Food Science and Technology Letters
ISSN: 0976-982X & E-ISSN: 0976-9838, Volume 3, Issue 1, 2012, pp.-20-23.
Available online at http://www.bioinfo.in/contents.php?id=66

HIGH-THROUGHPUT ASSAY FOR REAL-TIME DETECTION AND QUANTIFICATION OF LOW CONCENTRATION, VIABLE *Escherichia coli* O157:H7 IN APPLE JUICE

PACKARD M.M. AND ALOCILJA E.C.*

Nano-Biosensors Laboratory, Michigan State University, East Lansing, MI USA
*Corresponding Author: Email- alocilja@msu.edu

Received: June 06, 2012; Accepted: June 18, 2012

Abstract- Increases in production and worldwide distribution of fruit juices have been correlated with greater variability in food supply chain protocols and efficacies. Previously considered exempt from bacterial contamination, high-acid juices such as apple juice have been recently recognized as sources of enterobacteria including the pathogenic *Escherichia coli* O157:H7. Although rare, failures in sterilization efficacy can lead to widespread risks to public health. A novel, high-throughput application of an established resazurin metabolism assay provides real-time detection and quantification of bacterial contaminants representing less than one cfu/mL.
Key words- *Escherichia coli* O157:H7, high-throughput, real-time viability, apple juice.

Citation: Packard M.M. and Alocilja E.C. (2012) High-Throughput Assay for Real-Time Detection and Quantification of Low Concentration, Viable *Escherichia coli* O157:H7 in Apple Juice. Food Science and Technology Letters, ISSN: 0976-982X & E-ISSN: 0976-9838, Volume 3, Issue 1, pp.-20-23.

Introduction

Worldwide production of apple juice has increased to well over one million metric tons per year [1-3]. As demand increases, so does international distribution, increasing variability in source, manufacturing and post-production preservation within the food supply chain. Although apple juice was previously thought to be exempt from risk of bacterial contamination due to its acidic nature (pH<4.5), multiple outbreaks and spoilage occurrences have been associated with this beverage [4]. A variety of preservation methods including pasteurization, freezing, high pressure processing, are successfully employed to inactivate microorganisms in juice. Failures in sterilization efficacy, though rare, can have disastrous results[4]. The objective of this study was to develop a mechanism for assessment of sterility through modification of an existing method of metabolism assessment to offer high-throughput detection and quantification of bacterial contaminants in apple juice in real-time.

Escherichia coli O157:H7 is an enterohemmorhagic bacterial pathogen expressing virulent cytotoxins associated with hemorrhagic colitis, thrombotic thrombocytopenic purpura and hemolytic uremic syndrome (HUS) [4]. Its documented tolerance of acidic environments makes *E. coli* O157:H7 capable of surviving the low pH of apple juice. Several outbreaks have been reported since its recognition as a pathogen in 1975, including those associated with beverages including unpasteurized milk, apple juice and apple cider.

Screening for foodborne bacterial contamination must confirm a negative answer to two distinct questions if microorganisms are detected: (1) are they viable and (2) are they pathogenic. Either the viability or species determination can be made first, but each condition without the other poses no threat. For food products where sterilization is included in processing, the traditional bacterial culture screening methods, which are used to confirm viability and species identification, require extensive laboratory resources to report hundreds of negative results in search of rare anomalous contamination events. Standard protocol for bacteriological contamination as outlined by the USDA Food Safety and Inspection Service requires sample enrichment and culture requiring several days [5] and minimal potential for automation. Additionally, for samples where sterility is standard, several alternative procedures

including polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), potentiometric detection and fluorescent antibody staining, have been reported in the literature [4, 6-13]. The majority of these, however, are incapable of directly differentiating live and dead bacterial presence and may therefore report a false positive result for properly sterilized samples. This report describes a novel screening method for low level bacterial contamination indicative of sterilization failure. A key advantage of this approach is that it allows PCR-like sensitivity for confirming viability and only confirmed positive samples need to be subsequently tested for species identification.

The CellTiter-Blue® Cell Viability Assay (Promega Catalog # G8080) is typically used to determine viability through endpoint detection of cellular reduction products. The assay protocol as suggested by the manufacturer involves the addition of an optimized resazurin reagent to suspicious samples followed by incubation at 37°C for 1-4 hours. Live, metabolically active bacteria will reduce blue resazurin to pink resorufin, a highly fluorescent metabolic product produced in quantities proportional to live cell concentrations (Fig. 1). Results can be determined colorimetrically via absorbance or fluorescently. Although this assay non-specifically quantifies all present bacteria, it is not destructive to assayed cells and, upon observation of a positive signal, confirmation of species identity can be immediately determined with a now enriched bacterial sample.

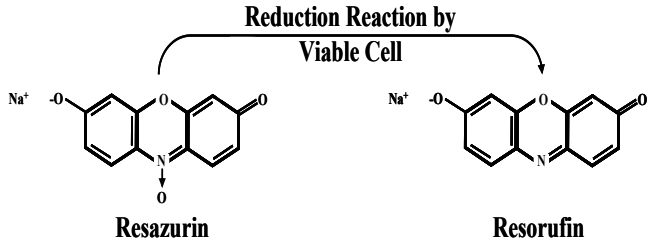

Fig. 1- CellTiter-Blue® Cell Viability Assay [14]

Reported here is a novel method for monitoring live bacteria propagation over time with the commercially available CellTiter-Blue® Cell Viability Assay modified for high throughput quantitative screening by kinetic measurements. By increasing the incubation time and performing multiple, periodic fluorescent readings throughout the incubation period, we were able to detect and quantify concentrations ranging from less than 1 cfu/mL to greater than 10,000 cfu/mL from apple juice samples.

Materials and Methods

Bacteria

To assess the efficacy of the assay under optimal conditions, twenty-four hour cultures of *Escherichia coli* O157:H7 (ATCC #43895) were obtained by incubation in nutrient broth at 37°C and serially diluted in triplicate in 10 mL nutrient broth (Difco #234000). To assess the system's ability to detect low concentrations of viable bacteria, dilutions ranging from 10^{-4} to 10^{-10} were tested. To confirm applicability to other likely contaminants, the gram-negative coliforms *Shigella flexneri* (ATCC #12022) and *Klebsiella pneumonia* (ATCC #13883) as well as the gram-positive *Enterococcus faecalis* (ATCC #19433) were similarly prepared. Samples were then centrifuged at 10,000 rpm for 10 min and resuspended in 100 uL, allowing assay performance on the entire population of bacterium present in each of three samples. Identical samples were also plated directly on typticase soy agar to extrapolate the true concentration of bacterium. Negative controls included killed bacteria obtained by treating full concentration samples with 70% isopropanol and autoclaved nutrient broth.

Detection and Quantification of Viable Bacteria

Each triplicate sample was prepared in accordance with manufacturer protocol at a ratio of 20 uL CellTiter Blue reagent per 100 uL bacteria suspended in nutrient broth and the mixture was distributed to wells in a white 96 well plate (Perkin Elmer #6005290). To minimize evaporation and cross contamination, plates were covered with an optically clear seal designed for fluorescent detection (Excel Scientific TS-RT2-100). Serial dilutions (10^{-2}-10^{4} cfu/mL) of *Escherichia coli* O157:H7 were assayed in nutrient broth at 37°C and fluorescent signal (excitation 560 nm, emission 590 nm) was recorded in ten minute increments for ten hours using kinetic observation on a Victor 3 fluorometer (Perkin Elmer 1420). Results were then exported to excel where time-to-threshold (TTT) was determined as the time in minutes required for signal to reach a threshold of 100,000 RFU or greater. This threshold is indicative of approximately four times the average of the background signal emitted by negative nutrient broth controls.

Post-screening Differentiation of Bacterial Species

Upon detection, all samples were plated by streaking on selective ChromAgar for detection and differentiation of *Escherichia coli and* other coliforms (ChromAgar #EE222) immediately after performance of the CellTiter Blue assay to confirm species identity. This method was also repeated in the absence of the CellTiter Blue assay to assess the effect of the resazurin reagent on total cell counts.

Detection of *E. coli* O157:H7 in Apple Juice

To assess the ability of the assay to detect *Escherichia coli* O157:H7 in apple juice, bacteria was serially diluted in 100 mL to contain between 0.1 and 1000 bacteria per mL Samples were then centrifuged and resuspended in 100 uL nutrient broth prior to performance of the modified CellTiter Blue Assay as described above.

Results and Discussion

Detection and Quantification of Viable Bacteria

As the bacteria grew at an exponential rate, the fluorescent signal emitted by metabolic product resorufin similarly increased (Fig. 2). By reporting the time taken to cross the threshold signal, the initial bacterial concentration can be extrapolated. One sample containing 10^{-2} cfu/mL crossed the threshold after 9 hours incubation, suggesting a possibility of detection with further incubation, however, these results were not included for predictive modeling.

A significant quantitative relationship (R^2=0.964) was shown correlating initial bacterial concentration to TTT by setting a threshold at 100,000 rfu, or approximately four times that of the broth negative control (Fig. 3). Low concentrations of bacteria reached threshold levels in as few as 1.5 hours for 10^{4} cfu/mL and just under 9 hours for 10^{-1} cfu/mL. Signal for cells inactivated with 70% isopropanol and autoclaved nutrient broth remained below threshold through-

out detection.

Results from additional bacterial species yielded similar trends when compared to the predictive model based on *Escherichia coli* O157:H7 data (Fig. 4). Although further exploration to address variability in growth rates cross-species may be required to confirm applicability of quantitative capabilities, these results proved the ability of this novel approach to detect viable bacteria of several types.

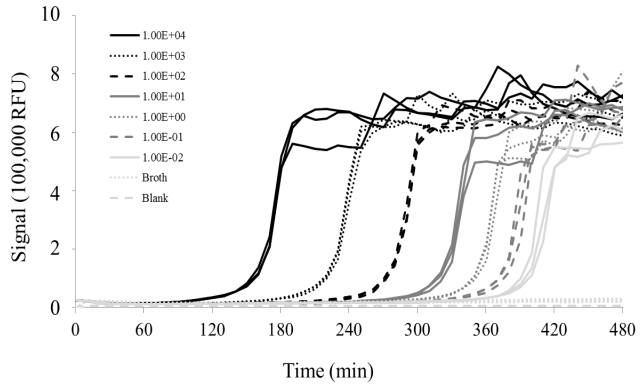

Fig. 2- Exponential growth over time of resorufin fluorescent signal product as a result of metabolism of resazurin by *Escherichia coli* O157:H7 (10^{-2}–10^4 cfu/mL). Signal from negative controls remained less than the set threshold after eight hours incubation

Post-screening Differentiation of Bacterial Species

All samples detected as positive for bacteria growth were immediately plated for differentiation. Incubation on ChromAgar plates produced expected mauve colonies indicative of *Escherichia coli* O157:H7 growth. *Escherichia coli* O157:H7 samples grown in nutrient broth only after Cell Titer Blue assay were indistinguishable in appearance and relative colony concentration. *Shigella flexneri* and *Klebsiella pneumonia* samples showed minimal blue colonies associated with gram-negative, non-*Escherichia coli* species. Isopropanol-inactivated bacteria and gram-positive *Enterococcus faecalis* showed no growth.

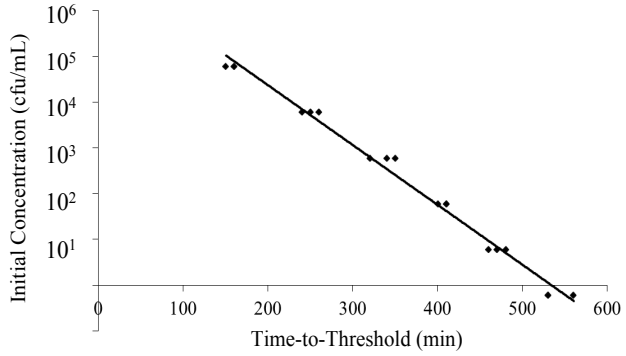

Fig. 3- Predictive model derived from *Escherichia coli* O157:H7 serially diluted in nutrient broth with final concentrations ranging from less than 1 cfu/mL through greater than 10^4 cfu/mL ($y=10^7e^{-0.03x}$, R^2=0.9888). Time-to-threshold increased as initial bacterial concentration decreases.

Detection of *E. coli* O157:H7 in Apple Juice

Apple juice samples spiked with 1-10^3 cfu/mL of viable *Escherichia coli* O157:H7 were successfully detected in less than nine hours by CellTiter Blue screening (Fig. 5). Of the triplicate apple juice samples containing 10^{-1} cfu/mL, only one crossed the signal threshold. TTT values for all samples were plotted against the predicative model previously developed revealed a high correlation between initial bacterial concentrations expected by the model (Fig. 6) and those observed (R^2=0.9845).

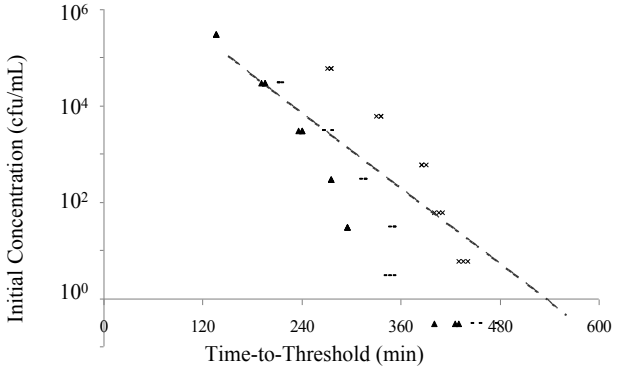

Fig. 4- Relationship between TTT and initial bacterial concentration for *Shigella flexneri* (x), *Klebsiella pneumonia* (▲) and *Enterococcus faecalis* (-) showed similar trends to that of Escherichia coli O157:H7 (---).

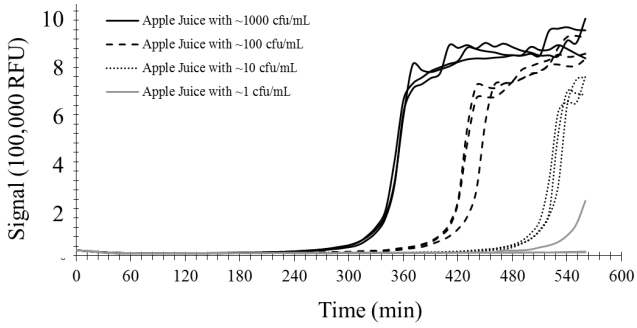

Fig. 5- Exponential growth over time of resorufin fluorescent signal product as a result of metabolism of resazurin by *Escherichia coli* O157:H7 (1 – 10^3 cfu/mL) in apple juice. Signal from negative controls remained less than the set threshold after eight hours incubation

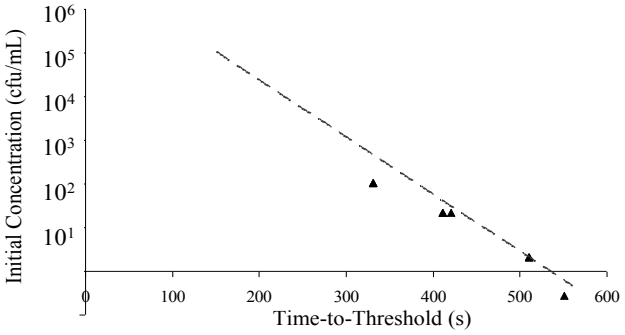

Fig. 6- Initial bacterial concentration as determined by total plate counts of *Escherichia coli* O157:H7 in apple juice were statistically similar to concentrations derived by predictive model from *Escherichia coli* O157:H7 serially diluted in nutrient broth (R^2=0.9845).

Conclusions

The assay reported here provides quantitative detection of low-level bacterial contaminants including *Escherichia coli* O157:H7. Although additional testing is required to specifically characterize a contaminant once detected by this high-throughput method, the real-time screening capabilities of the reported assay make it ideal for samples, such as apple juice, where sterility is expected and contamination is a rare anomaly. Rather than invest resources to specifically test hundreds of samples to reveal one or two positive results, this inexpensive and labor-saving application allows a significant increase in throughput with no loss of sensitivity.

In addition to the reported utility of this method for apple juice monitoring, future applications of this platform will prove invaluable for a number of inquiries including but not limited to clinical monitoring of blood and other sterile bodily fluids, specific detection of viable bacteria using selective broths and real-time studies of propagation of bacteria in the presence of antibiotics and other toxic additives.

Acknowledgements

Special thanks to Maxim Shusteff for editorial support, to the Science, Mathematics, and Research Transformation (SMART) scholarship for funding and to Lawrence Livermore National Laboratories for materials and technical support.

References

[1] FAS/USDA (2005) *World Apple Juice Situation: Global Apple Juice Production Continues to Set New Record, Trade To Remain Strong in MY*. *Horticultural & Tropical Products Division,* 1-5.

[2] FAS/USDA (2008) *Global Concentrated Apple Juice. World Markets and Trade*, 1-4.

[3] Fonsah E.G., Muhammad Andrew (2008) *Journal of Food Distribution Research*, 39(1), 57-61.

[4] Tribst A.A., Sant'Ana Ade S., de Massaguer P.R. (2009) *Crit. Rev. Microbiol.*, 35(4), 310-339.

[5] Dey B.P.L.C.P. (1998) *Microbiology Laboratory Guidebook*, 1, 3*rd edn*.

[6] Zelada-Guillen G.A., Bhosale S.V., Riu J., Rius F.X. (2010) *Anal. Chem.*, 82(22), 9254-9260.

[7] Nyquist-Battie C., Frank L.E., Lund D., Lim D.V. (2004) *J. Food Prot.*, 67(12), 2756-2759.

[8] Nyquist-Battie C., Mathias L., Freeman L.E., Lund D., Lim D.V. (2005) *J. Immunoassay Immunochem.*, 26(4), 259-271.

[9] Yamaguchi N., Sasada M., Yamanaka M., Nasu M. (2003) *Cytometry A.*, 54(1), 27-35.

[10] Ripp S., Jegier P., Johnson C.M., Brigati J.R., Sayler G.S. (2008) *Anal. Bioanal. Chem.,* 391(2) 507-514.

[11] Rompre A., Servais P., Baudart J., de-Roubin M.R., Laurent P. (2002) *Journal of Microbiological Methods*, 49(1), 31-54.

[12] Hsu C.F., Tsai T.Y., Pan T.M. (2005) J. Clin. Microbiol., 43(6), 2668-2673.

[13] DeCory T.R., Durst R.A., Zimmerman S.J., Garringer L.A., Paluca G., DeCory H.H., Montagna R.A. (2005) *Appl. Environ Microbiol.*, 71(4), 1856-1864.

[14] Promega CellTiter-Blue® (2009) *Cell Viability Assay. In: Technical Bulletin*., TB317.