CENTER for PRODUCE SAFETY

CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Rapid bacterial testing for on-farm sampling

Project Period
January 1, 2015 – December 31, 2016 (extended to February 28, 2017)

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Objectives
1. ***Phage screening:*** *In this objective, bacteriophages will be screened for several performance characteristics. These factors, such as specificity and replication time, will improve the overall performance of the detection system. The screening will allow the identification of a bacteriophage(s) which will allow peak performance of the detection kit. Simultaneously, we will continue collecting produce rinse water from farms throughout New England. This water will be used as a sample matrix in all objectives.*

2. ***Sample preparation and separation:*** *Researchers will be investigating the detection of Salmonella spp. from agricultural water. Because agricultural water may contain plant materials, we plan to release internalized or surface-adhered bacteria using enzymatic digestion. The preliminary data has shown this method to liquefy foods within 10-15 minutes. Formerly surface-adhered and/or internalized pathogens are then able to be separated using magnetic particles.*
3. ***Dipstick assembly and validation:*** *The dipstick development will utilize the established technology which has demonstrated ultra-low detection limits. The complete assay, starting from the sampling of the agricultural samples, will be conducted and compared to current methods using local laboratories. From these experiments we will demonstrate the reliability and performance of the entire detection system.*

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FINAL REPORT

Abstract

There is a continuing need to develop new methods that can provide early detection of bacterial contamination in produce. In order for these new technologies to be pragmatic, attention must be paid to the assay time, cost, and robustness. We have developed a detection method starting from sample to results. The assay can be broken into three parts: 1) separation of bacteria from leafy greens into liquid samples, 2) concentration of bacteria in liquid samples, and 3) detection of bacteria from concentrated liquid samples. The three components were designed to be performed in sequence but can also be modularly incorporated into new or existing assays. Leafy greens were surface inoculated with bacteria (*Salmonella*) to simulate field contamination. The greens were then mixed and incubated with liquid enzyme cocktails that digested the greens, resulting in approximately two times the recovery of bacteria compared to samples without enzymes. Engineered bacteriophages, conjugated to magnetic particles, were designed to capture and separate bacteria from liquid samples. The use of phages demonstrated a significant improvement in the capture efficiency of bacteria as compared to traditional immunomagnetic separation. These differences were especially evident in non-biological conditions with respect to temperature, pH, and salinity. The phages also were engineered to overexpress a reporter enzyme containing an affinity tag during the infection of the bacteria. The affinity tag allowed the attachment of the reporter enzyme to a simple dipstick where the sample was placed. A colorimetric substrate allowed the visual indication of the reporter enzyme, and therefore initial host bacteria, on the dipstick via a transition from white to blue. This streamlined approach allows a low-cost sample-to-results test that can be used for leafy greens or agricultural water.

Background

Bacteria separation from a complex food matrix is a critical step prior to the detection of foodborne pathogens [1]. It is difficult to conduct a rapid and sensitive bacteria detection in food matrices without an efficient isolation means to remove and concentrate the analytes into a relatively clean sample [2,3]. Currently the food and agricultural industries rely on the standard microbiological methods to detect the presence of bacteria. Food samples are typically pre-enriched to allow bacteria present to grow to high numbers. This increases the possibility of removing some of the bacteria for detection. The sample is then plated on the selective or differential media and enumerated after an additional incubation [4]. The need for multiple enrichment steps lengthens the total assay time into days. A more pragmatic system would allow the potential for in-shift analysis. In addition, the new and rapid methods have shown the potential to detect relatively low numbers of the bacteria in pure samples, but they might not work effectively when applied to complex samples such as those encountered in food and agriculture.

The abundance of methods that can reliably detect bacteria in clean, concentrated samples makes it clear that the true problem in rapid detection is sample preparation [1,5]. Therefore, there is a significant demand for new strategies for the rapid and efficient separation of food pathogens for food safety. An ideal separation method would provide the benefits to the rapid detection of bacteria.

Bacterial cells can form relatively strong attachment to the surface and interior of a food or plant, which also makes bacterial separation from food matrices become a big issue. Organisms, such as *Salmonella* spp., are able to infiltrate the tissues of the agricultural product that provides the protection for bacteria against the chemical and physical removal methods [6,7]. Strong adhesion to the food matrices can result in false negatives in the downstream detection. The persistence and attachment of bacteria is affected by the bacterial strains, leaves, and blade roughness and so on [8-10]. Currently the commonly used methods to separate the bacteria from solid food matrices are

physical methods such as stomaching. However, most physical methods only remove the part of bacteria attached on the surface and are not able to isolate the interior bacteria or have the potential to kill the bacteria, such as ultraviolet radiation [10]. Immunomagnetic separation is also one of the most prevalent methods for a relatively rapid, clean and specific separation. The magnetic beads conjugated with specific antibodies are added into the food sample solution and will capture the specific organisms [11,12]. In the presence of a magnet, the beads can pull the bacteria to the side wall of the tube. After the discard of the suspension, the beads-analyte conjugates are then resuspended in the buffer and used for further detection. However, this was used to capture the bacteria existing in the buffer, and was ineffective to the strongly attached and interior bacteria. Therefore, there is increasing need for developing an effective strategy to isolate the bacteria, both surface-attached and internalized.

Research Methods and Results

1. Phage screening & engineering

The research team has evaluated the host ranges for phages that display specificity to either *Salmonella* spp. or *E. coli*. We have determined cocktails necessary for the detection both pathogens and indicator groups, respectively. From the cocktails, we have selected model phages which we engineered with the following modifications: 1) a biotin tag on the capsid protein to allow conjugation to magnetic particles, and 2) insertion of a gene for an engineered alkaline phosphatase with a tag for affinity to cellulose. From the first modification, we tagged the capsid gene (*gp17* for T7 phage) with a DNA sequence coding for a biotin acceptor peptide (BAP). The *E. coli* endogenous enzyme BirA naturally modifies this tag with available biotin. We initially found that during the T7 infection only ~9% of the 415 capsid proteins on the phage capsid were biotinyated, resulting in a poor efficiency. Attempts to incorporate additional biotin tags to the phage post-infection were unsuccessful, most likely due to access of the BAP following the folding of the capsid protein. We then added an additional gene for BirA to the *E. coli* which could be overexpressed during infection. This additional modification resulted in ~33% of the 415 capsid proteins on one the phages being tagged with biotin. When the modified phages were incubated on magnetic particles (2.8 µm diameter) for 12 hours, we found an immobilization of ~248 phages per magnetic particle (see Appendix: Figure 1). We also were able to design a reporter enzyme with an affinity tag to cellulose. The gene for this reporter enzyme was inserted into the T7 phage and the functionality was validated.

2. Sample preparation and separation

We looked at several strategies to accomplish separation in “dirty” samples. Traditional methods of separating bacteria from food routinely involve stomaching, blending, and shaking. However, these methods may not be efficient at removing all the bacteria from complex matrices. We investigated the benefits of using enzyme digestion, followed by immunomagnetic separation to isolate *Salmonella* from spinach and lettuce. Enzymatic digestion using pectinase and cellulase was able to break down the structure of the leafy green vegetables, resulting in the detachment and release of *Salmonella* from the leaves. Immunomagnetic separation of *Salmonella* from the liquefied sample allowed an additional separation step to achieve a more pure sample without leaf debris, which may benefit additional downstream applications. We have investigated the optimal combination of pectinase and cellulase for the digestion of spinach and lettuce to improve sample detection yields. The concentrations of enzymes used to digest the leaves were confirmed to have no significant effect on the viability of the inoculated *Salmonella*. The recovery of the *Salmonella* from the produce after enzyme digestion of the leaves was significantly higher ($P < 0.05$) than traditional sample preparation methods to separate bacteria (stomaching and

manual shaking). The use of enzyme digestion of produce allows a more efficient separation of the adhered bacteria into a liquid sample matrix (see Appendix: Figure 2).

In order to separate bacteria from liquid samples, magnetic separation of the bacteria using phages for attachment was developed for both magnetic nanoparticles as well as micro-scale particles. The particles were compared to the gold standard immunomagnetic separation (IMS) in both municipal and agricultural water. It was clear that outside of biological conditions, the phages had significantly higher capture efficiency (see Appendix: Figure 3). This finding is important because most food and food-related matrices are typically outside of biological conditions with regard to pH, temperature and salinity. Therefore, our phage-based magnetic particles will provide a greater assurance of the capture of indicator and pathogenic bacteria.

3. Dipstick assembly and validation

The newly accepted scheme for detection using genetically engineered bacteriophages makes use of reporter enzymes expressed with an affinity tag that binds onto cellulose pads. We have performed optimization assays which quantify the enzymatic color change directly on the dipstick pad. Plastic sheets and cellulose pads were selected from a large sampling based on performance, cost and ease of manufacturing (see Appendix: Figure 4).

The signal on the cellulose pad is a result of a reaction between the alkaline phosphatase (produced during the phage infection with the host bacteria) and the substrate NBT/BCIP, which forms a blue precipitate following the reaction. In order to maximize the signal on the pad, we needed to reduce the amount of precipitate that adsorbs to the plastic baking. We tested several blocking agents, including polyvinyl acetate (PVA), casein, Triton X100 as well as combination of these blockers (see Appendix: Figure 5). PVA (1%) in combination with casein (1%) was shown to greatly block substrate binding on the plastic. In addition to visual observation, we quantified the images with ImageJ to better determine an optimal blocking solution.

The dipsticks were optimized with respect to temperature and time required for binding of the reporter enzyme. Additionally, the quantification method was optimized to allow for a direct reading of the results without the need for user interpretation.

The enzymatic reaction on the dipstick was optimized and characterized. With the aid of the Talbert Research Group (Iowa State University) we have characterized the enzyme dynamics immobilized on the cellulose pads via the affinity tag. It was found that an engineered Alp without the fusion tag had a k_{cat} and K_m of 1540 s^{-1} and 0.39 mM, while the engineered ALP with the fusion tag was 918 s^{-1} and 0.46 mM, respectively. We found that while the activity of the enzyme decreased slightly, the affinity tag allowed the localization of the signal on the cellulose pad and therefore improved performance.

While we have optimized the individual steps of the proposed assay, new materials (enzymes, phages, substrates) are continuously becoming available. Therefore, this ongoing process results in an ongoing improvement in performance. The limit of detection has been performed on the individual steps of the assay utilizing the proper controls. It was not possible to inoculate low concentrations of bacteria onto the spinach/lettuce leaves to determine a limit of detection for the overall assay. This is because a significant proportion of the bacteria are killed during the drying step following spotting onto the leaves. Unfortunately, because the bacteria are adhered to the leaves in a multilayer, it was not possible to determine the number if “viable” bacteria at the start of testing. Rather, we are currently performing a direct comparison to current methods in order to determine the improvements over the current state of the art.

Outcomes and Accomplishments

We have demonstrated tangible improvements in the separation, concentration and detection of bacteria from leafy green and/or agricultural water. These methods establish a platform for which further customizations will allow bacteria detection in additional matrices relevant to produce safety. We have published five peer-reviewed research papers containing our findings and we are currently preparing several additional papers.

Summary of Findings and Recommendations

The research project has resulted in several findings that can convey a significant benefit to produce safety. Some key takeaway points are listed below:

- Enzymes significantly improve the efficiency of removing adhered bacteria from produce. By digesting the produce that bacteria are adhered to, the bacteria are more likely to enter into the liquid matrix. Additionally, the digestion of the plant material results in a partial liquefaction (~40%) of the material, which means less material is able to trap the bacterial cells during the subsequent filtration step.
- Phages offer an improved method for capturing bacteria. The ability of phages to adhere to the bacterial surface in non-biological conditions results in improved capture efficiency when compared to the current state of the art (antibody-based capture). Phages therefore offer a lower-cost and more reliable magnetic separation than currently available.
- Phages allow the determination of viability during detection. Although phages can adhere to non-viable bacteria, they can only infect bacteria with functional replication machinery. Therefore, by using engineered phages for the detection of bacteria, it can be assumed that the bacteria are viable and able to reach log-phase.

APPENDICES

Publications and Presentations

Several publications are currently in preparation while five have already been published with acknowledgements:

- Wang D, Chen J, Nugen SR. Electrochemical detection of *Escherichia coli* from aqueous samples using engineered phages. *Analytical Chemistry* 2017; 89(3): 1650-7.
- Wang Z, Wang D, Chen J, Sela DA, Nugen SR. Development of a novel bacteriophage based biomagnetic separation method as an aid for sensitive detection of viable *Escherichia coli*. *Analyst* 2016; 141(3): 1009-16.
- Wang D, Wang Z, He F, Kinchla AJ, Nugen SR. Enzymatic digestion for improved bacteria separation from leafy green vegetables. *J Food Prot* 2016; 79(8): 1378-86.
- Jackson AA, Hinkley TC, Talbert JN, Nugen SR, Sela DA. Genetic optimization of a bacteriophage-delivered alkaline phosphatase reporter to detect *Escherichia coli*. *Analyst* 2016; 141(19): 5543-8.
- Wang Z, Wang D, Kinchla AJ, Sela DA, Nugen SR. Rapid screening of waterborne pathogens using phage-mediated separation coupled with real-time PCR detection. *Analytical and Bioanalytical Chemistry* 2016; 408(15): 4169-78.

Budget Summary

All funds have been used as scheduled in the project proposal.

Tables and Figures

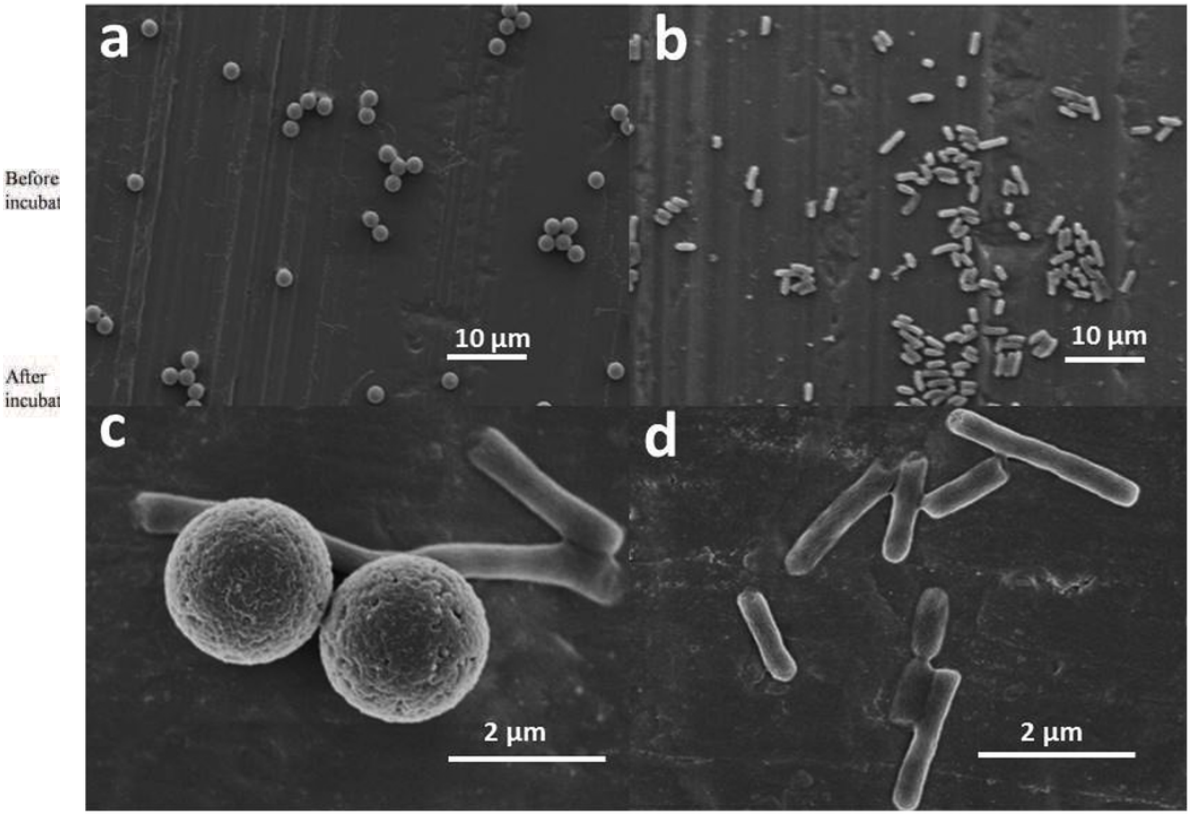

Figure 1: Scanning electron microscopic images. (a) Phage based magnetic beads (biosorbents). (b) *E. coli* cells (appear as rods). (c) High magnification of *E. coli* cells captured by the biosorbents. (d) High magnification of *E. coli* cells.

Figure 2: The physical appearance of the leaves of lettuce (a and c) and spinach (e and g) before the incubation. The physical appearance of the leaves of the lettuce (b) and spinach (f) without enzyme digestion and lettuce (d) and spinach (h) with enzyme digestion after 10 s of mixing and 1.5 h of incubation. The concentration of the enzyme used was 7.5 U/g pectinase and 2.5 U/g cellulase for lettuce and 22.5 U/g pectinase and 7.5 U/g cellulase for spinach.

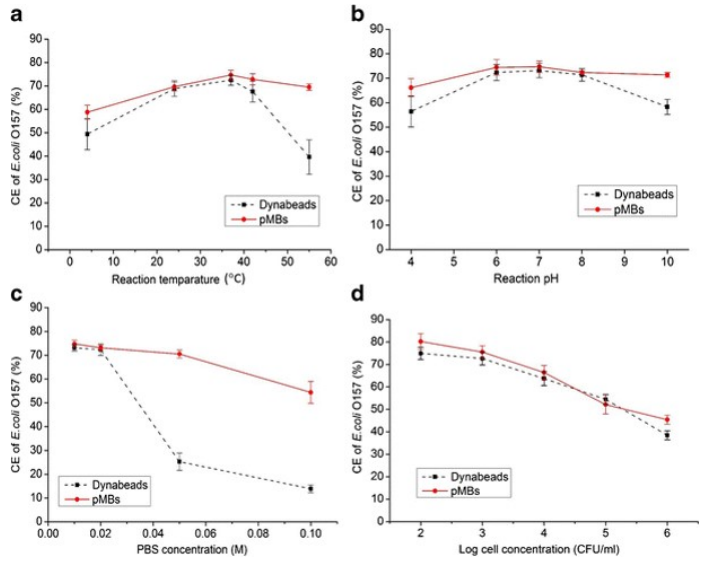

Figure 3: Phages were compared to antibodies for the magnet separation of bacteria. The influence of a) temperature, b) pH, c) salinity, and d) bacterial concentration were evaluated. The data suggest that phage binding at non-biological conditions was significantly better than commercially available antibody-based kits.

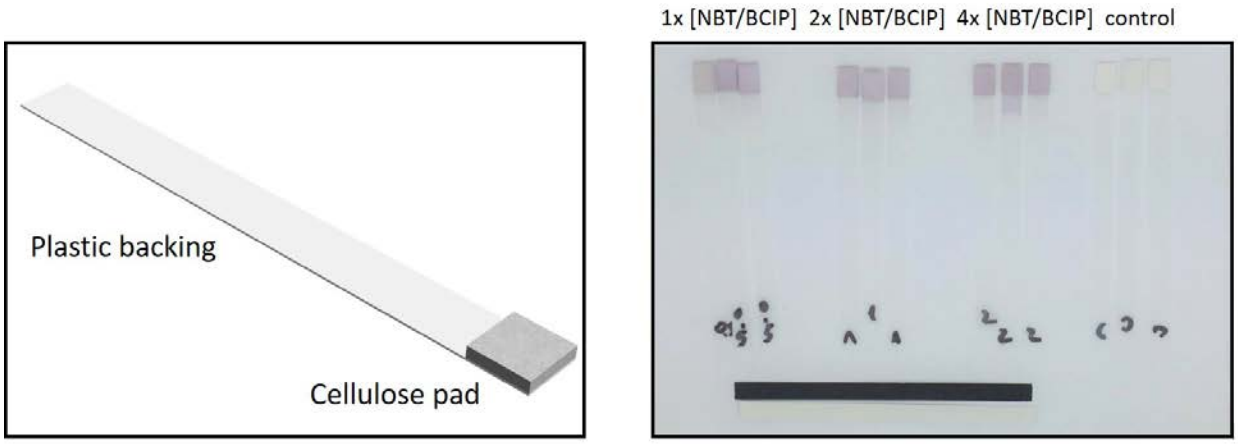

Figure 4: Dipsticks for bacterial testing. a) The dipsticks were constructed from a plastic backing and a cellulose pad. The cellulose served as the enzyme immobilization site for the assay. b) The concentration of the substrate NBT/BCIP was determined for optimal signal:noise.

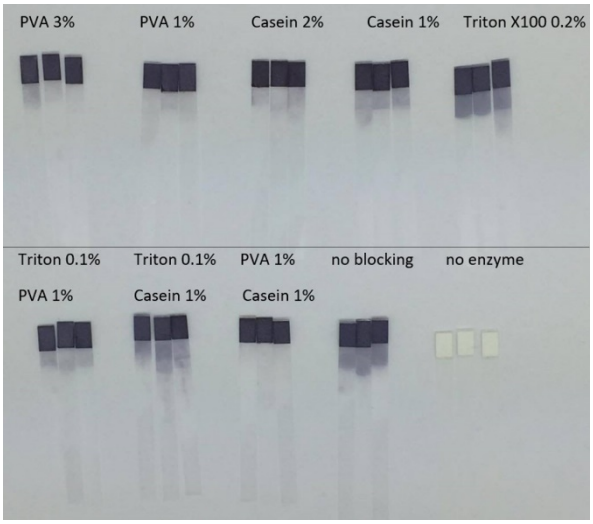

Figure 5: Blocking of the dipsticks. Various blocking solutions were evaluated for the reduction of substrate deposition on the plastic backing. This maximized the substrate deposition on the cellulose pad.

Suggestions to CPS

I recommend additional exploration of advanced, yet pragmatic technologies in the area of rapid detection. Specifically, exploration of the bottleneck, which is sample preparation, is of utmost importance.

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