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A novel microfluidic mixer-based approach for determining inactivation kinetics of *Escherichia coli* O157:H7 in chlorine solutions

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ABSTRACT

Determination of the minimum free chlorine concentration needed to prevent pathogen survival/cross-contamination during produce washing is essential for the development of science-based food safety regulations and practices. Although the trend of chlorine concentration-contact time on pathogen inactivation is generally understood, specific information on chlorine and the kinetics of pathogen inactivation at less than 1.00 s is urgently needed by the produce processing industry. However, conventional approaches to obtain this critical data have been unable to adequately measure very rapid responses. This paper reports our development, fabrication, and test of a novel microfluidic device, and its application to obtain the necessary data on pathogen inactivation by free chlorine in produce wash solution in times as short as 0.10 s. A novel microfluidic mixer with the capability to accurately determine the reaction time and control the chlorine concentration was designed with three inlets for bacterial, chlorine and dechlorinating solutions, and one outlet for effluent collection. The master mold was fabricated on a silicon wafer with microchannels via photopolymerization. Polydimethylsiloxane replicas with patterned microchannels were prototyped via soft lithography. The replicas were further assembled into the micromixer on glass via O_2 plasma treatment, and the inlets were connected to a syringe pump for solution delivery. To determine the kinetics of free chlorine on pathogen inactivation, chlorine solutions of varying concentrations were first pumped into the micromixer, together with the addition of bacterial suspension of *Escherichia coli* O157:H7 through a separate inlet. This was followed by injection of dechlorinating solution to stop the chlorine-pathogen reaction. The effluent was collected and the surviving bacteria cells were enumerated using a modified 'Most Probable Number' method. Free chlorine concentration was determined using a standard colorimetric method. The contact time was experimentally set by adjusting the solution flow rate, and was estimated by computational fluid dynamics modeling. Results showed that 1) pathogen inactivation was significantly affected by free chlorine concentration ($P < 0.0001$) and subsecond reaction time ($P < 0.0001$) and their interactions ($P < 0.0001$); and 2) the current industry practice of using 1.0 mg/L free chlorine will require more than 1.00 s total contact to achieve a 5-$\log_{10}$ reduction in an *E. coli* O157:H7 population, whereas a 10.0 mg/L free chlorine solution will achieve 5-$\log_{10}$ reduction in as little as 0.25 s. Information obtained from this study will provide critical insight on kinetics of bacterial inactivation for a broad range of sanitizers and produce wash operational conditions, thus facilitating the development and implementation of science-based food safety regulations and practices for improving food safety.

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1. Introduction

Since the 1993 *Escherichia coli* (*E. coli*) O157:H7 outbreak, when nearly 10,000 people were sickened, foodborne illness has become a major threat to the public health (CDC, 2011; Gould et al., 2013).

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Almost two decades later, microbial contamination remains one of the most serious challenges for assuring the safety of food supplies (FDA, 2012a; FDA, 2012b). In 2011, the Centers for Disease Control and Prevention (CDC) estimated that roughly 48 million people are sickened by food-borne pathogens each year, including 3000 cases ending in deaths. Among all food categories, fruits and vegetables have emerged as significant vehicles of foodborne bacterial pathogens because they are frequently consumed raw (CDC, 2011). Washing is an important step during fresh-cut produce processing as it removes the debris, soils, and produce latex released from the cut edges and maintains quality and shelf life of the final products, and can reduce 1–2 $\log_{10}$ CFU/g microbial loads. Sanitizers such as chlorine and peroxyacetic acid are often used during produce washing. Although the efficacy of those sanitizers on pathogen reduction is limited, the presence of sufficient sanitizer concentration in the fresh and fresh-cut produce wash water is critical for preventing pathogen survival in the wash water and its consequential transfer to the clean produce washed in the same tank/flume of water (Lopez-Galvez et al., 2009, 2010; Luo et al., 2011; Shen et al., 2013; Zhang et al., 2009).

Maintaining a high level of sanitizer in wash water is a practical challenge to the produce industry due to the rapid reaction of organic matter with sanitizers, especially the widely used hypochlorous acid (chlorine). Sanitizer, especially free chlorine, concentration usually declines rapidly during fresh produce wash operation directly as a result of its reaction with organic materials present in the wash water. It is important to note that as the wash process progresses the organic materials and the chlorine demand accumulates in the wash water (Zhou et al., 2014). Therefore, although chlorine can be replenished to certain degree by adding more concentrated sodium hypochlorite, large quantities of sodium hypochlorite will be needed to restore the required level. Thus it is critical to establish a minimum chlorine concentration that is effective for preventing pathogen cross-contamination, and also achievable by the industry. While a plethora of information is available regarding chlorine concentration on pathogen inactivation, specific information regarding the minimum chlorine concentration for preventing pathogen cross-contamination is scarce. We (Luo et al., 2011) reported that although no pathogen survival was noted in the wash water with at least 1.0 mg/L free chlorine and 30 s of leaf to chlorine exposure time, pathogen cross-contamination between adjacent leaf surfaces was observed during produce wash until the free chlorine level water reached 10 mg/L. Similar findings were also obtained during a pilot plant study (Luo et al., 2012). One major reason for this discrepancy may be related to reaction time as results on pathogen survival are often obtained with 30.00 s or longer exposure time, while pathogen cross-contamination could occur within a much shorter time frame. Therefore, it is important to determine the minimum chlorine concentration that results in the inactivation of pathogens quickly enough to avoid cross-contamination.

The effect of chlorine concentration and contact time on pathogen inactivation has drawn increasing research interest. Several studies have investigated the contact time from 5 s to 120 min using different chlorine concentrations (0.1–100.0 mg/L) (Gil et al., 2009; Gómez-López et al., 2014; Lopez-Galvez et al., 2009, 2010; Luo et al., 2011; Uyttendaele et al., 2004; Zhang et al., 2009). One study found that low chlorine concentration (0.1–0.5 mg/L) cannot reach 5-$\log_{10}$ reduction in water even after 120 min of treatment (Gil et al., 2009; Uyttendaele et al., 2004). Most recently, Shen et al. (2013) determined that at least 2 mg/L is required to inactivate pathogens with at least 5 s exposure time. In another study, a minimum of 5.0 mg/L of chlorine was found to be effective to inactive *E. coli* O157:H7 under industrial conditions (Gómez-López et al., 2014). The differences in minimum chlorine concentration in these studies can be attributed to different contact times. However, as a result of manual methods used in these studies, the shortest contact time that could be controlled was 5 s, and the study results include many other factors that at the scale of the study could not be easily controlled, including time variations associated with thorough mixing of the chlorine with pathogen, time variation associated with the mixing of chlorine stop solutions, and the time variations caused by manual handlings. Therefore, an approach is needed to better control the reaction time and chlorine concentration, in order to obtain the critical time-dose information for pathogen inactivation. Therefore, developing other ways to control and manipulate liquids in short time frames (<1 s) without other confounding factors is highly desirable.

The stopped-flow technique (Stocker et al., 2008; Sasaki et al., 2012; Wessel et al., 2013) has been widely used to provide rapid mixing of two or more solutions to study millisecond (e.g., 10^{-4}–10^{-2} s) kinetics of irreversible reactions, including bacterial inactivation. However, this technique has limited capabilities for studying pathogen inactivation in food processing systems, where the detection and quantification of food-borne bacteria requires both high throughput and low limit of detection (e.g., 10 CFU/g of below). Stopped-flow technique can only handle small sample flow rate (Gomez-Henz and Perez-Bendito, 1991) of up to 0.50 mL/min or 0.0083 mL/s of the solution. The flow rate was insufficient for collecting enough sample size for pathogen enumeration in food systems, and the improvement of throughput capacity requires either a larger mixing chamber or a parallel multiple channel system, which can substantially increase the mixing time and the cost of the system respectively. In addition, to detect or quantify changes of bacterial survival rate, this technology has mostly relied on spectrometric or photometric detectors for qualitative and quantitative analysis (Wessel et al., 2013), which are not cost-effective and have a high limit of detection (at least 10^4 CFU/g). Therefore, it is important to develop a novel micromixer that will fulfill the requirements of studying pathogen inactivation in food processing systems.

On the other hand, microfluidic mixers (micromixer) have been widely used for biological and biotechnological applications that require very brief controlled reaction times (Stroock et al., 2002). A micromixer has two unique advantages compared to the traditional vortex-based or manual mixing method: solutions can be mixed effectively in very short mixing length when passing through a microscale fluid channel (Kim et al., 2005; Stroock et al., 2002); the mixing time (contact time) can be set by adjusting the flow rate of the steady pressure-driven flows (Boskovic et al., 2011; Stroock et al., 2002). Different mixing microchannel designs were developed to improve mixing efficiency. In this study, we adopted three commonly used designs, Y-injection, Dean's vortex (serpentine), and chaotic mixers (Kim et al., 2005; Stroock et al., 2002; Whitesides, 2006) in our microfluidic mixer (Fig. 1A). In such devices, sample mixing is achieved by enhancing the diffusion effect between the laminar flows of two different fluids by incorporating Y-injection and Dean's vortex mixers (Kim et al., 2005; Stroock et al., 2002). The modification and design of the chaotic mixer can enhance folding, stretching and breaking of the laminar flows, which improves their mixing efficiency (Whitesides, 2006). The diminutive scale of the flow channels in microfluidic systems increases the surface-to-volume ratio, and provides instant and adjustable contact time (<1.00 s) by adjusting flow rate, which is therefore advantageous for investigating cross-contamination and sanitization in wash water. Therefore, the main objectives of this study were to design and fabricate a microfluidic mixer and to use it to determine pathogen inactivation kinetics via chlorine solution in sub-second range.