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Assessment of sanitation efficacy against *Escherichia coli* O157:H7 by rapid measurement of intracellular oxidative stress, membrane damage or glucose active uptake

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ABSTRACT

To reduce cross contamination with foodborne pathogens during washing procedures, sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) are widely used in the fresh produce industry. To ensure food safety and minimize excessive use of sanitizers and water during sanitation, it is critical to develop a rapid method to assess sanitation efficacy. This study examines the potential for employing oxidative stress, membrane damage and glucose uptake measurements to assess the antimicrobial efficacy of NaOCl (0–70.4 ppm) and H_2O_2 (0–1.6% v/v) toward *Escherichia coli* O157:H7. The relative amount of intracellular reactive oxygen species (ROS) was measured using Aminophenyl fluorescein (APF) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for cells treated with NaOCl and H_2O_2 respectively. Results from these ROS sensitive probes revealed a limited correlation between these oxidative stress measurements and inactivation of bacteria measured using the plate counting method. Sanitation experiments, conducted with contaminated pre-cut lettuce leaves in water, were also carried out. Measurement of the bacterial membrane integrity was assessed using the membrane permeable probe propidium iodide (PI) and by evaluating effects on active transport by the glucose transport system using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG). The results demonstrate that the relative increase in membrane permeability using PI correlated with NaOCl, but did not correlate with H_2O_2 induced reduction of bacterial survival quantified by the plate counting method. In contrast to the other approaches, monitoring the loss of glucose transport system function by measuring the uptake of 2-NBDG displayed strong correlation with the reduction of bacterial survival for both NaOCl and H_2O_2 treatment. Overall, the study demonstrated potential of glucose uptake measurements with 2-NBDG to serve as surrogate method for the traditional plate counting, which is the current gold standard used by the Food and Drugs Administration, to provide a faster analysis of sanitation processes.

1. Introduction

Sanitation of process water and equipment is a critical step to ensure the safety and quality of food (Gil, Selma, López-Gálvez, & Allende, 2009). The significance of sanitation is highlighted by the fact that numerous foodborne outbreaks have been traced back to pathogens surviving in food process facilities, resulting in cross-contamination of the food (Luo et al., 2011). Therefore, rapid assessment of sanitation efficacy is highly desirable for many operations in food processing. These rapid assays can improve food safety, operational efficiency, and reduce the excessive use of sanitizers and water during sanitation.

Food process operations are designed to be time and cost efficient with strict attention to maintaining safety. For fresh ready-to-eat products, such as produce, the timeline from harvest through processing, packing and shipment is often 1–2 days. Within these operations sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) are very effective oxidizers commonly used as sanitizers (Gil et al., 2009). To measure the efficacy of these sanitizers, the current monitoring approaches, such as the standard plate count, provide

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only retrospective assessments for bacterial contamination that often take several days to complete. Chemical based methods are available to measure sanitizer concentration, total carbon content, oxidation reduction potential (ORP), turbidity and pH of the aqueous phase (Le, Zhang, Lim, McCarthy, & Nitin, 2015; Suslow, 2004). However, these measurements are not effective in measuring bacterial reduction in complex environments because of potential fouling of electrodes as well as interference due to organic matter (Suslow, 2004). Improvement would be gained by rapid assays that directly measure the effectiveness of sanitizers on target bacteria based on robust technologies that function in complex environments such as wash water flumes and associated produce sanitation equipment. A rapid assay that would measure the real biological effect on the bacterial target rather than simply a chemical parameter of the wash water, would be definitely well accepted by the market. With this motivation, prior studies have measured changes in various physiological markers in bacterial cells using fluorescence spectroscopy, flow cytometry, and imaging (Baatout, De Boever, & Mergeay, 2006; Kramer & Muranyi, 2014; Wang, Claeys, van der Ha, Verstraete, & Boon, 2010). The physiological markers evaluated in prior studies included membrane damage (membrane permeabilization, membrane potential, and intracellular pH) and reduction in enzymatic activity such as esterase activity. These results were correlated with standard plate counting method. Based on these outcomes, it was suggested that physiological measurements have a potential to predict the efficacy of sanitation, but the key challenge was in the identification of an optimal biomarker for a selected sanitizer compound (Wang et al., 2010). Prior studies measured the effect of sanitizers on membrane damage and enzymatic activity, but the knowledge from studies that directly measured bacterial oxidative stress induced by sanitizers in bacteria is limited (Baatout et al., 2006; Kramer & Muranyi, 2014; Wang et al., 2010). Since antimicrobial activity of most of the current sanitizers including NaOCl and H_2O_2 is based on oxidative stress induced in bacteria, direct assessment of oxidative stress induced by sanitizers may be an indicator of sanitizer efficacy.

The objectives of this study were to evaluate a range of potential physiological measurements that may strongly correlate with reduction in bacterial survival by: (a) measuring bacterial oxidative stress induced by NaOCl and H_2O_2 using *Escherichia coli* O157:H7; and (b) measure changes in membrane damage and active uptake of glucose metabolite upon treatment of *E. coli* O157:H7 with the selected sanitizers with and without the presence of pre-cut lettuce. These measurements were compared with the standard bacterial plate counting method to evaluate the potential of developing one of these approaches into a technology for predicting reduction in bacterial survival, defined here as the ability to form a colony on standard growth medium. Currently, these oxidative stress measurements using free radical sensitive fluorophores such as 2′,7′-dichlorofluorescein and MitoSOX red have been mostly used in mammalian cells with limited application in bacteria (Bergamini, Moruzzi, Sblendido, Lenaz, & Fato, 2012; Hosoki et al., 2012; Nakazato et al., 2007). To investigate intracellular generation of reactive oxygen species (ROS) in bacteria, the Aminophenyl fluorescein (APF) dye or the hydroxyl radical sensitive dye 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCFDA) were chosen. APF is a fluorescein derivative, which is specific for measuring levels of $^{-}$OCl and *OH in cells upon exposure to HOCl or NaOCl (Choi & Hu, 2008). This ROS reporter dye has been previously used in mammalian cells to measure ROS generated by endogenous hypochlorous acid (Nakazato et al., 2007). Upon interacting with $^{-}$OCl ions and *OH radicals, the non-fluorescent APF dye is activated resulting in an increase in fluorescence signal intensity. Similar to the APF dye, H_2DCFDA is also a fluorescein derivative and is a cell permeable dye with specificity for hydroxyl radicals (Lyon, Brunet, Hinkal, Wiesner, & Alvarez, 2008). After permeating into cells, this dye molecule is cleaved by intracellular esterases which prevents the release of the entrapped dye from bacterial cells. Upon reaction with hydroxyl radicals, the non-fluorescent dye molecules in the bacteria are converted to a fluorescent DCF (2′,7′-dichlorodihydrofluorescein) form.

In addition to measuring oxidative stress in bacteria, two physiological markers were also selected in this study in order to compare changes in their signals and reduction in bacterial survival. The structural integrity of bacteria was assessed based on changes in membrane permeability by propidium iodide (PI). The intracellular uptake of glucose metabolite was selected as an indicator of metabolic activity, using a fluorescent analog of deoxyglucose. Specificity of intracellular uptake of this analog through glucose transporters has been validated in both bacterial and mammalian systems (Nitin et al., 2009; Yoshioka et al., 1996). Metabolic activity was selected in this study as previous studies in both bacterial and mammalian systems have indicated the significance of changes in metabolic activity to predict reduction in cell viability (Luo et al., 2014; Yoshioka et al., 1996). The results of this study will aid in development of rapid assays for the measurement of the efficacy of standard sanitizers in environments such as wash water of fresh produce avoiding time consuming procedures and costs associated with expensive food recalls.

2. Material and methods

2.1. Bacterial culture

A Rifampicin-resistant Shiga toxin negative derivative *Escherichia coli* O157:H7 ATCC#700728 (*E. coli* O157:H7) was kindly provided by Dr. Linda Harris (University of California, Davis, USA) and stored in liquid nitrogen with 50% v/v glycerol. For selective growth of *E. coli* O157:H7, Rifampicin (TCI America, Portland, OR, USA) was added to the medium at 50 μg/mL. The liquid nitrogen *E. coli* stock was streaked onto a Luria Bertani Agar (LBA) (Fisher Scientific, Pittsburg, CA, USA) plate and grown overnight at 37 °C. A colony was picked from the agar plate, cultured in Luria Bertani (LB) broth (Fisher Scientific, Pittsburg, CA, USA) for 16 h and then sub-cultured in LB broth for 16 h at 37 °C, 250 rpm agitation, before use in each experiment. In the subculture step, *E. coli* was grown to the stationary growth phase (OD = 1.4 at 600 nm). The bacteria were washed twice with phosphate buffered saline (PBS) (Fisher Scientific, Pittsburg, CA, USA) by centrifuging the sample at 3,100 × *g* for 10 min at room temperature and resuspending the sample in PBS for further analysis.

2.2. Exposure of Escherichia coli O157:H7 to sodium hypochlorite or hydrogen peroxide

The stationary growth phase bacteria were diluted in PBS to OD = 1 at 600 nm (1×10^8 Colony forming units (CFU)/mL) before each experiment. Sodium hypochlorite (NaOCl) (Sigma-Alrich, St. Louis, MO, USA) or hydrogen peroxide (H_2O_2) (Fisher Scientific, Pittsburg, CA, USA) were used as sanitizers for the inactivation. The final concentrations of NaOCl used were 0, 2.2, 4.4, 8.8, 17.6, 35.2, and 70.4 ppm, while the final concentrations of H_2O_2 used were 0, 0.1, 0.2, 0.4, 0.8, 1.6% v/v in 1 mL of reaction volume. These concentrations were chosen based on previous works that showed similar ranges being effective to achieve non-lethal, sub-lethal and lethal activity in water against *E. coli* (Le et al., 2015; Luo et al., 2011). After treatment with NaOCl for 10 min, sodium thiosulfate (Sigma-Alrich, St. Louis, MO, USA) with a final concentration of 0.1% w/v was added to the *E. coli* O157:H7 suspension to stop further activity of NaOCl. Similarly, catalase (Sigma-Alrich, St. Louis, MO,