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Investigation on chlorine-based sanitization under stabilized conditions in the presence of organic load

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ABSTRACT

Chlorine, the most commonly used sanitizer for fresh produce washing, has constantly shown inferior sanitizing efficacy in the presence of organic load. Conventionally this is attributed indirectly to the rapid chlorine depletion by organics leading to fluctuating free chlorine (FC) contents. However, little is known on whether organic load affects the sanitization process directly at well-maintained FC levels. Hereby, a sustained chlorine decay approach was employed to study the inactivation of *Escherichia coli* O157:H7 under stabilized washing conditions. Chlorine solution was first incubated with organic load for up to 4 h, modeling the chlorination in produce washing lines. The FC level was then stabilized at five targeted values for sanitization study. Our study showed decreased sanitizing efficacy as the organic load increased. At 5 s residence time and pH 6.5, a minimum of 0.5 and 7.5 mg/L FC were needed to achieve a 5 log reduction at 0 and 900 mg/L chemical oxygen demand (COD), respectively. The decrease was more pronounced at lower FC, higher COD, higher pH, and shorter residence time values. The organics-associated interference with FC measurement and disruption of chlorine/bacteria interaction, together with the chlorine demand of concentrated inoculum per se, collectively resulted in inadequate sanitization. Finally, our results were compared with existing studies conducted under dynamic conditions in the context of different experimental settings. This study provided a feasible method for studying the bacteria/sanitizer interaction while ruling out the confounding effect from fluctuating FC levels, and it indicated the direct, negative impact of organic load.

1. Introduction

Fresh produce has emerged as substantial vehicles of foodborne bacterial pathogens, leading to increasing risk of illness and death in the US (Painter et al., 2013). Water washing is an essential step in the fresh and fresh-cut produce processing for removing the debris, soils, and produce latex released from the cut edges, thus maintaining the quality and shelf life of the final products (Simons, 2001). Sanitizers are commonly used during washing to prevent the release and transmission of pathogens (Gómez-López et al., 2014). Chlorine is the most widely utilized sanitizer due to its low cost and rapid bacterial inactivation. However, the efficacy of chlorine is significantly reduced by the presence of organic matters released from the produce (Gómez-López et al., 2014; Shen, 2014; Tomás-Callejas et al., 2012). A number of studies have been conducted under constantly changing conditions (i.e., produce and FC are fed batchwise or continuously) to simulate industrial washing and find out the underlying mechanisms (Luo, 2007; Zhang et al., 2009; Zhou et al., 2015). According to those studies, the inferior efficacy is largely attributed to the rapid depletion of FC by accumulating organic load, followed by periodical replenishment of FC in excess, which collectively lead to considerably fluctuating FC levels (Toivonen and Lu, 2013) (Weng et al., 2016). As a result, the FC level could potentially drop below the effective values, leading to inadequate sanitization efficacy.

However, it remains unclear whether organic load poses a direct impact on chlorine-based sanitization or, in another word, whether FC performs equally effectively at a stabilized level, with or without the organic load. Studies conducted under stable conditions enable the identification of roles for a specific parameter of interest, which is otherwise confounded by the irrelevant parameters that fluctuate under dynamic experimental settings. This provides valuable insights into the interaction among chlorine, organic load, and the bacteria. Ultimately, this would help identifying the key compound or interaction that compromises the sanitizing efficacy significantly, which benefits the

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technological advancement in preventing water-mediated cross-contamination. A recent study by Gómez-López et al. (2014) was a successful attempt with this regard, which demonstrated lower sanitization efficacy of chlorine at higher organic load at a same, stabilized FC level. However, knowledge gap remains since the sanitization time could not be precisely controlled with such an experimental design. In addition, it remains unknown whether the negative effect of organic load exists at different pH levels.

In this study, a feasible method based on sustained chlorine decay was developed to achieve precisely controlled and stabilized working parameters, including FC, chloramine, COD, and pH, all of which were representative for the conditions for fresh produce washing. The effects of organic load and pH on the depletion and stabilization of FC levels were firstly evaluated, using lettuce extract (LE) as a model source of organic load. This allowed us to find optimal strategies for stabilizing the working parameters. Thereafter, a series of sanitization experiments were performed under these well-controlled conditions, using non-pathogenic *Escherichia coli* O157:H7 as model bacteria. Two major questions were to be answered in this study. (1) Within a short time period, does free chlorine perform equally effectively, with or without the presence of organic load when maintained at the same level? (2) Is the impact of organic load on the sanitization pH-dependent? Finally, the data obtained from this study were compared with previous ones measured under dynamic washing conditions, and possible mechanisms underlying our observation were discussed in detail.

2. Materials and methods

2.1. Preparation of lettuce extract

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) was purchased from a local grocery store in College Park, MD. The lettuce was cut manually into pieces, pressed through a household juicer, and filtered through eight layers of cheesecloth. The filtrate was further centrifuged (9000*g*, 30 min, 4 °C) and then passed through a syringe filter with 1 μm pore size (Pall Corp., Port Washington, NY, USA). These procedures yielded a clear, dark green dispersion defined as lettuce extract (LE), which was frozen at − 20 °C for subsequent assays.

2.2. Chlorine decay test

Prior to the test, LE was thawed and determined for its chemical oxygen demand (COD) using the reactor digestion method (Hach method 10236). Sodium hypochlorite solution was diluted in water (deionized, same hereinafter) according to previous literature (Shen et al., 2012; Zhou et al., 2014a) to achieve various initial free chlorine levels (FC_0) ranging from 50 to 320 mg/L (as Cl_2, measured by DPD [N,N-diethyl-*p*-phenyldiamine] free chlorine photometric method [Hach method 10069], same hereinafter). The resultant solution was adjusted to preset pH (3.0, 5.0, and 6.5) using 1 M H_3PO_4. The selection of the three pH values was based on the recommendation in a previous study (Yang et al., 2012). The two higher pHs (6.5 and 5.0) are typically found in lettuce processing facilities based on our recent field study (data to be published), while the lower one (3.0) represents an extremely acidic condition that was occasionally observed, due to poor pH adjustment or insufficient agitation. The purpose for using phosphorous acid was to avoid the interference from oxidant (e.g., HNO_3), unintended chlorine demand (e.g., from citric acid), or chloride (e.g., from hydrochloric acid). In addition, phosphorous acid-based acidulants such as T-128 have already been applied in commercial production of fresh produce (Yang et al., 2012).

After equilibrium at room temperature for 20 min, proper amounts of LE (typically 30 to 70 mL per liter chlorine solution) was introduced to achieve desired COD levels (450, 600, and 900 mg/L) and initiate the depletion process. Our preliminary study showed that the change in free/total chlorine composition followed a very similar pattern at 4 or 25 °C, although the rates of reaction were greater at higher temperatures. The pH of the mixture was monitored and maintained at the desired values (3.0, 5.0, and 6.5) in the first 10 min of experiment using 1 M NaOH, and it remained stable throughout the whole incubation process afterwards (preliminary data not shown). Samples of 2 mL were withdrawn periodically from the mixture and analyzed as follows. The DPD photometric analysis was performed as a conventional method to determine both free (Hach method 10069) and total chlorine (Hach method 8167) levels. Total chloramine was calculated as the difference between total and free chlorine (Zhou et al., 2014b). Meanwhile, the indophenol method (Hach method 10241) for FC was used for comparison, because it was significantly less interfered by chloramines than DPD method according to Hach Company.

Upon completion of the depletion test, the FC level was plotted against incubation time. For each COD/pH combination, various FC_0 levels were tested, and the FC_0 that resulted in stabilized FC levels at 10 (with a tolerance of fluctuation by 5%, same hereinafter), 7.5, 5, 2.5, and 1.25 mg/L for at least 10 min was chosen for the following sanitization test.

2.3. Bacterial culture

A three-strain cocktail of *Escherichia coli* O157:H7 (RM4406, ATCC 43895, and ATCC 700728) with ampicillin resistance and green fluorescence protein marker was used in this study. The isolate RM4406 (lettuce outbreak isolate) was kindly provided by Robert Mandrell (U.S. Department of Agriculture, Agricultural Research Service, Albany, CA, USA). Isolates ATCC 43985 and ATCC 700728 were obtained from the American Tissue Culture Collection. The cells were cultured in 35 mL tryptic soy broth (TSB, Neogen, Lansing, MI, USA) containing 100 mg/L ampicillin at 37 °C in a reciprocal water bath for 20 h. Thereafter, the cell suspension was centrifuged twice at 3000 *g* for 5 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 30 mL of PBS (Zhang et al., 2015). A cocktail of inoculum with approximately 10^8 CFU/mL (determined by turbidity that had been correlated to standard plate counts) *Escherichia coli* O157:H7 was obtained.

2.4. Inactivation of Escherichia coli O157:H7 at different FC and COD levels

A batch of chlorine/LE mixtures prepared with the pH, COD, and FC_0 chosen in the "*Chlorine decay test*" section was incubated under constant stirring. As the FC level declined to 10 ± 0.5 mg/L, samples were drawn and tested by a method from Zhou et al. (2015) with slight modifications. In specific, four samples of 1.9 mL were transferred to four sterile scintillation vials (22 mL capacity). After 30 s of equilibration, 0.1 mL of the bacterial inoculum was introduced to each vial using a mechanical pipette and incubated for 5 or 20 s. Afterwards, 1 mL of neutralizing reagent (sodium thiosulfate, 50 mg/mL in water) was added to each vial, which reduced the free and total chlorine levels to zero instantly (preliminary data not shown). All these procedures were performed under constant stirring (1000 rpm) with a magnetic stirrer. The test was finished within 10 min, after which the FC level of the chlorine/LE mixture was re-checked to ensure successful maintenance. The same experiment was carried out as the FC level of the mixture declined sequentially to 7.5 ± 0.25, 5 ± 0.25, 2.5 ± 0.1, and 1.25 ± 0.08 mg/L. Negative (LE with same COD and pH as the treatment group, no FC) and positive (0.5 mg/L FC in pure water, same pH, no LE) controls were performed in parallel to validate the experimental procedures.

After the abovementioned treatments, bacterial survival was evaluated using a previously described most-probable-number (MPN)