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INACTIVATION OF ESCHERICHIA COLI O157:H7 ON GREEN BELL PEPPER BY CHLORINE DIOXIDE SIMULATING PACKINGHOUSES PROCESS

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ABSTRACT

In this study, packinghouses spraying disinfection process was simulated to compare the effectiveness of aqueous chlorine dioxide and sodium hypochlorite in reducing *Escherichia coli* O157:H7 on bell pepper surface. Bell peppers inoculated with 7.74 Log_{10} CFU of *E. coli* O157:H7 were placed in a rotary mixer (60 rpm), and sprayed with chlorine dioxide and sodium hypochlorite. Chlorine dioxide at 5 mg L^{-1} during 20-30 s reduces from 7.74 Log_{10} CFU of *E. coli* O157:H7 adhered on bell pepper to undetectable numbers, while the exposition with chlorine dioxide at pH 6 and 8 during 10 s was necessary to reduce 4.25 and 3.90 Log_{10} CFU, respectively. On the other hand, sodium hypochlorite at 200 mg L^{-1} at pH 6 reduced 2.89, 3.25 and 3.54 Log_{10} CFU/bell pepper at 10, 20 and 30 s, respectively. Spraying disinfection process using aqueous chlorine dioxide was more effective than sodium hypochlorite applications, therefor chlorine dioxide is an effective alternative to reduce *E. coli* O157:H7 on bell pepper surface.

Keywords: Disinfection, Sanitizers, Spray washing, Vegetables, Food safety

INTRODUCTION

The increasing worldwide tendency of including fresh produce on the diet has also been related to the occurrence of foodborne illnesses due to consumption of contaminated produce. In the United States, every year occurs 9.4 million episodes of foodborne illnesses, 55,961 hospitalizations and 1,351 deaths; much of these cases, reported as outbreaks, have been related to the consumption of contaminated fruits and vegetables, (**Scallan *et al.*, 2011; CDC, 2006; CDC, 2008; CDC, 2013**). Several studies using microbial source tracking indicate that cross-contamination offers to bacteria, parasites and virus a pathway to reach fruits and vegetables (**Beuchat, 1996; Ravaliya *et al.*, 2014; Harwood *et al.*, 2014**). Microbial contamination throughout the production chain may occur as a consequence of inadequate or scarce sanitation process during production chain, resulting in diverse punctual contamination sources, especially when Good Agricultural and Manufacturing Practices are not strictly followed (**Cummings *et al.*, 2001; CDC, 2002; CDC, 2003; CDC, 2005; CDC, 2008**).
Pathogenic bacteria, such as *Escherichia coli* O157:H7 has been responsible for at least 18 foodborne outbreaks in the last ten years in the United States, and nine of these were related to vegetables (**CDC, 2016**) causing economic losses up to $271 million dollars per year (**Hoffmann *et al.*, 2015**). In order to eliminate pathogens from fruits and vegetables surface, the Food and Drug Administration (FDA) published in the Code of the Federal Regulations (21CFR 173 315) a list of chemicals allowed for washing or assisting the peeling of fruits and vegetables (**US FDA, 2008**) in which chlorine-based disinfectants appear as an alternative for this application.
At present, sodium hypochlorite is the widest sanitizer used for washing fruits and vegetables in packinghouses. Several studies confirm the inactivation effect of chlorine. **Beuchat *et al.* (1998)** reduced the presence of *E. coli* O157:H7 on tomato surface in 2.08 Log_{10} colony forming units per cm^2 (CFU/cm^2) by spraying with chlorinated water at 200 mg L^{-1} at pH 6.8. **Chaidez *et al.* (2007)** were able to reduce 3 Log_{10} CFU/tomato of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) under laboratory conditions when chlorinated water was sprayed (sodium hypochlorite) at 200 mg L^{-1} at pH 7. However, chlorine has some undesirable characteristics, since alkaline pH and organic matter reduce its effectiveness; in addition, toxic by-products are generated when reacting with organic matter (**Beuchat, 1998**). Therefore, chloramines, ultraviolet light, gamma rays, ozone, peracetic acid and chlorine dioxide have been evaluated as an alternative to disinfect water and fresh produce external areas (**Rudd and Hopkinson, 1989; Banach *et al.*, 2015**).
Several studies about chlorine dioxide tested on fruits and vegetables surfaces reported reductions of pathogens from 4 to 7 log_{10} CFU (**Han *et al.*, 2000a; Du *et al.*, 2003**). Also, **Singh *et al.* (2002)** showed reductions of 1.72 Log_{10} CFU/g of *E. coli* O157:H7 when applied chlorine dioxide at 20 mg L^{-1} for 15 min on lettuce surface. In another study by **Pao *et al.* (2007)** evaluated chlorine dioxide at 20 mg L^{-1} on tomatoes surface by the immersion method, reaching reductions up to 5 Log_{10} CFU/cm^2 of *Salmonella enterica* and *Erwinia carotovora*. Recently, **Pao *et al.* (2009)** reduced *Salmonella* in 5 Log_{10} CFU/cm^2 from tomato surface by immersing in 5 mg L^{-1} of aqueous chlorine dioxide for 10 s, aided by rotating brushes. Even though aqueous chlorine dioxide is highly effective against pathogenic bacteria, its application in the fresh produce industry is scarce. We hypothesized that aqueous chlorine dioxide applied by spraying on green bell peppers has a better effect on microbial reduction than sodium hypochlorite. Therefore, the objective of this research was to compare the spraying application of chlorine dioxide and sodium hypochlorite in reducing *E. coli* O157:H7 inoculated on bell pepper surface simulating packinghouses disinfection operation.

MATERIALS AND METHODS

Green bell peppers (*Capsicum annuum* L.)

Large-size of green bell peppers (GBP) free of physical injuries, were directly collected from fields provided by a local grower. In order to avoid natural microbial presence, GBP were immersed in 300 mg L^{-1} of chlorine for 2 min as described by **Chaidez *et al.* (2007)**. Subsequently they were immersed in sodium thiosulfate solution (1%) for 1 min. Finally, the bell peppers were rinsed with sterile distilled water and dried inside of a biosafety cabinet for 30 min at room temperature (~ 28°C), and then placed in sterile bags and stored at 10°C for 24 h prior to the experiment.

Inoculum preparation

A strain of *E. coli* O157:H7 (CECT 4076) was used for the assays. Previous to the experiment, the strain was subjected to confirmation by multiplex PCR, amplifying the genes *hly*A, *eae*A, *fli*C, *stx*1 and *stx*2 as described by **Wang *et al.* (2002)**. Once confirmed, *E. coli* O157:H7 was grown in Trypticase Soy Broth (BD Bioxon, Mexico) and incubated at 37°C for 24 h with constant agitation and centrifuged at 13,800 *x g* for 10 min at 4°C. The supernatant was discarded and the cell pellet was washed twice to minimize non-cellular components associated with the solution and after diluting using phosphate buffered solution (PBS) 0.01 M. Appropriate dilutions were spread plate on mFC agar (DB Difco Laboratories, USA) with rosolic acid [10 mL of a 1% solution in 0.2N NaOH per litter], and incubated at 37°C for 24 h yielding an inoculum of 9.3 Log_{10} CFU/mL.

Disinfectants preparation

First, chlorine dioxide gas was generated with a Chlorine Dioxide Plant (Bello Zon® type CDKc 170, Prominent®) generator, using as precursors hydrochloric acid at 30% and sodium chlorite at 25% under the manufacturer specifications. Second, chlorine dioxide gas was dissolved in 20 L of sterile distilled water in an amber airtight container, and subsequently adjusted to 2 and 5 mg L^{-1}; whereas for the preparation of chlorine solutions, sodium hypochlorite (Clorox® 5%) was used and adjusted to 200 mg L^{-1} by diluting it in sterile distilled water. The concentrations of chlorine dioxide and sodium hypochlorite were determined by using DPD (N,N-diethyl-p-phenylenediamine), method 10126 and 8021, respectively, with a spectrophotometer 2010 DPD colorimetric method (DR-2010, Hach Co. Ames, IA, USA) as described by **APHA, (1998)**.
The pH solutions were adjusted with phosphoric acid to 6 and 8 (Pinnacle 530 pH meter, Corning, USA). Phosphate buffered solution was used as a control treatment in order to determine the dragging spray effect in both disinfectants and pH levels. The solutions were prepared 1 h before use.

Green bell peppers inoculation

Disinfected GBP were placed in a sterile bag containing 50 mL of a *E. coli* O157:H7 suspension (9.3 Log_{10} CFU/mL) and manually agitated for 1 min, then let them dry in a biosafety cabinet for 1 h at 25°C and stored at 4°C overnight to allow bacterial adherence to surface. In order to determine *E. coli* O157:H7 adhered concentration, the inoculated GBP were rubbed with a sterile sponge (*Nasco* Whirl-Pak® Speci-Sponge 18 oz) for 1 min, then each sponge was placed in a bag containing 25 mL of sterile distilled water and vigorously massaged for 1 min. Subsequently, an aliquot of 1 mL was taken to perform the microbial enumeration as described in the inoculum preparation section (**APHA, 2001**). This procedure was repeated three times.

Disinfectant´s treatments evaluation

Disinfection was carried out to simulate the passage of GBP through a washing conveyor belt in a packinghouse. Briefly, bell peppers were inoculated as described in the green bell peppers inoculation section. The bell peppers were placed in a rotary mixer spin (Rotamix RKVSD, ATR, MD, USA) at a speed of 60 rpm and sprayed from a distance of 30 cm, which is the distance that the sprinkler is placed in the conveyor belt at the packinghouses (**Chaidez *et al.*, 2007**). Each disinfectant solution was applied for 10, 20 and 30 s at 25°C and the spent volumes sprayed were 19.5, 38.3 and 57.45 mL, respectively (Table 1). In order to stop the disinfectant bactericidal activity and to quantify the bacterial concentration left on each GBP after treatment these were individually rubbed with a sponge containing sodium thiosulfate (0.1%), which were then placed in a sterile bag. Later, a 1 mL-aliquot was taken from the bag (as described in green bell peppers inoculation section) to perform the microbial enumeration as described in the inoculum preparation section (**APHA, 2001**). This procedure was done in triplicate.

Table 1 Treatments applied on bell pepper inoculated with *E. coli* O157:H7.

Treatment	Concentration (mg L^{-1})	pH		Contact time (s)/Volume (mL)		
Control	0					
Chlorine dioxide	2	6	8	10/19.5	20/38.3	30/57.45
Chlorine dioxide	5					
Sodium hypochlorite	200					

Statistical Analysis

A three-factor completely randomized design in triplicate was established. The analysis of variance (ANOVA) was performed using the MINITAB Software version 14.0. Comparisons between means were determined using Tukey test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Disinfectant activity of sodium hypochlorite on GBP

The use of sodium hypochlorite and chlorine dioxide for reduction of *E. coli* O157:H7 from bell pepper surface, showed different and significant results according to disinfectant product (p=0.000), pH (p=0.013) and time of contact (p=0.000), furthermore, the interaction pH-disinfectant showed significant differences, indicating that lowering pH values can negatively affect the bactericidal effect of both disinfectants [Table 2].

Table 2 Interaction pH-disinfectant for the reduction of *E. coli* O157:H7

Product	pH	Reduction (Log)	p Value
NaClO	6	3.23	0.0000
	8	1.72	
ClO_2	6	6.58	0.0000
	8	6.56	

The application of sodium hypochlorite at 200 mg L^{-1} and pH 6 on inoculated bell peppers showed reductions of 2.89, 3.25 and 3.54 Log_{10} CFU/GBP at contact times of 10, 20 and 30 s, respectively. However, at pH 8 the bactericidal activity of sodium hypochlorite against *E. coli* O157:H7 was negatively affected, being necessary 30 s of contact time to achieve maximum reductions of 2.82 Log_{10} CFU/GBP (Figure 1), suggesting that pH 6 favours better conditions to obtain free chlorine and disinfectant effect. Results for sodium hypochlorite at pH 6 were similar to that obtained by **Chaidez *et al.* (2007)** whom achieved 3 Log_{10} CFU reduction of *S.* Typhimurium attached to tomato surfaces by spraying 200 mg L^{-1} of sodium hypochlorite for 30 s at pH 7. In this work, a higher reduction was achieved probably because of the acidic pH of the solution, which exerted lethal activity of chlorine solutions given by the amount of hypochlorous acid (HOCl) as free chlorine at pH closer to 6 (**Beuchat, 1998**).
In a similar study, **Beuchat *et al.* (1998)** inoculated *E. coli* O157:H7 on tomatoes disinfecting them by spraying 5 or 6 times with chlorinated water at 200 and 2000 mg L^{-1} at pH 6.8 letting chlorine to act for 1, 2, 3, 5 and 10 min, achieving a maximum reduction level at 5 min (2.08 and 2.06 Log_{10} CFU/cm^2 at 200 and 2000 mg L^{-1}, respectively), showing that chlorine provides a limited reduction of no more than 3 Log_{10} CFU/cm^2 of *E. coli* O157:H7. In this study, the reduction was higher than mentioned above despite that sodium hypochlorite was inactivated with sodium thiosulfate after disinfectant application, suggesting that pH 6 and the prolonged spraying could help to reduce the pathogen, since the reduction achieved was 3.25 and 3.54 Log_{10} CFU/GBP at pH 6 and contact time of 20 and 30 s, respectively. This may be attributed to the measuring dimensions by cm^2 by **Beuchat *et al.* (1998)** and measures by complete green bell pepper in our study.

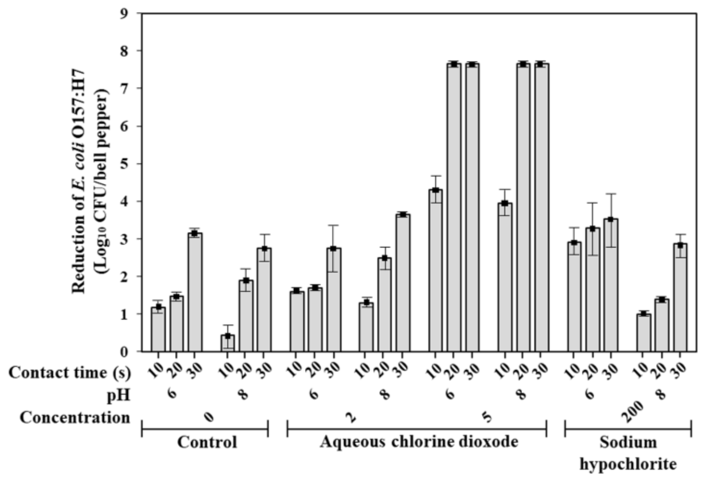

Figure 1 Effect of sodium chlorine and aqueous chlorine dioxide in reducing *E. coli* O157:H7 on green bell pepper. Control = 0; Chlorine dioxide = 2 mg L^{-1} and 5 mg L^{-1}; Chlorine = 200 mg L^{-1}. Values are mean ± standard error (n=3).

Disinfectant activity of chlorine dioxide on GBP surface

The use of chlorine dioxide resulted in the best option for microbial reduction on bell pepper surface. In this regard, the application of 5 mg L^{-1} chlorine dioxide achieved the major reductions of *E. coli* O157:H7, showing at pH 6 and pH 8, reductions of 7.74 Log_{10} CFU/GBP at contact times of 20 and 30 s, respectively. Interestingly, the time of 10 s showed favourable reductions of 4.25 and 3.9 Log_{10} CFU/GBP at pH 6 and 8, respectively. Sodium hypochlorite treatments at 30 s and pH 8 reduced more than 2 Log_{10} CFU/GBP, as well as chlorine dioxide at 2 mg L^{-1} at pH 8 in 20 s. Based on results, the main factor affecting *E. coli* O157:H7 viability was the contact time (p=0.000), because the longer spraying time the greater reduction of the microorganism obtained. In regard to acidity, pH 6 was more effective (p=0.0126) in reducing the microorganism, however this effect was only observed in sodium hypochlorite but not in chlorine dioxide and control buffer solutions. Results indicate that disinfectant concentration levels, contact time and pH influenced the effectiveness of both disinfectants. It was also observed that aqueous chlorine dioxide at 2 mg L^{-1} can reduce *E. coli* O157:H7 from GBP surface at pH 6 in 30 s close to 3 Log_{10} CFU/GBP, while at pH 8 and contact time of 20 s were achieved more than 2 Log_{10} CFU/GBP of reduction. The effectiveness of chlorine dioxide treatments was improved using 5 mg L^{-1}, with noticeable effect by the pH of the solution.
Han *et al*. (2000b) inoculated *E. coli* O157:H7 on GBP and its population had a maximum reduction of 8.04 Log_{10} CFU when gaseous chlorine dioxide was applied at ,0.60 mg L^{-1} concentrations at 20°C for 30 min with a relative humidity of 90-95%. They concluded that gaseous chlorine dioxide concentration was an important factor in reducing the bacteria adhered to fresh produce surface. It is important to mention that subchronic effects (failure in thyroid metabolism by decrease of serum thyroxine) were observed in African Green monkeys when chlorine dioxide was administered in drinking water during 30-60 days, however these effects were observed after the fourth week of exposure at high dose of chlorine dioxide (100 mg/L); on the other hand, no toxic effects were observed in human volunteers who ingested 1 mg/L of chlorine dioxide in drinking water **(Bercz *et al*., 1982)**, suggesting that the exposure to low dose do not show significant adverse effects. It is important to mention also that in this investigation, the exposure was in the GBP at low concentrations of 2 and 5 mg/L during 10 to 30 seconds, further reducing the possible adverse effects in humans.
On the other hand, the phosphate buffered solution (control treatments) showed significant reductions. The highest reduction obtained with control treatment was 30 s at pH 6 achieving a reduction of 3.11 Log_{10} CFU/GBP, followed by treatment 30 s at pH 8 which reduced 2.72 Log_{10} CFU/GBP; however, there was no significant difference between both treatments (p=1), which can be a "dragging effect" of spraying water. However, during "dragging" the bacteria is only detached from the surface, however, it does not mean a bactericidal effect that ensure bacterial elimination, which is necessary to ensure the absence of pathogenic bacteria in rinsing water, given it probable re-usage for packinghouse activities or releasing in the environment, becoming in a potential source of contamination. As shown by **Pao *et al*. (2007)** whom proved that washing with water can remove the microbial load from the fruits surface, but these are able to survive in water, becoming it a contamination route.
A few studies have been done on washing fruits and vegetables simulating the spraying process in agricultural packinghouses. In this study both disinfectants achieved higher bacterial reductions than those established by the scientific advisory group of **US EPA, (1997)** which states that, in order to consider the effectiveness of a disinfectant, it has to reduce at least 2 Log_{10} CFU of the microorganism adhered to the surface of fruits and vegetables. In this study, reductions of *E. coli* O157:H7 were up to 7.74 Log_{10} CFU/GBP by chlorine dioxide at 5 mg L^{-1} and a contact time of 20 s regardless of pH. These results suggest that chlorine dioxide can be considered as a viable alternative to chlorine in green bell peppers by using the spray-washing technique in agricultural packinghouses.
The differences in reduction of this study compared to the previously mentioned may be due to different inoculation methods, as well as initial microbial concentration, contact time, type of fruit, disinfection system, disinfectant forms, and enumeration method. More research is needed to assess aqueous chlorine dioxide simulating spray-washing technique of fruit and vegetables against various microorganisms such as Gram (+) bacteria, plant pathogens, protozoan of importance in public health, and enteric viruses.

CONCLUSION

Chlorine dioxide can be considered as a viable alternative to conventional disinfection using Sodium hypochlorite in green bell peppers by the spray-washing technique in agricultural packinghouses. However, it is necessary to perform additional research focused on different vegetables and other public health-related pathogens, in order to validate the disinfection process using chlorine dioxide.

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