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Climate.UAR Final Report

Low-Cost, Sustainable, and Rapid Detection of *E. coli* in Drinking Water

The UN estimates that at least 1.8 billion people globally are at risk of diarrhea-causing disease from water contamination[1], a leading cause of death especially for young children living in poverty.[2] Additionally, water supply contamination is a common problem faced by many farmers that can lead to loss of crops, a particularly serious issue for lower-income farms.[3] Water quality testing is a sustainability and climate issue as well as a public health crisis, and is central to one of the UN Sustainable Development Goals.[4] As the impacts of climate change worsen, higher temperatures will exacerbate pathogen contamination in water and extreme weather events that lead to flooding or other infrastructure damage will increase contamination risk, in both residential and agricultural areas of communities.[5]

E. coli is considered a good indicator of microbiological contamination in water supplies and is therefore often used as a metric of water quality to help ensure access to clean water. However, current tests are expensive, difficult to use, and unsustainable to manufacture, reducing the extent to which these technologies can be used in low-income areas affected by water contamination. Additionally, most low cost tests take one to two days of incubation time to yield results, which is too late for immediate intervention. Furthermore, the ideal test design should not just be affordable to use, but be sustainable to manufacture and dispose of as well.

The project is primarily based on previous research to develop and improve the design of the ECC Vial[6], an existing *E. coli* detection test that has been manufactured and used in Nepal. This research was conducted with input from members of two Nepali organizations, ENPHO[7] and EcoConcern[8], who are involved in work around the ECC Vial and other water quality and sanitation projects. Additionally, the research at MIT was supported by guidance and resources from Professor Benedetto Marelli and other members of the Laboratory for Advanced Biopolymers, Professor Susan Murcott at the MIT D-Lab, and the MIT Climate and Sustainability Consortium Climate Scholars Program.

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[1] "UNICEF Target Product Profile Rapid E. Coli Detection Tests." UNICEF, December 2019. https://www.unicef.org/innovation/sites/unicef.org.innovation/files/2020-10/Rapid-coli-detection-TPP-2019.pdf.
[2] "Water Quality Testing and Decontamination." Accessed May 10, 2024. https://d-lab.mit.edu/academics/student-showcases/d-lab-fall-2021-student-showcase/water-quality-testing-and.
[3] MIT SOLVE. "IRRIGaTE." Accessed May 10, 2024. https://solve.mit.edu/challenges/indigenous-communities-2022/solutions/62920/application.
[4] "Goal 6 | Department of Economic and Social Affairs." Accessed May 10, 2024. https://sdgs.un.org/goals/goal6.
[5] "Water – at the Center of the Climate Crisis." United Nations. Accessed May 10, 2024. https://www.un.org/en/climatechange/science/climate-issues/water.
[6] "Low Cost Water Testing and Disinfection | Abdul Latif Jameel Water and Food Systems Lab (J-WAFS)." Accessed May 10, 2024. https://jwafs.mit.edu/projects/2020/low-cost-water-testing-and-disinfection.
[7] "Enpho – Environment and Public Health Organisation." Accessed May 10, 2024. https://enpho.org/.
[8] "Ecoconcern." Accessed May 10, 2024. https://www.ecoconcern.com.np/.

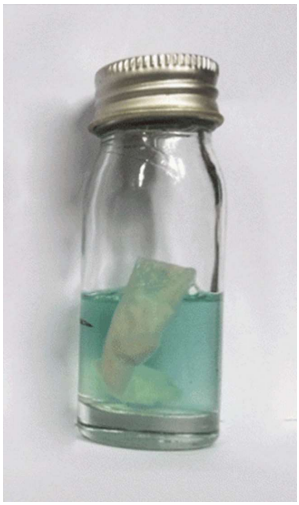

Figure 1. ECC Vial test, showing positive test result.

Objectives

This research project is simply a piece of an ongoing effort, and as such, the goal of this report is to share the work I have done while bringing future researchers up to speed, noting successes and challenges I faced in this work, and reflecting on potential future directions and impact this work could have in a lab, commercial, or startup environment.

This project was designed to advance progress towards an *E. coli* water test that is sustainable, affordable, fast, and easy to use. New elements of test design were built on principles from other existing bio-based *E. coli* tests, primarily the ECC vial, as well as utilizing the UNICEF Target Product Profile for Rapid *E. coli* tests[1]. Current development is focusing on identifying Beta Glucuronidase (β-Glu), an enzyme that is widely accepted as a relatively specific indicator of the bacteria.[9] Many tests to identify β-Glu use 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide, a substrate also commonly known as X-Gluc, which changes from colorless to blue after being cleaved by the enzyme. However, most tests sensitive enough to detect pathogenic concentrations of bacteria involve culturing water samples in media containing X-Gluc. This technique requires anywhere from 8 to 48 hours of incubation at 37C, the ideal growth temperature of *E. coli*[10]. The incubation time and temperature is a severe burden due to the long delay and the need for electricity, which might mean that remote communities must transport samples to the laboratories of organizations testing water samples. My research aimed to increase the accessibility of these water quality tests by reducing the need for high incubation temperatures and decreasing required the incubation time.

In addition to testing methods to increase the speed of the test, formulations of a paper-based, silk protein-protected assay were explored as an alternative to existing formats.

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[9] Frampton, Elon W., Lawrence Restaino, and Nancy Blaszko. "Evaluation of the β-Glucuronidase Substrate 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide (X-GLUC) in a 24-Hour Direct Plating Method for Escherichia Coli." Journal of Food Protection 51, no. 5 (May 1, 1988): 402–4. https://doi.org/10.4315/0362-028X-51.5.402.

[10] Gunda, Naga Siva Kumar, Saumyadeb Dasgupta, and Sushanta K. Mitra. "DipTest: A Litmus Test for E. Coli Detection in Water." PLOS ONE 12, no. 9 (September 6, 2017): e0183234. https://doi.org/10.1371/journal.pone.0183234.

While the ECC Vial uses a glass vial, a paper-based test that similarly changes color upon detection of *E. coli* holds the potential advantages of using readily available materials, being biodegradable, and offering a clearer visual marker of a positive test. Additionally, some previous researchers noted that the ECC Vial potentially had an insufficient shelf-life, experiencing discoloration and producing false positives after just 3-6 months in storage. Conversations with ENPHO, the partner organization, resulted in initial recommendations to protect the vial from light, as X-Gluc is a photosensitive substrate. Beyond this, the biodegradable protein silk fibroin, which can be extracted at low cost from waste silk cocoons, was investigated as a medium to protect X-Gluc in the test formulation.

Background and Approach

According to the UNICEF Target Product Profile for rapid *E. coli* detection tests, acceptable designs must detect as little as 10 colony forming units (CFU) per 100 mL of water sampled. Additionally, an acceptable product would deliver test results in under 6 hours, operate under a range of temperature and humidity conditions, and minimize consumables and excess packaging. This project aimed to primarily improve the time required to detect the bacterial threshold, with a secondary focus on temperature and consumable requirements.

Previous lab research to develop the ECC Vial, in Professor Murcott's group in the MIT D-Lab, had considered some alternatives to X-Gluc during the development of the ECC vial. One set of substrates, called RUG and REG, showed promise in the literature[11]. However, this substance has since been determined to be extremely toxic and thus has been avoided for use.

To continue this work and determine if new options had been discovered since this project began 6 years ago, initial literature review was conducted to better understand existing methods of bacterial detection, particularly for E. coli. We began by looking into different detection and visual reporting methods for specific molecules, as we wanted to see if other methods of detecting bacteria could be more effective than the X-Gluc used in the ECC Vial. Additionally, we looked specifically into lateral-flow assays as a model comparable to the now popular at-home COVID tests. This literature search included looking at alternative technologies such as magnetic bead technology[12], zwitterionic peptides in gold nanoparticle suspensions[13], and antibody conjugations to gold nanoparticles[14], potentially with quantum dots. Some synthetic

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[11] Magro, Germinal, Robert E. S. Bain, Claire A. Woodall, Robert L. Matthews, Stephen W. Gundry, and Anthony P. Davis. "Synthesis and Application of Resorufin β- d -Glucuronide, a Low-Cost Chromogenic Substrate for Detecting Escherichia Coli in Drinking Water." Environmental Science & Technology 48, no. 16 (August 19, 2014): 9624–31. https://doi.org/10.1021/es502319n.

[12] MIT News | Massachusetts Institute of Technology. "Tiny Magnetic Beads Produce an Optical Signal That Could Be Used to Quickly Detect Pathogens," August 25, 2023. https://news.mit.edu/2023/tiny-magnetic-beads-quickly-detect-pathogens-0825.

[13] Jin, Zhicheng, Yash Mantri, Maurice Retout, Yong Cheng, Jiajing Zhou, Alec Jorns, Pavla Fajtova, et al. "A Charge-Switchable Zwitterionic Peptide for Rapid Detection of SARS-CoV-2 Main Protease." Angewandte Chemie International Edition 61, no. 9 (February 21, 2022): e202112995. https://doi.org/10.1002/anie.202112995.

[14] Deng, Jinqi, Mingzhu Yang, Jing Wu, Wei Zhang, and Xingyu Jiang. "A Self-Contained Chemiluminescent Lateral Flow Assay for Point-of-Care Testing." Analytical Chemistry 90, no. 15 (August 7, 2018): 9132–37. https://doi.org/10.1021/acs.analchem.8b01543.

biology approaches were also studied. However, many of these methods required external equipment to store, prepare, or read the test, making them unsuitable for use in remote areas.

Focusing more specifically on low-cost diagnostics, various studies were reviewed that included detection of *E. coli* with simpler designs. We looked at a paper-based point-of-care biosensor[15] and a centrifugal microdevice[16] utilizing nucleic acid detection to identify the bacteria. However, both of these methods had limits of detection 10 to 1000 times higher than the limit of detection required for drinking water in addition to the extra reagents necessary for nucleic acid amplification.

In addition, we found a couple of promising papers showing very simple designs, although the limits of detection varied from meeting international standards to being orders of magnitude higher. One paper described a paper chromatography test formulated with X-Gluc and another colorimetric reagent, Red-Gal, utilized for identifying coliforms[17]. Another paper test, dubbed the DipTest, utilized Red-Gal as its substrate as well[18]. The DipTest also included a bacterial protein extraction reagent to lyse *E. coli* cells and release the enzyme that specifically cleaves Red-Gal, and successfully showed results in as little as 30 minutes. However, this paper did not show if the test was sensitive enough to meet the product profile. Finally, we looked at a method utilizing filtration of water and addition of an enzyme inducer with colorimetric substrates X-Gluc and REG. The paper showed that for both substrates, as little as 10 CFU/mL could be detected within 6-7 hours[19]. While these time points and sensitivities were higher than the targets for this project, together, these papers demonstrated the possibility for paper-based tests with additional reagents to rapidly and sensitively detect *E. coli* in drinking water.

Focusing on the concept of extracting enzymes from cells to increase reaction speed, initial experiments intended to characterize the reaction of β-Glu with X-Gluc using purified enzymes extracted from *E. coli*, including identifying limits of detection and working within a wider temperature range. Later work then utilized *E. coli* cultured in the laboratory, with pure enzyme included in experiments as a positive control. In addition to visual observation of the color change from clear to blue, absorption spectroscopy was used to quantify the reaction time.

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[15] Choi, Jane Ru, Jie Hu, Ruihua Tang, Yan Gong, Shangsheng Feng, Hui Ren, Ting Wen, et al. "An Integrated Paper-Based Sample-to-Answer Biosensor for Nucleic Acid Testing at the Point of Care." Lab on a Chip 16, no. 3 (February 7, 2016): 611–21. https://doi.org/10.1039/c5lc01388g.

[16] Sayad, Abkar, Fatimah Ibrahim, Shah Mukim Uddin, Jongman Cho, Marc Madou, and Kwai Lin Thong. "A Microdevice for Rapid, Monoplex and Colorimetric Detection of Foodborne Pathogens Using a Centrifugal Microfluidic Platform." Biosensors and Bioelectronics 100 (February 15, 2018): 96–104. https://doi.org/10.1016/j.bios.2017.08.060.

[17] Hossain, S. M. Zakir, Cory Ozimok, Clémence Sicard, Sergio D. Aguirre, M. Monsur Ali, Yingfu Li, and John D. Brennan. "Multiplexed Paper Test Strip for Quantitative Bacterial Detection." Analytical and Bioanalytical Chemistry 403, no. 6 (June 1, 2012): 1567–76. https://doi.org/10.1007/s00216-012-5975-x.

[18] Gunda, Naga Siva Kumar, Saumyadeb Dasgupta, and Sushanta K. Mitra. "DipTest: A Litmus Test for E. Coli Detection in Water." PLOS ONE 12, no. 9 (September 6, 2017): e0183234. https://doi.org/10.1371/journal.pone.0183234.

[19] Wu, Jianyong, Jill R. Stewart, Mark D. Sobsey, Chris Cormency, Michael B. Fisher, and Jamie K. Bartram. "Rapid Detection of Escherichia Coli in Water Using Sample Concentration and Optimized Enzymatic Hydrolysis of Chromogenic Substrates." Current Microbiology 75, no. 7 (July 1, 2018): 827–34. https://doi.org/10.1007/s00284-018-1454-8.

In this project, we also explored the use of biologically-derived materials as a testing medium, particularly the use of silk fibroin as a preservative for the X-Gluc. Paper tests were also a format to be considered due to their minimal use of synthetic materials. Proof-of-concept tests with paper coated in silk fibroin were prototyped and tested with pure enzyme, both for ability to report results and to determine if silk fibroin provided protection from degradation. The prototype tests were also demonstrated to successfully report contact with *E. coli* bacteria.

Methods and Results

Reaction Sensitivity and Time Experiments

Initial experiments focused on utilizing *E. coli* purified β-Glu enzyme[20] as a more direct method of testing the planned formulations and improvements. This method allowed us to provide a quick and easy proof-of-concept and determine the effectiveness of X-Gluc based assays directly, initially removing potential confounding factors from using whole bacteria.

The first experiments utilized liquid-based assays to determine the sensitivity of the X-Gluc to β-Glu enzyme. Correlations between X-Gluc concentration, enzyme concentration, and time required for a visible color change were determined. Spectrophotometric methods were used to measure the absorbance of samples, and visually corroborated to determine what value of absorbance correlated to visible color changes. This information was used as a guide for the concentrations and times used in future experiments, to ensure that a timely reaction could take place with sensitivity to diluted enzyme concentrations.

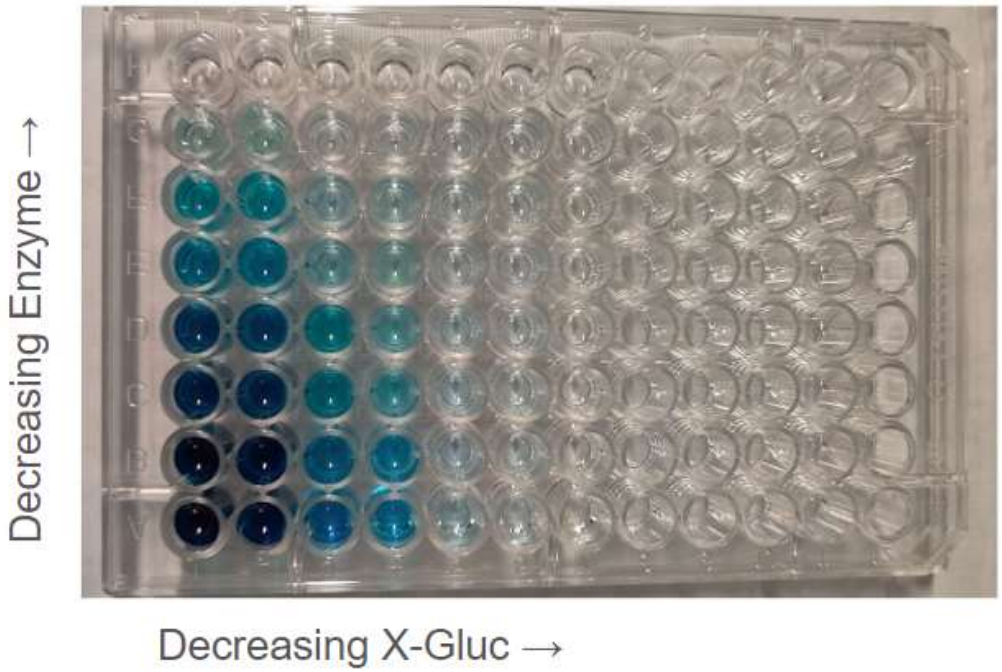

Figure 2. Visible differences in reaction shade as correlated to different concentrations of X-Gluc and β-Glu.

[20] "B-Glucuronidase Type VII-A, Lyophilized Powder, Main 5,000,000-20,000,000units/g Protein 30 Min Assay 6.8 9001-45-0." Accessed May 10, 2024. http://www.sigmaaldrich.com/.

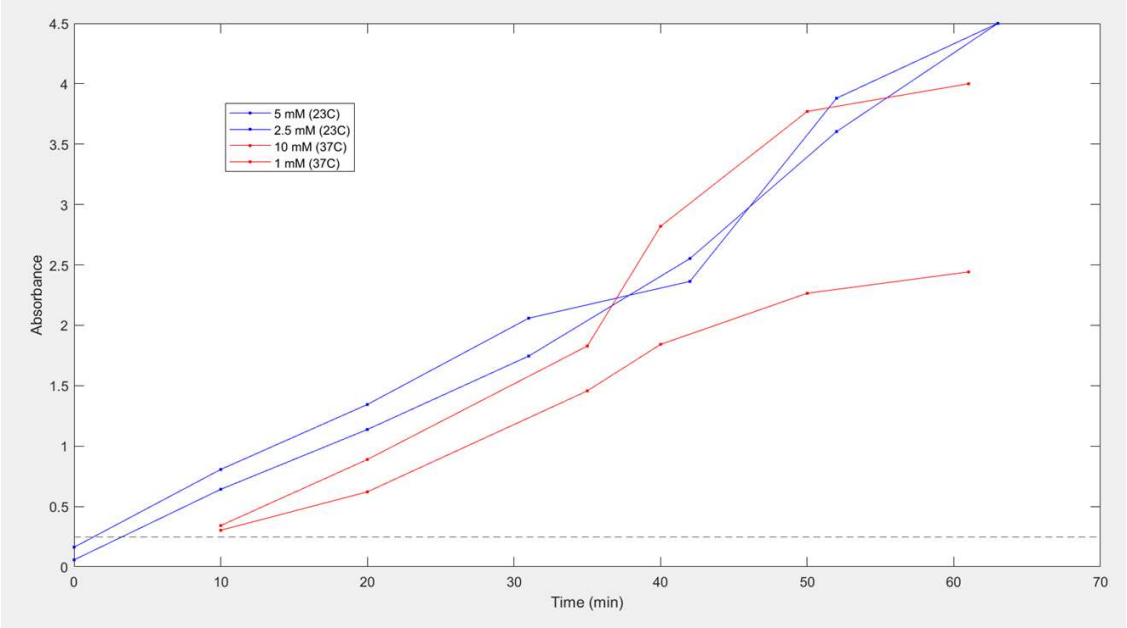

Figure 3. At the same magnitude of X-Gluc concentrations (legend) added to the same concentration of β-Glu, rate of reaction as measured by absorbance was comparable at room temperature (blue) and body temperature (red). In all conditions shown, the reaction crossed the visible threshold within 10 minutes of the experiment start.

To translate the results of pure enzyme assays to the behavior of *E. coli*, liquid assays were conducted to compare sensitivity. Initial experiments demonstrated that the enzyme experiments in liquid did translate to results in *E. coli*, with visible color changes after incubation at 37C. However, the amount of enzyme produced by the bacteria seems to be lower than expected, as the color change was minimal under 10^5 CFU of *E. coli* per mL of water. Additionally, the time required for this reaction was similar to that seen in traditional X-Gluc based assays, with a color change visible after approximately 8 hours at the highest concentrations of bacteria. These results were in contrast to the reactions observed in under 30 minutes for various concentrations of pure enzyme.

Figure 4. From left to right, decreasing concentrations (10^8, 10^7, 10^6, 10^5, and 10^4 CFU) of E. coli *in reaction with X-Gluc.*

Initial literature reviews to determine the localization of β-Glu in *E. coli* indicated that the enzyme may be a soluble protein in the cytoplasm of the cell. Therefore, we hypothesized that lysis of the bacteria would release soluble protein into the lysate solution. We expected that this

process would improve the reaction speed, as the release of enzyme would allow the reaction to proceed similarly to the experiments with pure enzymes.

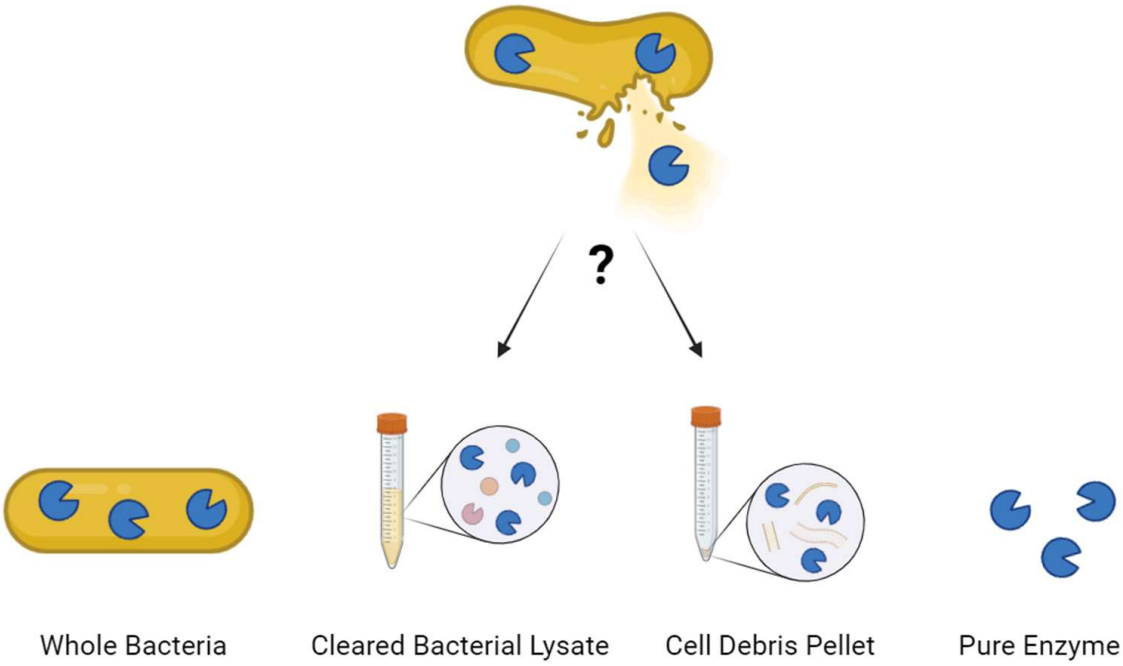

Figure 5. Schematic representation of whole bacteria, cleared bacterial lysate, cell debris pellet, and pure enzyme groups tested.

Experiments were designed to compare the reaction time required for whole, unlysed bacterial cells with lysate released from cells and cleared of cell debris. The whole bacterial cells were expected to contain β-Glu within the cells. Cells lysed using a standard protocol with lysis buffer and enzymes were separated by centrifugation into a cleared lysate solution and a resuspended solution of pelleted cell debris. The lysate solution was expected to contain soluble proteins, including β-Glu, while other membrane-bound proteins would be found in the cell pellet. Three experimental groups were tested, with decreasing cell concentrations of 10^7,10^5, and 10^2 cells per mL. Pure enzyme was also included as a positive control. To quantitatively obtain data, absorbance measurements were taken at 610 nanometer wavelength and normalized with negative controls (no added X-Gluc), and were observed to be visibly blue at approximately 0.25 Absorbance Units.

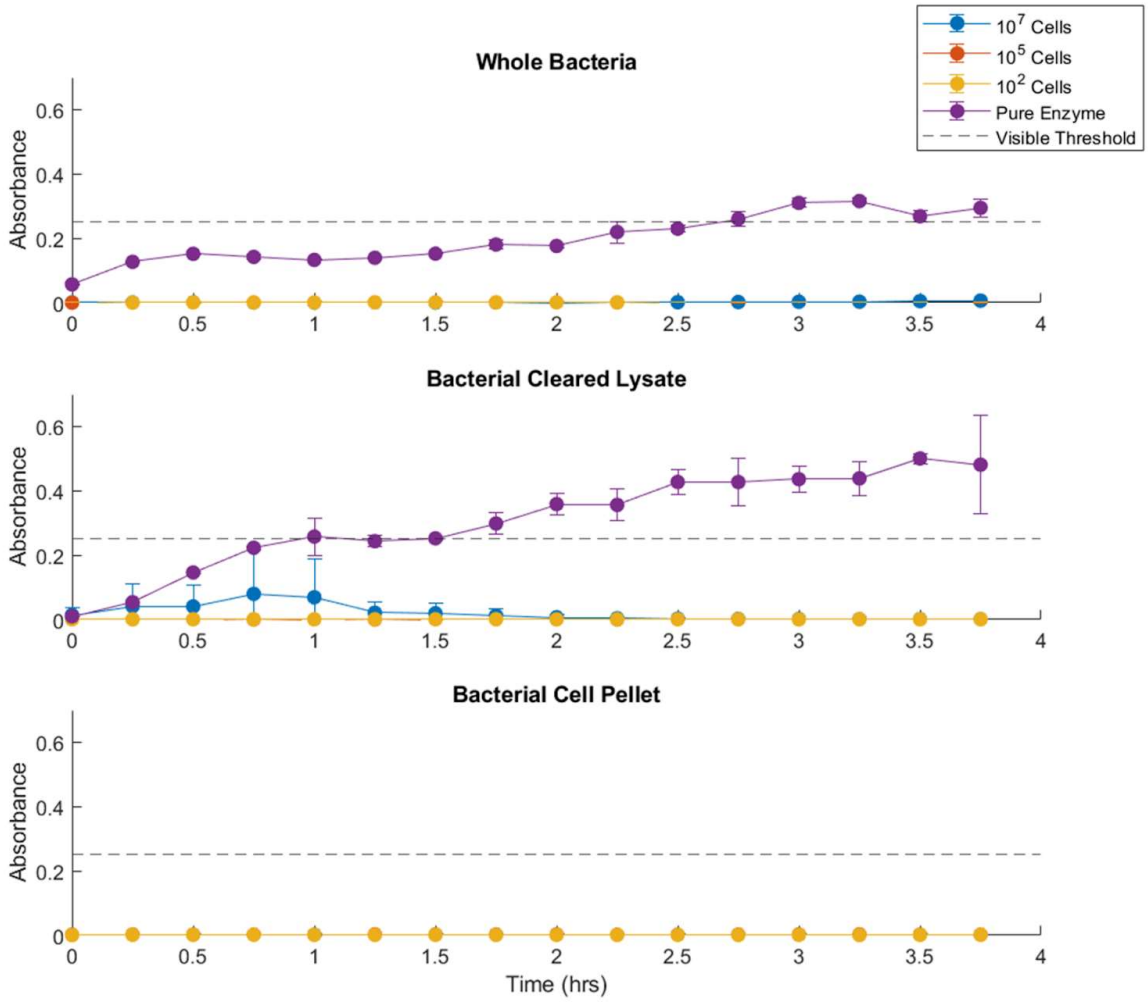

Figure 6. Absorbance of whole bacteria, cleared lysate with soluble proteins, and bacterial cell pellet with membrane-bound components reacting with X-Gluc. Three concentrations of bacteria and pure enzyme as a positive control were included in triplicate.

We observe visible color changes for pure enzyme reacting with X-Gluc after approximately 2.5 hours in unlysed bacterial conditions, and approximately 1 hour in the bacterial lysate conditions. This indicates that lysis buffer components do not reduce X-Gluc or β-Glu activity, and may even improve reaction speed. However, for whole cells, lysate released from the cells, and pelleted cell debris, at all bacterial concentrations, we see no increase in absorbance within the first four hours of the experiment. The initial increase in absorbance for the bacterial lysate from the highest cell concentration, followed by a decrease after one hour, is likely due to residual bubbles in the solution rather than an indication of X-Gluc cleavage. Our findings show that lysis of bacterial *E. coli* cells does not necessarily lower the detection time using X-Gluc, as compared to whole bacterial cells. Additionally, we did not find that lysis improved the sensitivity of the assay.

To test if the enzymatic and chemical lysis processes were interfering with β-Glu in unexpected ways, lysis through freezing of cells was also tested. We compared live cells with cells killed and broken open at -20C, conditions intended to preserve enzyme activity as best as possible. However, the results from this experiment showed similar results, with visible positives at the highest bacterial concentration after 8 hours for whole cells and 20 hours for frozen cells.

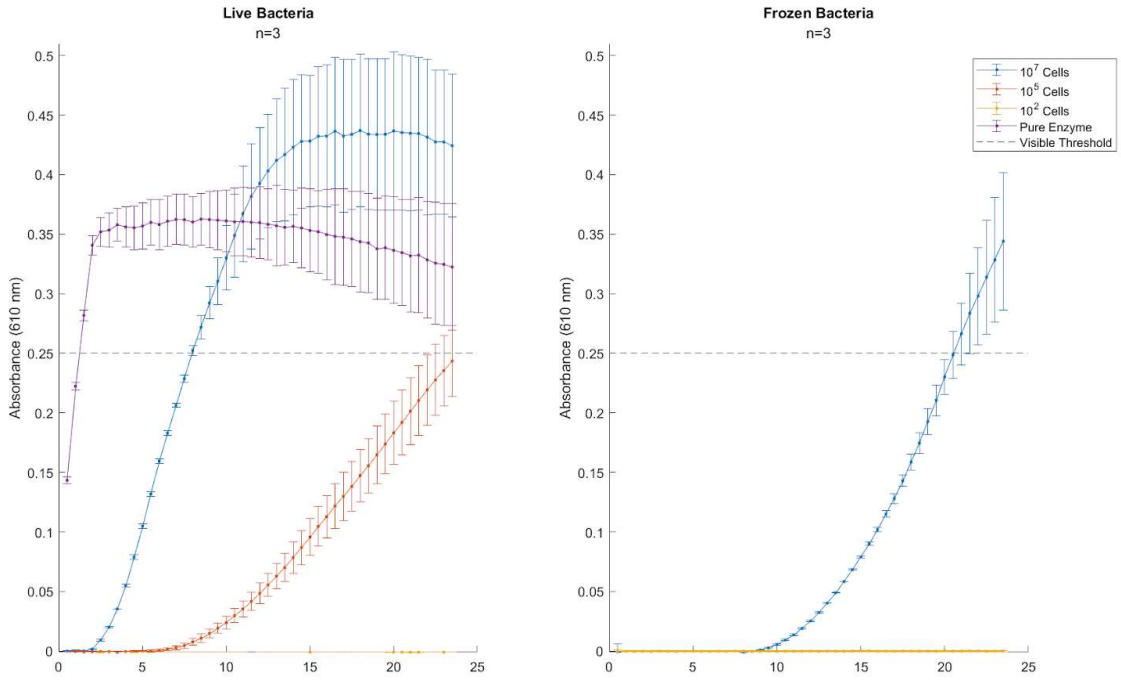

Figure 7. Absorbance of whole bacteria and frozen bacteria with ruptured membranes reacting with X-Gluc. Three concentrations of bacteria and pure enzyme as a positive control were included. X-axis represents elapsed time, in hours.

These results indicate that lysing bacteria is not an effective method of decreasing the reaction time with X-Gluc. One potential explanation is that the bacteria may produce minute quantities of β-Glu, in contrast to the larger concentrations of enzyme in even diluted positive control samples. Addition of X-Gluc to live bacteria may even induce the production of β-Glu, which may explain the lag time before changes in absorbance are observed. These explanations may be consistent with the literature review demonstrating rapid results with X-Gluc when an inducer for the enzyme was added to bacterial samples, or with the ability of cell lysis to increase speed of detection for *E. coli* with Red-Gal, which detects a different enzyme.

Paper-Based Assay Experiments

Another set of experiments explored the development of paper-based assays, to determine the feasibility of delivering the X-Gluc test in a different format than the ECC Vial or traditional media-based assays. We first attempted to determine if silk fibroin could be utilized as a method to preserve the X-Gluc added to a paper-based test, and we tested different formulations including layers of silk fibroin and silk fibroin gels. These gels were created through

ultrasonication of the silk fibroin solution, and different parameters including sonication time, intensity, and order of adding X-Gluc were tested as well.

To determine if any structural differences in the silk film were created with the addition of X-Gluc and with various parameters of sonication to create the gel, fourier transform infrared spectroscopy (FTIR) was utilized to analyze changes in beta-sheet crystallization[21] across different silk film preparations with X-Gluc. With sonication, we expected to see shifts in maxima, from the absorption band characteristic of random coils to that characteristic of antiparallel beta sheets and indicative of crystallization. Silk film samples were prepared, dried, and analyzed with FTIR to determine if X-Gluc was disrupting or enhancing the formation of crystalline structure through sonication, a process being utilized in this case to manipulate the water solubility and permeability of the film formed.

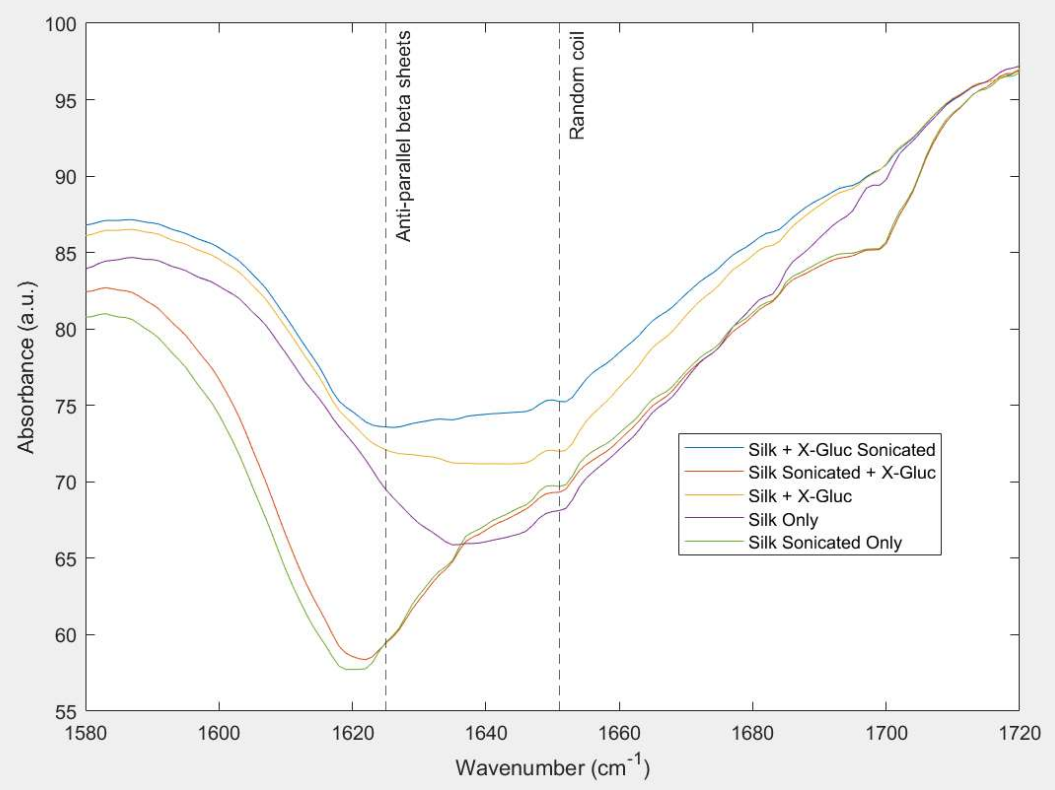

Figure 8. FTIR absorbance spectra (absorbance units, a.u.) versus wavenumber for films prepared with silk fibroin mixed with X-Gluc and sonicated, silk fibroin sonicated before mixing with X-Gluc, and silk fibroin solution mixed with X-Gluc. Additional control films were included with silk fibroin only, unsonicated and sonicated.

These results demonstrate that the addition of X-Gluc prior to the sonication of silk may prevent the crystallization of the silk fibroin film, as the absorbance spectra of this film is similar to the patterns of unsonicated silk fibroin with X-Gluc and control silk fibroin. However, when

[21] Hu, Xiao, David Kaplan, and Peggy Cebe. "Determining Beta-Sheet Crystallinity in Fibrous Proteins by Thermal Analysis and Infrared Spectroscopy." Macromolecules 39, no. 18 (September 1, 2006): 6161–70. https://doi.org/10.1021/ma0610109.

X-Gluc is added after the sonication of the silk, the beta-sheet characteristic band strengthens in a comparable pattern to the sonicated silk only film.

We then moved to testing various silk formulations with β-Glu. These experiments validated that addition of silk fibroin to the test did not impede the X-Gluc reaction with β-Glu. Initial experiments demonstrated the feasibility of a paper-based test format, with consistent and rapid positive results affirming the compatibility of silk fibroin and X-Gluc with detection of pure enzyme. Two studies were also conducted with paper tests stored at room temperature for an extended period, one for approximately a month and the other for approximately 3 months.

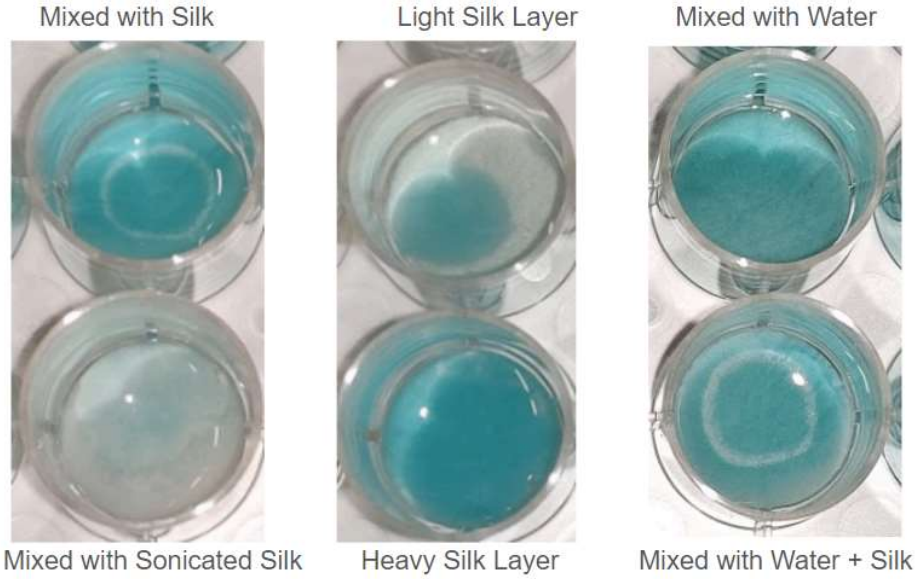

Figure 9. Paper tests of various formulations were stored at room temperature for approximately 3 months. Formulations included water-based X-Gluc solution only, silk fibroin mixed with X-Gluc, silk fibroin layered over X-Gluc, and silk fibroin-based gel with X-Gluc. Pure enzyme was then added to the tests, and images were taken for up to 90 minutes (pictured) to compare intensity and time required for the reaction.

The goal of these experiments was to determine the effectiveness of silk formulations on X-Gluc preservation and identify any changes in time required for a visible, positive result to be observed. However, results from both these tests did not show any quantifiable difference in reaction time and intensity for silk-based formulations compared to basic water-based formulations. Generally, formulations mixed with silk fibroin or coated in silk fibroin performed better than the sonicated silk fibroin gel formulations, likely due to the low water solubility of the gel-based films preventing X-Gluc from interacting with β-Glu.

Finally, the paper-based test format was tested with *E. coli* bacteria in varying concentrations, incubated at 37C for 48 hours. We qualitatively demonstrated that the paper-based test format was compatible with detection of *E. coli* cells, and that the paper assay performed slightly better than the liquid-based assay when considering reaction time and intensity of colorimetric reaction.

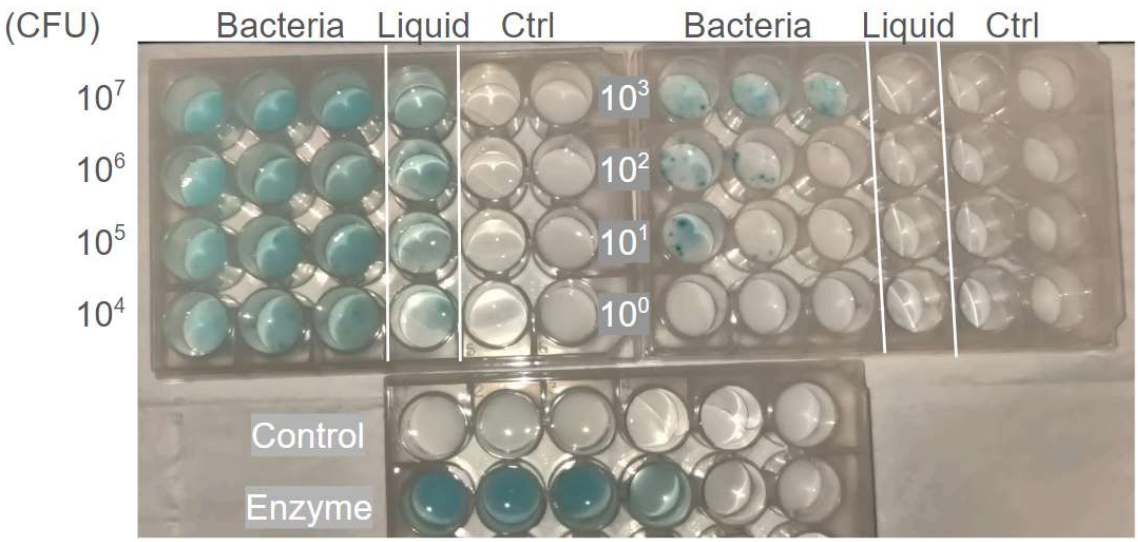

Figure 10. Comparison of paper-based test (bacteria) to liquid-based test (liquid) for E. coli *detection after 48 hours of incubation. Each concentration of bacteria (CFU) was tested in triplicate, with a single liquid control and two negative control samples (Ctrl). Additionally, a negative control with no bacteria and a positive control with pure enzyme was included.*

Pure enzyme positive results were visible in the paper test format within 10 minutes of adding the sample, a few minutes earlier than the liquid result color change was visible. Only the highest bacteria concentration tested, 10^7 CFU/mL, showed a visible color change in both tests within 2 hours. Within 24 hours, as little as 10^4 CFU/mL showed a positive test result in the paper-based format. After 48 hours, all samples with greater than 10 CFU/mL showed some color change, although with some variation in intensity across replicates. In all samples with less than 10^3 CFU/mL, however, the liquid test results were not visibly positive in comparison to the paper-based test results. While these results did not show significant improvements in assay time compared to existing X-Gluc based tests, the paper-based test format did outperform the liquid assays and demonstrated the potential for this medium to be developed further.

Future Work

This project provides various directions for future researchers to continue improving the ECC Vial test design to detect *E. coli* in drinking water. Further experimentation is required to identify effective methods of liberating and increasing activity of β-Glu, as a method of decreasing the time and incubation temperature of X-Gluc based *E. coli* detection. Utilizing additional substrates such as enzyme activity inducers, as described by Wu et al.[19], may increase the quantity of β-Glu produced and close the gap between reaction speed in pure enzyme as compared to whole bacteria. Additional replication of experiments can also provide more insight into whether the results shared here are artifacts of experimental design, or give reliable insight into the methods of X-Gluc and β-Glu interaction.

There is certainly a need for more baseline research on β-Glu activity, especially in the context of its reaction with X-Gluc. More studies on the fundamental enzyme kinetics, localization and expression in the cell, and other aspects of β-Glu activity can help pinpoint methods of increasing the speed and sensitivity of the assay, such as whether the addition of X-Gluc to live *E. coli* induces the production of β-Glu. Furthermore, testing should be extended to field samples, where conditions such as pH, alternative pathogens, and various other factors are not controlled. These results can be verified using known methods of *E. coli* quantification such as Most Probable Number counting, and quantification of test sensitivity and specificity can be conducted based on these experiments.

Future work should also expand on the potential advantages of using a silk-coated, paper-based test, including its effectiveness in preserving X-Gluc performance after storage as well as its ease of use as a test format. Accelerated shelf-life studies can be a tool to study the test longevity more rigorously, utilizing models of reaction kinetics and the effect of temperature, time, and humidity on substrate degradation[22]. Additionally, a more robust and quantitative comparison of the ECC Vial as a liquid-based assay with the paper-based assay formulation can provide more insight into whether this is a worthwhile transition to pursue. If this paper-based format can also be applied to low-cost filters, the sensitivity of the test can be greatly improved as bacteria from larger water samples can be concentrated for the assay. It would also be important to conduct user trials and interviews with the new format, to determine if the appearance of blue on white paper indicating a positive test result is more intuitive and easy to read for the average user when compared to the ECC Vial's liquid-based color change from clear to blue.

Through numerous conversations with investors and lab members, I have also found that this project may lend itself to a future startup or other collaboration beyond academia. There is a need for a low-cost *E. coli* test to become widely available, especially in low- and middle-income countries. One major challenge may lie in sustainably scaling the technology; larger-scale manufacturing processes must be accounted for in the research process and developed in tandem with local communities to ensure both cultural and environmental sustainability. Currently, this process is successfully progressing through the work of ENPHO and EcoConcern in Nepal. If the test function can be significantly improved through future research, a larger-scale collaboration or venture will bring us closer to delivering life-saving clean water technology to millions of affected people across South Asia and the rest of the globe.

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[22] Clancy, Don, Neil Hodnett, Rachel Orr, Martin Owen, and John Peterson. "Kinetic Model Development for Accelerated Stability Studies." AAPS PharmSciTech 18, no. 4 (May 1, 2017): 1158–76. https://doi.org/10.1208/s12249-016-0565-4.