure 3: transformed *E. coli* colonies growing in antibiotic (Kanamycin) selection LB agar plate.

After successfully transforming *E. coli* using heat shock, the adapted protocol for *K. xylinus* was tested, without any success. Therefore, we decided to try different variations of the experiment to find the appropriate conditions for the chemical transformation of *K. xylinus*. Through attempting multiple alterations of the transformation process, data was collected to direct changes to the protocol that would help produce a successful transformations method in the future. The main variations tried were: temperature gradient, heat shock time, growth media, and cells recovery time.

Temperature gradient: The temperature gradient experiment used two sets of eight samples of competent *K. xylinus* cells that were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at temperatures of 30.2°C, 30.8°C, 32.3°C, 34.5°C, 37.5°C, 39.8°C, 41.3°C and 41.9°C. Following the incubation period, none of the cells appeared to be damaged or killed. This meant that none of the temperatures used were so high that they killed or denatured the cells. Thus, optimal heat shock temperature was chosen to be 42°C. This was the highest temperature at which cells were viable on the plates, suggesting that the temperature is sufficiently high enough to transmit the DNA across the membrane without permanently denaturing the cell membrane. We plated the cells in non-selective plates as well, to make sure the procedure was not killing them, as shown in Figure 4 below.

Figure 4: *K. xylinus* colonies after temperature gradient transformations on control HS-only plates.

Heat shock time: After determining an acceptable heat shock temperature, heat shock time was studied. For this study, three sets of two *K. xylinus* cells and transforming DNA samples in PCR tubes were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at 42°C for 15, 30 and 60 seconds respectively. Because none of the heat shock times gave successful transformations, there was not one correct heat shock time to pick. None of the heat shock times killed the cells either so a heat shock time was determined based off of other factors. Because 30 seconds is the standard heat shock time used for *E. coli*, 15 seconds was determined to be too short of a heat shock time for *K. xylinus*. It was determined that 30 seconds would be sufficient heat shock time as long as it did not kill the cells.

Growth media: One other modification to the transformation protocol was modifying the *K. xylinus* growth media by adding Mg^{2+} cations. HS media with 20 mM of Mg^{2+} was used for the initial growth of *K. xylinus.* The transformation protocol adapted for *K. xylinus* was then carried out using a heat shock temperature of 42°C for 30 seconds, with a recovery time of 45 minutes. This modification did not result in a successful transformation, leading us to next explore recovery time.

Recovery time: After determining acceptable heat shock temperatures and times, post-heat shock recovery time was varied. Given that *K. xylinus* has a longer doubling time than *E. coli*, we hypothesized that prolonging the recovery time would give transformed *K. xylinus* cells more time to express the Kanamycin resistance gene that they need to survive in the selection plates. Three recovery periods were studied: 45 minutes, 4 hours and 24 hours. Three sets of two *K. xylinus* cells and transforming DNA samples in PCR tubes were heat shocked using the Eppendorf MasterCycler Pro Thermal Cycler at 42°C for 30 seconds. Unfortunately, these experiments were not completed as they were in the process of being tested when campus was shut down due to COVID-19.

In addition to making adaptations to an *E. coli* transformation protocol, another protocol was adapted for *K. xylinus* from a transformation protocol for *Salmonella typhimurium [15]*. One attempt to transform *K. xylinus* using the *Salmonella typhimurium* adapted protocol was performed. The first attempt did not produce a successful transformation, but the cells plated without antibiotics were not killed. More transformations were planned using the Salmonella method again, paired with the results from the recovery time experiment, but neither were able to be completed due to the COVID-19 shut down of campus. The below picture shows the results of the HS selection plate with no transformed *K. xylinus* colonies on the left, and the HS-only plate with colonies on the right (to show that the protocol was not lethal to the bacteria).

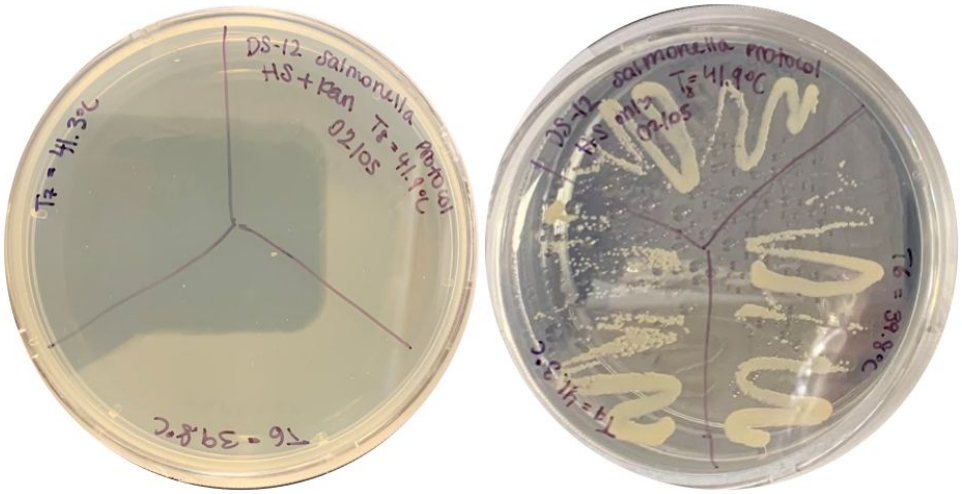

Figure 5: Results from the adapted Salmonella transformation protocol, HS selective plate on the left, and HS-only plate on the right (with *K. xylinus* colonies present).

Section II: Metabolic Modeling of *K. xylinus*

The goal of this part of the project was to create an experimentally-based black-box metabolic model for the production of cellulose in *K. xylinus*. Unfortunately, due to the constraints imposed by the COVID-19 global crisis, we were unable to perform the necessary experiments to complete this part of the project. However, the plan was to ferment *K. xylinus* in HS media for 7-8 days, with different carbon sources: glucose, xylose, L-arabitol, and D-mannitol. The data collected would be the mass of cellulose at the end of the fermentation period, as well as concentrations of the substrate (carbon source) and by-products (ethanol and acetate) throughout the fermentation period. Even though we were not able to perform said experiments, we constructed some sample plots to show the expected results for this part of the project. All the plots and correlations use sample data and are only for illustration.

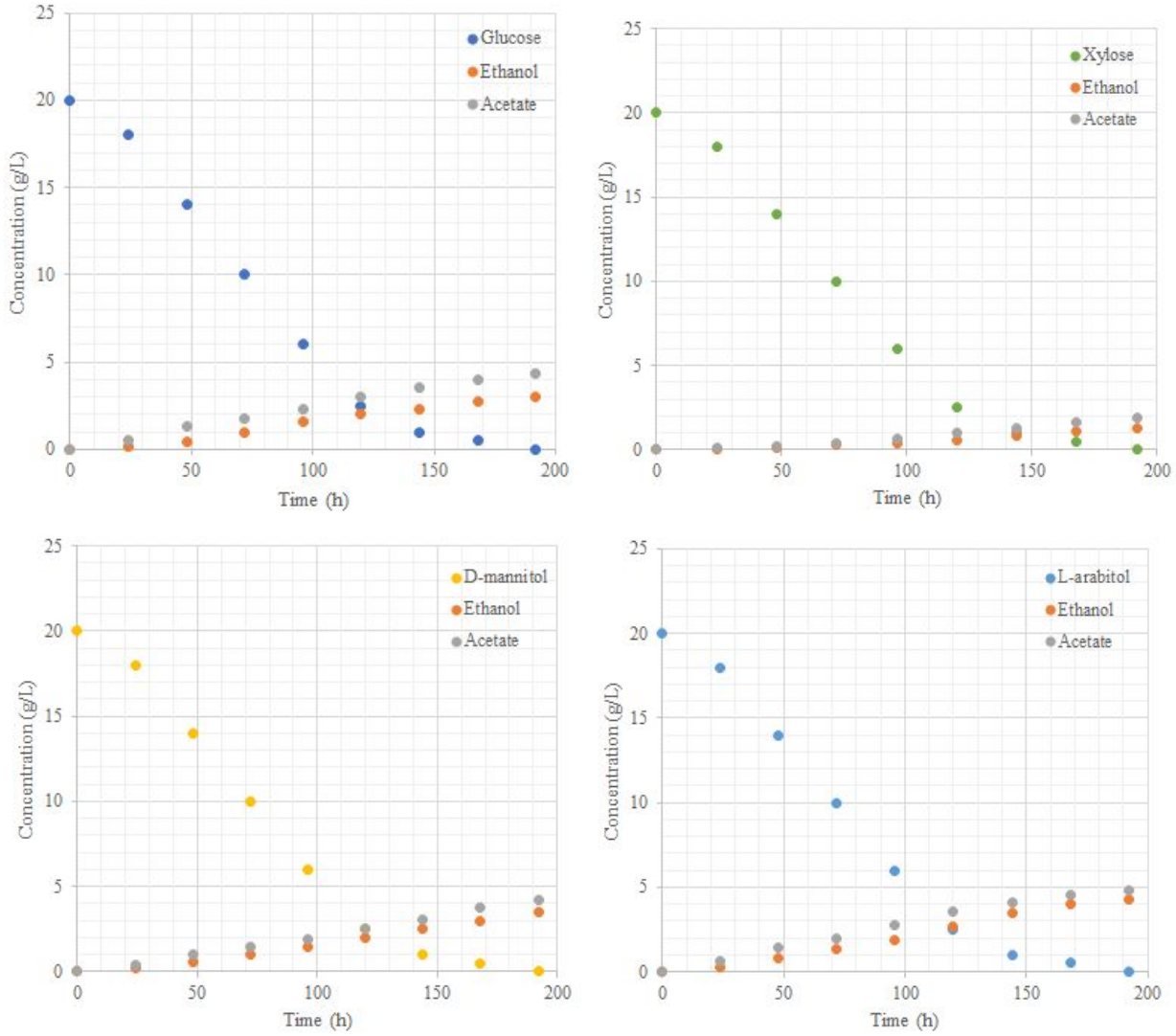

Figure 6: Plots showing sample raw data from enzymatic kits measuring the concentration of the carbon sources (glucose, xylose, L-arabitol, and D-mannitol) and by-products (ethanol and acetate) in the fermentation of *K. xylinus* in HS media. All plots contain sample data for demonstration purposes only.

As mentioned previously, the first set of data expected for the metabolic modeling experiments was the enzymatic kit measurements of the different substrates and products measured. Figure 6 displays the four plots that we would have been able to build with these data. There would be one plot for each of the sugars tested, with ethanol and acetate being measured in all of the fermentation variations. The carbon source was expected to be depleted over time, while the by-products were expected to increase slightly over time. We also expected to observe the differences in using varied carbon sources, for instance different by-product concentrations at the end of the fermentation.

Next, the dry weight of cellulose after 7-8 days of fermentation would have been measured for each of the carbon sources tested. This information, along with the final

concentrations of the carbon sources and by-products, would have let us calculate the yield of cellulose for each of the carbon sources. Figure 7 below shows a sample plot of the final results for cellulose yield would have looked like. This would have allowed us to compare the different carbon sources and determine which one results in the most efficient cellulose production.

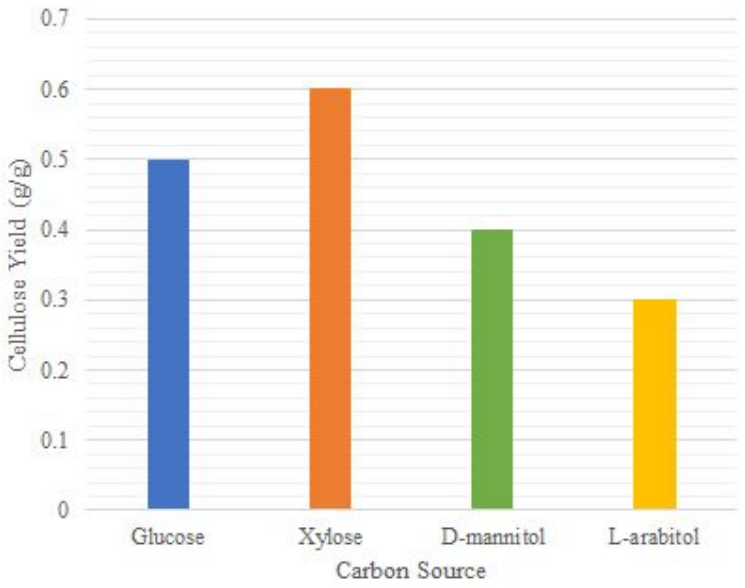

Figure 7: Cellulose yield (grams of cellulose produced per grams of carbon source consumed) in *K. xylinus* with different carbon sources for fermentation. This plot contains sample data for demonstration purposes only.

It is also important to note that the carbon source with the highest yield might not always be the best choice for *K. xylinus* fermentation. For example, observations from other project collaborators showed that feeding the cells with mannitol or arabitol instead of other more common sugars like glucose and xylose might have some benefits. The cellulose produced using these less common carbon sources had a particular transparency that could not be achieved when using more simple sugars. Therefore, depending on the purpose and use of the cellulose tissue needed, different considerations would have to be made about what carbon source to choose.

DISCUSSION

Overall, this project sought to increase our understanding of the cellulose-producing bacteria, *K. xylinus.* Our two tangible benchmarks for meeting this overarching and abstract goal were developing a high-throughput transformation protocol and a black-box metabolic model for *K. xylinus.* While neither of these goals were successfully achieved, our study and results contributed to a better understanding of this bacteria of interest.

Section I: Transformation of *K. xylinus*

While none of the attempted high-throughput transformation methods were successful, our results indicate that the chemical transformation of *K. xylinus* might be possible. Based on all of the information gathered and all of the studies we had planned to pursue, we hypothesize that the chemical transformation of *K. xylinus* was limited by two main factors. These two factors being information on the growth of *K. xylinus* and the understanding of the bacteria's membrane chemistry.

A plausible reason for the unsuccessful chemical transformation is that we transformed during suboptimal growth phases. Because we never defined growth curves or experimented with optimal starting ODs, we have no information regarding which growth phase the bacteria was in during each transformation. Furthermore, based on anecdotal accounts during experimentation, *K. xylinus* appeared to have inconsistent growth rates when inoculated in flasks. Under the same conditions, inoculated flasks would have highly-variable ODs. Before lab access froze due to the COVID-19 outbreak, we had planned on studying *K. xylinus* growth curves and optimal starting ODs for transformation. With well-defined growth curves and starting ODs, we hypothesize that our tested methods for transforming *K. xylinus* could have been successful.

The second hypothesis regarding the shortcomings of the chemical transformation is the lack of knowledge about *K. xylinus'* cell wall and membrane. Our results show that *K. xylinus* was not killed or severely damaged by any of the transformation methods attempted. This indicates that the bacteria's membrane was never breached. Based on these results compared to those of comparable bacteria like *E. coli* and *S. typhimurium*, we hypothesize that the *K. xylinus* membrane is either fortified by cellulose or composed in such a way that the heat shock formulations we tested were not sufficient to open the membrane. Again, before the COVID-19 outbreak, we had planned on running a 96-well plate transformation experiment to vary the salt concentrations in the buffer used. We planned on varying the $CaCl_2$ concentrations from less than 50mM to 100mM, in addition to testing different combinations of salts in each well. Based on literature, the chosen salt compositions for chemical transformation seem arbitrary. A more in-depth and systematic analysis of the concentration and characteristics of different salts in heat shock protocols is suggested for future studies.

Section II: Metabolic Modeling of *K. xylinus*

Even though we did not get the opportunity to perform the metabolic modeling experiments due to the COVID-19 outbreak, the results obtained would have aided in characterizing *K. xylinus* and determining which sugars result in the highest cellulose yield. The model can give a useful outline of the carbon flux through the system. This information is important for the industrial applications of *K. xylinus* because a generic scope of sugar feeds and by-products can guide the design of industrial-scale processes for the production of bacterial cellulose.

Although the overall carbon-flux information may be useful, a scale-up of *K. xylinus* cellulose production could magnify the minute details neglected by black black-box metabolic modelling. A black box metabolic model relies on simplified assumptions, such as all sugar consumed goes either to cellulose, ethanol, or acetate. In actuality this over-simplified model is often not the case and several by-products and internal reaction mechanisms are not taken into account. In industrial scale-up, the microscopic presence of side-products can be magnified by the order of 10^5 or greater. Therefore, it is important to supplement the black-box metabolic with more in-depth models of the *K. xylinus* metabolism.

Moving forward, these results could also be compared to a genome-scale metabolic model. This is a more complicated method, since it involves using a comparative genomics software, such as KBase (a systems biology-based platform), to analyze the genetic information of the cells to build a metabolic model like the one built experimentally [22]. Comparing both the genome-scale and black box metabolic models, can greatly improve our understanding of *K. xylinus* metabolism.

Final Conclusions

While this project did not meet its intended goals, it did act as a necessary stepping stone for advancing our understanding of the bacteria of interest. With these results, there is a much clearer direction for "next steps." For the chemical transformation of *K. xylinus*, we suggest defining growth curves and starting ODs, as well as varying the salt concentrations and composition for the heat shock protocols. For the metabolic model, we suggest developing a black-box metabolic model as described in our methodology as well as a genome-scale metabolic model using comparative genomics software. The groundwork, such as transformation and characterization, must be laid in order to perform higher level studies with *K. xylinus*.

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