A PAPER-BASED ANALYTICAL DEVICE FOR THE COLORIMETRIC DETECTION OF FOODBORNE PATHOGENIC BACTERIA

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ABSTRACT

We have developed a paper-based analytical device as a platform for colorimetric detection of pathogenic bacteria responsible for foodborne illness. The food industry currently relies on time-consuming and complex analysis techniques to determine the presence of live *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* in food products. Advantages of the paper-based design include low cost, simple fabrication and operation, portability, and disposability. Additionally, colorimetric assays allow for simple, rapid analysis. Using this novel method, enrichment time is reduced to 6 hr or less, with limits of detection on the order of 10^4 cfu/mL (colony forming units).

KEYWORDS: Foodborne Bacteria, Paper-Based Analytical Device, Colorimetric Assay, ImageJ

INTRODUCTION

An ongoing challenge in the food industry is the presence of pathogenic bacteria, with an estimated 47 million cases of food related illness occurring in the US each year [1]. While a variety of bacterial species are known to cause food-related illness, *E. coli* O157:H7, *S. enterica* serovar Typhimurium, and *L. monocytogenes* are among the more common and deadly [2, 3]. Current gold standard techniques for live bacteria detection require a time-consuming enrichment step (8-24 hr) prior to plating and counting. More advanced detection technologies such as polymerase chain reaction (PCR) can be tedious, require complex and costly instrumentation, and necessitate highly trained personnel [4]. Furthermore, many of these techniques cannot distinguish between live and dead bacteria, producing ambiguous results. We have developed a paper-based analytical device (μPAD) for the rapid determination of foodborne bacteria as a first-level of screening. The idea of using paper as a substrate material for a microfluidic device is very attractive from the perspective of cost, ease of fabrication, portability, disposability, and simplicity [5]. Moreover, using sensitive and selective enzymatic colorimetric assays, our paper-based pathogen sensor allows for reduced enrichment time and provides a simple, low-cost platform for live bacteria detection. The work presented here discusses the optimization of three enzymatic assays. We also demonstrate the efficacy of this method with live bacteria.

EXPERIMENTAL

An array of 7 mm circles, termed wells, is printed on Whatman #1 filter paper using a wax printer (Xerox Phaser 8860). The printer uses melted wax in place of ink and thus creates hydrophobic patterns on the paper. Once printed, the devices are placed on a 150˚C hot plate for approximately 2 min until the wax has melted through the paper generating a three-dimensional hydrophobic barrier. To complete fabrication, a section of clear packaging tape is place on the backside of the devices to enhance fluid control and prevent leaking. Device arrays are placed in petri dishes for easy transport in and out of a 37˚C incubator. For bacterial assays, reagent, buffer and sample are pipetted onto the wells where the enzymatic reaction occurs. Total volume for each well is 30 μL. Once the reaction is complete, the wells are allowed to dry and then imaged using a scanner. For semi-quantitative analysis, the images of the colorimetric results are analyzed by measuring the grey intensity using ImageJ software. A schematic of the device and data analysis is shown in Figure 1.

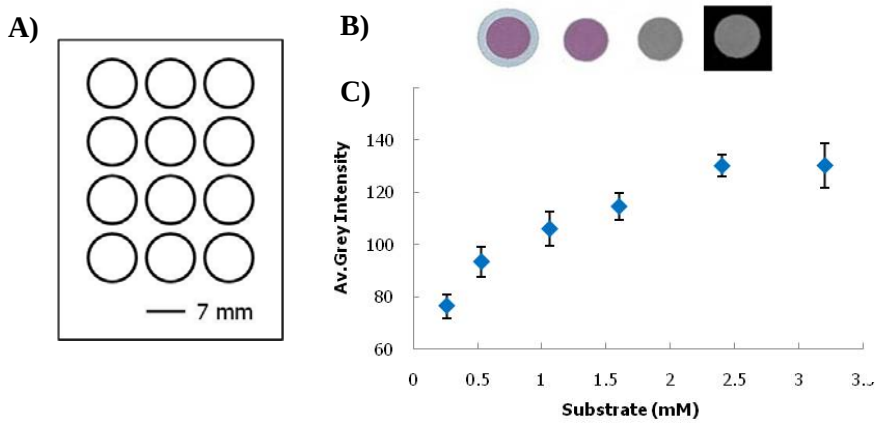

Figure 1. *(A) Diagram of an array of well devices, 7 mm in diameter. (B) Schematic showing data processing with ImageJ. From left to right: scanned image of a single well, color threshold adjusted with ImageJ to select only the colored well, image is converted to 32-bit grey scale, image is inverted. (C) Grey intensity of each well is measured with ImageJ and plotted versus substrate concentration.*

OPTIMIZATION OF ENZYMATIC ASSAYS

Colorimetric detection of each pathogen is achieved when a colorimetric substrate is enzymatically hydrolyzed to produce a visible color change on the μPADs. Selectivity of the different bacterial assays is achieved through the interaction of a species-specific enzyme with an appropriate colorimetric substrate. For example, the *L. monocytogenes* assay employs a phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme that cleaves the 5-bromo-4-chloro-*myo*-inositol phosphate (X-InP) substrate to produce an indigo product. For the detection of *E. coli* O157:H7, chlorophenyl red β-galactopyranoside (CPRG) is hydrolyzed by β-galactosidase to produce a yellow to red-violet color change. *Salmonella* Typhimurium is detected when esterase cleaves 5-bromo-6-chloro-3-indolyl caprylate (magenta caprylate) substrate generating a purple product. The optimal substrate concentration for each assay was determined by varying the concentration of substrate in an array of well devices while the amount of enzyme remained constant.

DETECTION OF LIVE BACTERIA

Live bacteria were grown on individual agar plates. For each bacterial species, a single, isolated colony was collected using a 10μL loop and placed in 1 mL of Tryptic Soy Broth (TSB) with yeast extract for enrichment. In order to determine the minimal enrichment time necessary for analysis, the bacterial assays were conducted on the paper devices at various enrichment time points. A 500 μL sample was collected in a centrifuge tube, sonicated for 20s to lyse cells, and tested on the paper devices. A limit of detection for each bacteria species was also determined. In this study, enrichment was carried out for 12 hr to ensure a high concentration of cells ($\geq 10^9$ cfu/mL). Then, a sample of each bacteria was collected and serial dilutions were made. Each serial dilution was tested on the paper device and plated on Tryptic Soy Agar (TSA) for method validation. Finally, cross-reactivity between assays was investigated. A concentrated sample of each bacterial species was tested with all three colorimetric substrates to ensure no false positives would result.

RESULTS AND DISCUSSION

Each enzymatic assay was first optimized using pure enzyme. Optimal concentrations of each colorimetric substrate were established by determining the maximum grey intensity measured as shown in Figure 2. Optimal concentrations of CPRG, X-InP, and magenta caprylate are 2.4 mM, 80 mM, and 9.6 mM, respectively. Then, using the optimal substrate concentration, a limit of detection was determined for each enzyme with an overall reaction time of less than 1 hr. The detection limits for PI-PLC, β-galactosidase, and esterase are 0.16, 0.21, and 0.06 μg/mL, respectively. While this detection limit does not compare directly with the analysis of live bacteria, it does provide information about the overall sensitivity of the device.

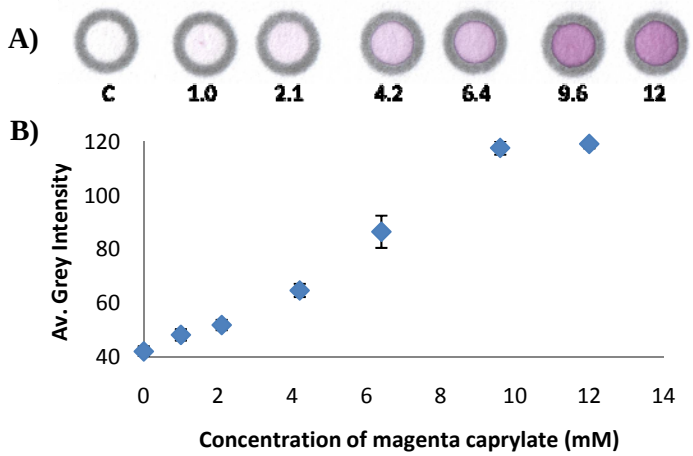

Figure 2. *(A) Image of a paper device consisting of multiple 7 mm wells. The experiment shown is the calibration of esterase activity with increasing concentrations of magenta caprylate substrate. The control, designated as "C," shows the color of the substrate when no enzyme is present. (B) Corresponding grey intensity measured for each well device (n=4) versus the concentration of magenta caprylate. Error bars represent ± standard deviation of the mean.*

Live bacteria were also analyzed on the paper device, and a minimum enrichment time was approximated for each assay. Since a single, isolated bacterial colony can vary greatly in the number of cells, the minimum enrichment time required for analysis may also vary. On average, the paper device is capable of detecting *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* within 4, 4.5, and 6 hr of enrichment, respectively. Also, a limit of detection was determined for each bacterial species. Analysis of live bacteria is shown in Figure 3 along with the average grey intensity measurements for each concentration of colony forming units. The limit of detection for *S.* Typhimurium is 10^4 cfu/mL, as determined from standard plating and counting, while the detection limits for *E. coli* O157:H7 and *L. monocytogenes* were higher at 10^6 and 10^8 cfu/mL, respectively. With a goal of 10^4 cfu/mL or less, we are continuing to improve upon detection limits as well as to reduce enrichment time.

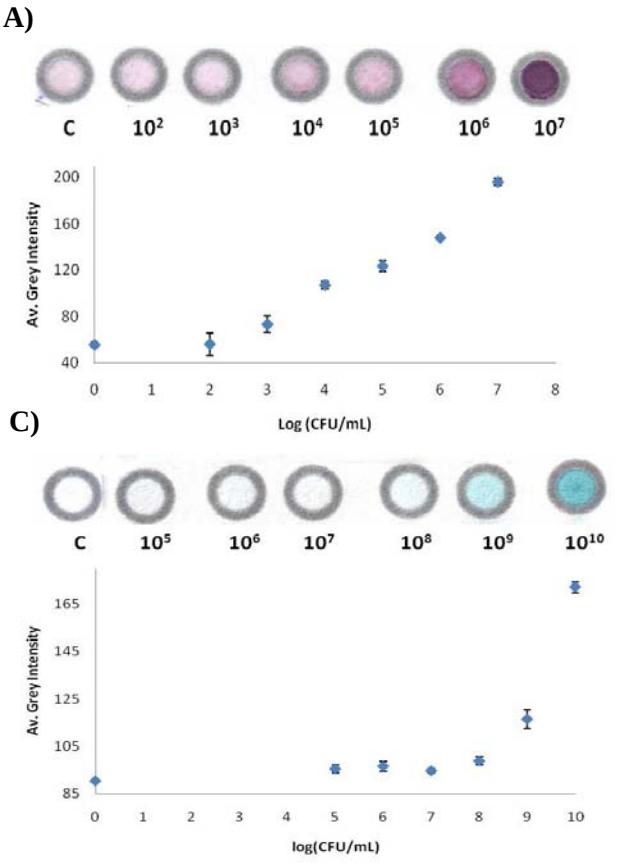

Figure 3. *LOD studies in cfu/mL for live (A)* S. Typhimurium *(B)* E. coli *O157:H7, and (C)* L. monocytogenes *with corresponding grey intensity measurements plotted as average grey intensity versus log(cfu/mL) where n = 4 for each data point. Error bars represent ± standard deviation of the mean. Controls in which no bacteria are present are represented as "C."*

Ultimately, this device will be employed for rapid testing of food samples, which may contain a wide variety of microorganisms. We examined the cross-reactivity of each assay among the three target bacterial species to ensure high selectivity of each assay. Figure 4 shows the results of this study in which a sample of each bacteria was tested against all three colorimetric substrates. In each case, a color change was only seen for the correct enzyme-substrate pair. For example, CPRG changes from yellow to red only in the presence of *E. coli*.

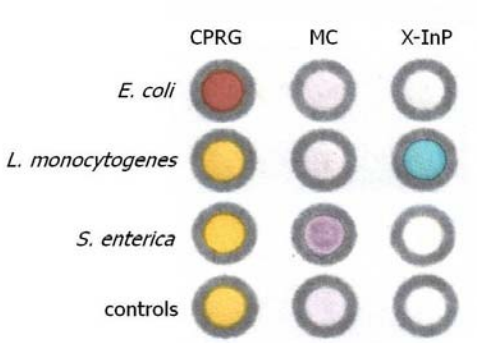

Figure 4. *Cross-reactivity study. Each row is labeled with the bacteria sample used and each column is labeled with the substrate used. The "controls" row shows the color of each substrate when no bacteria is present.*

CONCLUSION

A paper-based, pathogen sensor has been developed for the determination of foodborne bacteria. The colorimetric method is capable of detecting relevant concentrations of live bacteria with reduced enrichment time when compared to cell counting and PCR methods. Furthermore, the use of species-specific enzymes allows for a sensitive detection scheme with no cross-reactivity among the target bacteria. Utilizing a paper-based device, our sensor provides a simple, cost-effective means of pathogen detection as a first level of screening. The device shows great potential for reducing quality and control costs for the food industry. We continue to investigate means to improve detection limits and enhance assay selectivity as well as the efficacy of the device for real food samples.

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