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Evaluation of Sample Disruption Techniques for High Throughput Extraction of Live *Escherichia Coli* and Recombinant DNA from Spinach

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The Homogenizer Company™

Introduction

The detection and enumeration of pathogens in food is important to ensure the safety of foods throughout the food supply chain. Government agencies and food companies use microbiological analysis to monitor contamination and risks within their food supply. Traditional microbiological methods for detecting microorganisms in food are based on culturing followed by standard biochemical identification. These traditional methods are typically simple and inexpensive but can be time consuming and can require up to a week for preliminary and confirmation testing. PCR rapid methods with high sensitivity and specificity have been developed for pathogen detection. Samples are prepared by homogenizing in a stomacher and incubated in a selective enrichment media. Since the advent of bead mill technology, the time and efficiency of homogenizing a variety of sample matrices has drastically improved. This is achieved through the high speed shaking of a sample against small beads inside a sample vial. The high speed impact of the beads against the sample produces efficient grinding and can process a variety of sample matrices in less than half the time of traditional methods. Bead beating in conjunction with PCR has the potential to speed the sample testing process by processing the samples faster and eliminating the need for sample incubation.

Herein, spinach samples were inoculated with a serial dilution of known levels of recombinant *Escherichia coli* expressing green fluorescent protein (GFP) and processed using both bead mill and rotor stator homogenizers. Live cell recovery was evaluated via standard plate counts and limit of detection of both methods were analyzed through PCR amplification.

Methods

Transformation: Competent DH5 alpha E. coli cells were transformed with the pGLO plasmid containing the GFP gene. The cells were spread onto LB/Amp/Ara plates and later observed under UV light for detection recombinant colonies. Two recombinant colonies were inoculated into two separate LB/AMP broth cultures and incubated overnight at 37°C.

Dilutions and Processing: After incubation, each culture was serial diluted by the following dilution factors $1x10^{-2}$, $1x10^{-4}$,$1x10^{-5}$, $5x10^{-6}$, $2.5x10^{-6}$ and $1x10^{-6}$ to produce twelve total control colonies. ~5g of store bought spinach was added to each dilution (total 12 inoculums). Prior to processing, 100 µl of control dilutions 1 $x10^{-5}$ and 1 $x10^{-6}$ were plated on LB/Amp/Ara plates to confirm colony count. Six spinach inoculums ($1x10^{-2}$ to $1x10^{-6}$) were disrupted on the Omni TH rotor-stator homogenizer with Omni Hard Tissue tips for 1 minute at medium speed and the other six spinach inoculums ($1x10^{-2}$ to $1x10^{-6}$) were disrupted on the Bead Ruptor 24 bead mill homogenizer at 4 m/s for 30 s in a 30 ml tube containing 6.3 g of 2.8 mm ceramic beads. After processing, 100 µl of each spinach homogenate was plated on LB/AMP/Ara plates. All plates were grown over night at 37°C and counted to determine the number of viable recombinant colonies.

pGLO Extraction: The pGLO plasmid was extracted from each of the twelve spinach homogenates using OMEGA BioTek's E.Z.N.A Bacterial DNA Kit. The DNA concentrations of each dilution was analyzed using a NanoDrop Spectrophotometer.

GFP Gene Detection: 5 ng of each of the twelve spinach homogenates were amplified using the below primers. These primers are designed to amplify 99.2% of the GFP gene.

Figure 1: Primers

Instrument	Sequence
GFP-F	5'-ATGGCTAGCAAAGGAGAAGAA - 3'
GFP-R	5' - GTAGAGCTCATCCATGCCATGTG - 3'

Figure 2: PCR Program

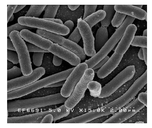
Amplifications were performed using the T100 Thermal Cycler by Bio-Rad.

	Instrument	Sequence
Hot Start/Denaturation	95	120
30 Cycles	95	30
	59	30
	72	60
Final Extension	72	5 min

PCR products were analyzed on a 2% agarose gel and stained using ethidium bromide. Spot volume intensities were analyzed via Bio-Rad's Gel DOC EZ Software.

Methods

pGLO

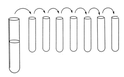


DH5α E.coli

E.coli Transformation

Grow up Recombinant E.coli O/N

Serial Dilute



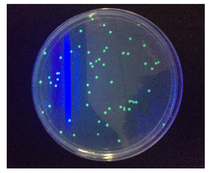
Process

Manual Homogenization
Omni Tissue Homogenizer (TH)

Automated Homogenization
Omni Bead Ruptor 24
Bead Mill Homogenizer

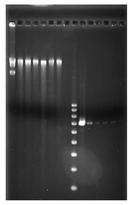
Bead Beating
2.8 mm ceramic beads

Standard Plating/Count



Extraction of pGLO plasmid

PCR Amplification/Gel Electrophoresis

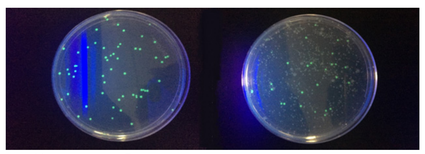


Results

Live Cell Recovery and Viability: Bead mill and rotor stator homogenizers are powerful mechanical homogenizers and are capable of vigorously homogenizing a variety of sample types. Here, an inoculated foodstuff was processed to complete homogenization while still maintaining microbial load and viability. After diluting, inoculation and processing, the homogenate was plated for standard plate count. The cell viability and recovery was compared to an unprocessed control. Recombinant GFP colonies fluoresce green under UV light. Both the rotor stator and bead mill can fully process spinach samples while also maintaining viable cells. Each processing technique recovered a similar amount of recombinant colonies as in the controls.

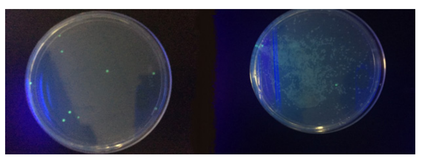
Rotor Stator Live Cell Recovery

Figure 4: $1x10^{-5}$ Dilution



Unprocessed Control Homogenate

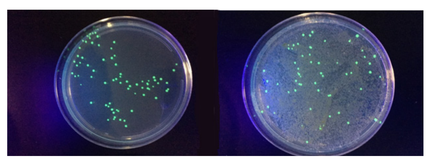
Figure 5: $1x10^{-6}$ Dilution



Unprocessed Control Homogenate

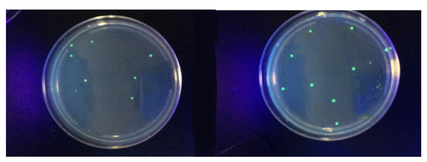
Bead Mill Live Cell Recovery

Figure 6: $1x10^{-5}$ Dilution



Unprocessed Control Homogenate

Figure 7: $1x10^{-6}$ Dilution



Unprocessed Control Homogenate

Results

Level of Detection: The pGLO plasmid was extracted and detected via end-point PCR after processing. Expected size of the pGLO plasmid was shown to be 714 bp. The CFU limit of detection was analyzed post amplification via gel electrophoresis. A decrease in spot intensity is shown with a decrease in number of CFUs.

Rotor Stator

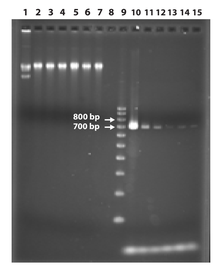


Figure 8: Lane 1: 2.5 kb Marker, 2-7: Genomic DNA, Lane 9: 100bp Marker, Lane 10: 10^{-2}, Lane 11: 10^{-4}, Lane 12: 10^{-5}, Lane 13: $5x10^{-6}$, Lane 14: $2.5x10^{-6}$, Lane 15: $1x10^{-6}$

Bead Mill

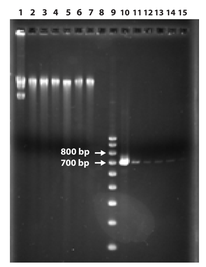


Figure 9: Lane 1: 2.5 kb Marker, 2-7: Genomic DNA, Lane 9: 100bp Marker, Lane 10: 10^{-2}, Lane 11: 10^{-4}, Lane 12: 10^{-5}, Lane 13: $5x10^{-6}$, Lane 14: $2.5x10^{-6}$, Lane 15: $1x10^{-6}$

Spot Volume Intensities: Spot volume intensities were analyzed via the Gel Doc EZ software (BioRad). The sensitivity was adjusted so that every band could be detected and analyzed.

Figure 10: Gel Spot Intensities

Dilution	Rotor Stator Spot Volume (Int)	Bead Mill Spot Volume (Int)
1 x 10^{-2}	1,246,320	1,554,420
1 x 10^{-4}	273,300	155,220
1 x 10^{-5}	125,880	58,230
5 x 10^{-6}	39,870	52,080
2.5 x 10^{-6}	51,960	41,730
1 x 10^{-6}	37,200	23,820

Spot Volume to CFU Analysis: The number of CFUs were analyzed by taking the log2 of the values and plotting against the gel spot volumes for each dilution sample. A best fit linear curve was applied. It is shown that the values are linear such that there is a correlation between the number of CFUs to the abundance of detected amplicon.

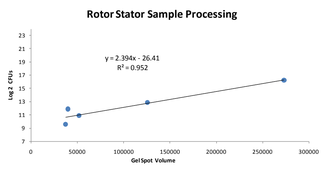


Figure 11: Rotor Stator CFU/Spot Volume Analysis

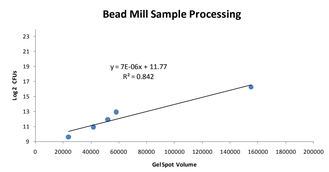


Figure 12: Bead Mill CFU/ Spot Volume Analysis

Conclusions

- Both the Bead Ruptor 24 and the TH are capable of fully homogenizing spinach without disrupting surface bacteria
- Both methods delivered a 76% cell recovery and a PCR detection limit of $1x10^{-6}$ CFUs/ml
- A linear relationship was demonstrated between the number of CFUs to quantity of DNA amplified via PCR, which is especially shown at the lower CFU limit
- The vigorous processing of the Bead Ruptor and TH potentially has the ability to eliminate the pre-enrichment step and allowing pathogen detection to be completed in half the time.