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ENHANCED CHLORINE EFFICACY AGAINST BACTERIAL PATHOGENS IN WASH SOLUTION WITH HIGH ORGANIC LOADS

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ABSTRACT

Chlorine is widely used as a sanitizer in commercial fresh-cut produce wash operations. However, chlorine reacts rapidly with organic materials, leading to potential pathogen survival when chlorine concentration nears depletion. This study evaluated a new process aid, T128, for its capacity to enhance chlorine efficacy against *Escherichia coli* O157:H7 in solutions with high organic loads. Chlorine solutions were prepared with either T128 or citric acid (control) to adjust pH. Survival of *E. coli* O157:H7 was assayed during washing of increasing amounts of shredded lettuce, or addition of lettuce juice as organic load. The application of T128 significantly reduced survival of *E. coli* O157:H7 when free chlorine in solution decreased to levels approaching depletion. In the presence of T128, no pathogen survival was observed with the free chlorine concentration as low as 0.05 mg/L, while 0.4 mg/L free chlorine was required to kill pathogens in the absence of T128 in the wash solution.

PRACTICAL APPLICATIONS

The foodborne illness outbreaks that plague the fresh-cut produce industry are a testament to the need for a more effective sanitizer to prevent pathogen survival and cross contamination in commercial produce wash systems with large influxes of organic matter. This paper reports the investigation of the effect of a new process aid, T128, on reducing the survival of *E. coli* O157:H7 in wash solutions in which chlorine is near depletion due to high organic load, and thus the potential to improve the safety margin of fresh-cut process control.

INTRODUCTION

While nutritional recommendations (USDA and USHHS 2010) have encouraged the public to increase their consumption of fresh fruits and vegetables, sporadic recurrence of nationwide foodborne illness outbreaks associated with leafy greens, melons and other fresh produce have led to consumer distrust with regard to some commodities (Calvin 2007; Fahs *et al.* 2009). Direct or indirect contamination by animal fecal material frequently has been cited as a source of bacterial pathogens on fresh produce during growth and harvest (Franz and van Bruggen 2008; Heaton and Jones 2008; Critzer and Doyle 2010). Contamination of fresh produce by bacterial pathogens can also occur after harvest if processing facilities are not adequately sanitized (Friesema *et al.* 2008; Hanning *et al.* 2008). Leafy green producers and processors traditionally have relied upon various sanitizers, in double or triple washes, to reduce potential contamination of fresh-cut produce by microbial pathogens. Several chemical sanitizers are commercially available for fresh-cut produce washes, including acidified sodium chlorite, peroxyacetic acid, ozone and chlorine dioxide; however, chlorinated water remains the most commonly used sanitizer for fresh-cut produce processing because of its minimal impact on the nutritional and aesthetic quality of the product, its established ability to kill pathogens in suspensions and low application cost (Gonzalez *et al.* 2004; Gil *et al.* 2009; Lopez-Galvez *et al.* 2009). The current sanitizer washing-based intervention strategies have had limited success for inactivating bacterial pathogens attached to fresh produce surfaces. Most of the available sanitizers typically reduced experimentally inoculated bacterial pathogens by

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1–2 $\log_{10}$ units (Parish *et al.* 2003; Gil *et al.* 2009). The strategic and critical importance of sanitizers to prevent potential pathogen cross contamination during produce processing has been emphasized repeatedly in recent years (Lopez-Galvez *et al.* 2009). Therefore, one of the primary benefits of sanitizer application during fresh-cut processing is to prevent potential in-plant cross contamination while maximally reducing existing contamination.

Hypochlorous acid is the most efficacious form of chlorine, also referred to as free chlorine (Connell 1996; Zagory 2000). It is critically important that a relatively constant free chlorine level be maintained in fresh-cut produce washing process to ensure the efficacy of the sanitizer for microbial reduction and to prevent potential cross contamination. However, like many other oxidant antimicrobial agents, free chlorine is highly reactive with organic substances and can be rapidly consumed by organic material present in the washing solution (Zagory 2000; Luo 2007; Gil *et al.* 2009). Fresh-cut processing of lettuce results in the release of considerable amounts of latex from the wounded tissues into the washing solution, leading to sudden surges in organic matter that rapidly deplete free chlorine. These temporary decreases in free chlorine levels create opportunities for bacterial pathogens to survive and cross contaminate produce in the wash system (Gil *et al.* 2009). Simply increasing chlorine input cannot satisfactorily address this problem, because repeated addition of chlorine to wash water that is high in organic content will result in increased formation of toxic by-products, and off-gas in the processing environment, which can be a respiratory hazard for workers (Suslow 2001).

The wash aid, T128 was recently formulated by scientists in the produce industry for the purpose of stabilizing chlorine in wash water (Lemons 2009). It is composed of chemicals with Generally Recognized as Safe status (FDA 2009), including a common inorganic acid and polyethers. For commercial fresh-cut leafy green processing, T128 is added to chlorinated wash water using an automated dosing system, which maintains the pH at a preset value, typically 5.0. We previously reported that T128 significantly attenuated the depletion of free chlorine in washing solutions containing up to 2% soil, and, to a lesser extent, in washing solutions containing high concentrations of lettuce extract (LE) (Nou *et al.* 2011). In comparison to traditional lettuce wash solutions with chlorinated water, the application of 0.05–0.1% T128 with chlorinated water reduced the likelihood of cross contamination by *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium, without negatively impacting the organoleptic quality of fresh-cut iceberg lettuce (Nou *et al.* 2011). The main objectives of this study were: (1) to investigate the effect of T128 on *E. coli* O157:H7 survival in chlorine solution during the washing of increasing amounts of shredded lettuce with periodic replenishment of free chlorine; and (2) to explore the possible mode of action of T128 for reducing pathogen survival in solutions containing low levels of free chlorine.

MATERIALS AND METHODS

Plant Materials

Fresh iceberg lettuce (*Lactuca sativa* L.) was obtained from a local fresh produce wholesale establishment and used within 24 h. In preparation for shredding and juicing, soiled and damaged outer leaves were removed and the stem end was trimmed and cored. Iceberg LE was prepared using a juicer (Breville, Model BJE200XL, Juice Fountain, Shanghai, China) and passed through a 0.33-mm perforated polyethylene filter (Nasco, Ft. Atkinson, WI) to remove coarse particles before being used to modulate organic content in wash solutions. Shredded lettuce was prepared using a vegetable cutter (Hallde RG-400, Paxton Enterprises LLC, Shelton, CT) to slice the lettuce into 0.125-inch strips.

Bacterial Strain and Inoculation

A spontaneous nalidixic acid-resistant mutant of the non-pathogenic *Escherichia coli* O157:H7 strain ATCC 700728 was selected by repeated subculturing of the strain on a nutrient plate containing a gradient of nalidixic acid (Sigma, St. Luis, MO) from 0 to 100 mg/L. This mutant was used throughout the experiments in this report, after tests showed that its growth pattern was indistinguishable from that of the parental strain, and, that resistance was stable following multiple subcultures in the absence of nalidixic acid. Cultures of *E. coli* O157:H7 were grown in tryptic soy broth (TSB) (Becton Dickenson, Sparks, MD) overnight at 37C. Cells were harvested by centrifugation and washed twice in sterile phosphate buffered saline. Bacterial cells were directly inoculated into wash solution to obtain the concentration of 10^5 most probable number (MPN)/mL.

Bacterial Enumeration

E. coli O157:H7 in various solutions were enumerated using a modified MPN procedure (Nou and Luo 2010). Briefly, eight 4-mL aliquots of sampled solution (after dechlorination) were mixed with 1 mL of 5× TSB supplemented with sodium pyruvate (0.1%) (TSBP) in a high-capacity deep-well microplate (Axygen, Inc., Union City, CA) and subsequently10-fold serially diluted in 1× TSBP. After overnight incubation at 37C, growth of *E. coli* O157:H7 in each well was confirmed by arraying 3 μL droplets of the cultures on sorbitol MacConkey plates supplemented with cefixime (0.05 mg/L), potassium tellurite (2.5 mg/L) (Invitrogen, Carlsbad, CA) and nalidixic acid (50 mg/L) (nctSMAC). MPN values were calculated using an MPN calculator (Curiale 2004). The calculated detection limit by the MPN procedure is −1.52 log cfu/mL. In different experiments, sometimes, a smaller volume of sample solution was used, resulting in a lower detection limit, as indicated in the presented data.

Effect of T128 on *E. coli* O157:H7 Inactivation in Spent Wash Water

Chlorinated wash water solutions containing 35 mg/L free chlorine were prepared using 6% sodium hypochlorite (NaClO) solution (Clorox, Oakland, CA). The pH of the solution was adjusted to 3.0 using T128 (New Leaf Food Safety Solutions, Salinas, CA) or 6.5 using citric acid (industry standard practice). Two-pound (lb) portions of freshly shredded lettuce were repeatedly washed in 40 L solutions for 30 s with moderate manual agitation. A maximum of 19 washes were conducted with a total of 38 lbs of lettuce. Twenty milliliters of sodium hypochlorite was added to the solutions after 7 (14-lb) and 14 (28-lb) washes to simulate periodic chlorine replenishment during commercial fresh-cut produce wash. A 10-mL aliquot of spent water was collected following each wash to determine residual free chlorine concentration. The water samples were also used to determine the antimicrobial potential of the wash solutions by inoculating with *E. coli* O157:H7 cells and allowing for 30 s exposure time.

Effect of T128 on *E. coli* O157:H7 Survival in Wash Solutions with Chlorine Concentration Degraded by Organic Materials

Chlorinated wash water solutions containing 20 mg/L of free chlorine were prepared and adjusted to pH 5.0, 3.0 using T128 (test solutions), or pH 6.5, 5.0, 3.0 using citric acid (controls). Freshly prepared LE was added in 0.5% (v/v) increments from 0.5 to 4.0% to the wash solutions to create conditions simulating those that lead to chlorine degradation associated with increasing amounts of organic materials. Solutions were inoculated with *E. coli* O157:H7 and allowed a 30-s exposure before adding sodium thiosulfate-based dechlorinating reagent (Hach, Loveland, CO) to neutralize the remaining free chlorine. Survival of *E. coli* O157:H7 in each wash solution was subsequently determined.

Effect of T128 on Bacterial Survival in Low Chlorine Solutions without Organic Load

The ability of T128 to enhance the sanitizer efficacy of low levels of free chlorine against bacterial pathogens was examined by assessing survival of *E. coli* O157:H7 after treatment in distilled water containing very low levels of free chlorine, with no additional organic materials. Wash solutions containing 0.8 mg/L free chlorine were prepared and pH was adjusted to 6.5, 3.0 or 5.0 using citric acid, or 3.0 or 5.0 using T128. Solutions were serially diluted in twofold increments to 0.025 mg free chlorine/L. Solutions were inoculated with *E. coli* O157:H7 and allowed a 30-s exposure before adding chlorine neutralizing reagent. Determination of *E. coli* O157:H7 survival in each wash solution followed.

Additionally, the hypothesis that T128 alters bacteria cells, thus increasing their susceptibility to extremely low levels of free chlorine in washing solutions, was tested by exposing *E. coli* O157:H7 cells to T128 treatment prior to free chlorine. *E. coli* O157:H7 cells were harvested by centrifugation and equal volumes were treated for 10 min in one of three 0.85% saline solutions adjusted to pH 3.0 with citric acid or T128. Following treatment, saline solutions were removed by centrifugation and cells were resuspended in distilled water, before adding to wash solutions (~5 log cfu/mL) containing low levels of free chlorine (0.005 to 1.0 mg/L) for 1 min. Survival of *E. coli* O157:H7 in each wash solution was subsequently determined.

Experimental Design and Statistical Analysis

The experiments were conducted using factorial designs with three replications. *E. coli* O157:H7 populations were subjected to log transformation before statistical analysis. Data presented in Fig. 1 were analyzed as a two-factor

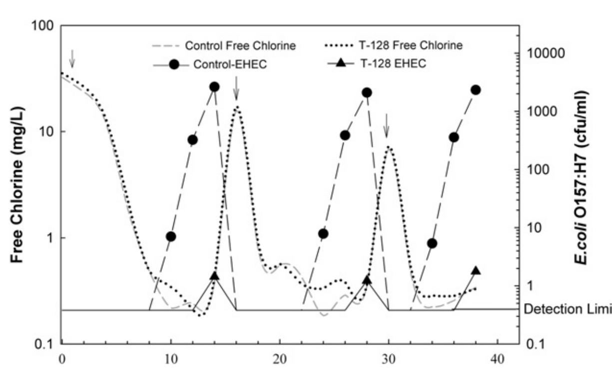

FIG. 1. EFFECTS OF ORGANIC LOADS ON FREE CHLORINE AND BACTERIAL SURVIVAL IN SPENT WASH SOLUTION. ORGANIC LOAD IS EXPRESSED AS AMOUNT OF SHREDDED LETTUCE (LBS) CONSECUTIVELY WASHED IN WASH SOLUTION. ARROWS INDICATE POINTS WHERE FREE CHLORINE WAS REPLENISHED BY ADDING NaClO. DATA POINTS WHERE CELL COUNT IS BELOW THE DETECTION LIMIT ARE NOT SHOWN. DATA REPRESENT THE AVERAGE OF THREE REPLICATIONS

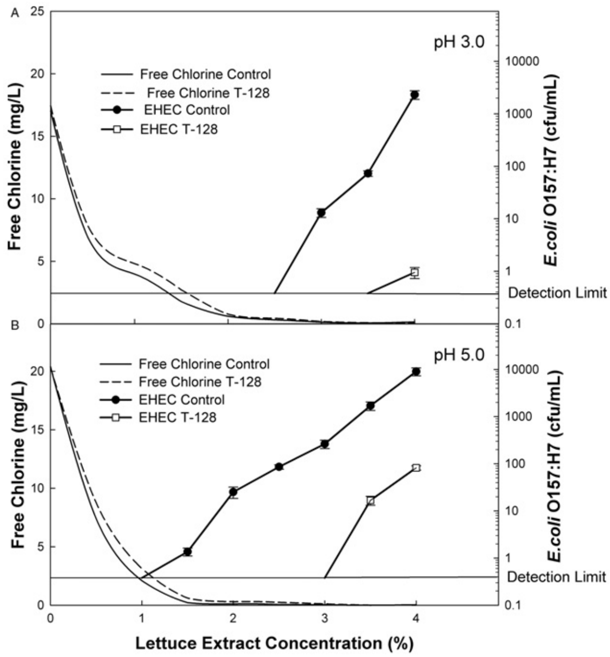

FIG. 2. EFFECT OF ORGANIC LOAD ON FREE CHLORINE AND BACTERIAL SURVIVAL IN WASH SOLUTIONS. ORGANIC LOAD IS EXPRESSED AS CONCENTRATION OF LETTUCE EXTRACT (%) IN WASH SOLUTION. DATA POINTS WHERE CELL COUNT IS BELOW THE DETECTION LIMIT ARE NOT SHOWN. DATA REPRESENT THE AVERAGE OF THREE REPLICATIONS

linear model with treatment and wash as the two factors. Because of the iterative nature of the washes, a repeated statement was included in the model using an unstructured (un) covariance parameter to relate the washes in each wash series. In each replication, the 7th, 14th and 19th washes were the only washes for which bacterial survival was found for all three treatments; hence, the statistical analysis focused on these three washes. Since bacterial survival for T128 treatments occurred only when free chlorine levels were at or below 0.025 mg/L, statistical analysis for T128 was performed only at these levels. Data presented in Fig. 2 were analyzed as one- or two-factor linear models using Proc Mixed with treatment as the first factor and LE% as the second factor, when two factors were used. Data were analyzed only where actual bacterial counts were available, i.e., for Fig. 2A at 4% LE for all three treatments (T128 pH 3, Control pH 3 and Control pH 6.5), and for the two controls at 3, 3.5 and 4% LE. For Fig. 2B, data were analyzed at 3.5 and 4% LE for all three treatments (T128 pH 5, Control pH 5, and Control pH 6.5), while bacterial counts for these two controls were compared at 2, 2.5, 3, 3.5 and 4% LE. Additional analysis was carried out to compare bacterial survival for the three controls at free chlorine levels of 0.2 mg/L and below. For all studies, assumptions of normality and variance homogeneity of the linear model were checked for the log-transformed data, and the variance grouping technique was used to correct for variance heterogeneity. When effects were statistically significant, means comparisons were performed with Sidak adjusted *P* values to maintain experiment-wise error $\leq$0.05.

RESULTS AND DISCUSSION

Survival of *E. coli* O157:H7 in Lettuce Wash Solutions as Impacted by T128 Application

Wash water is often recirculated and reused during commercial fresh-cut produce wash operations. Consequently, the water quality and free chlorine concentration decrease rapidly as increasing amounts of organic material, released from the cut produce, accumulate in the wash solutions (Luo 2007), thus leading to the potential for pathogen survival and cross-contamination as chlorine nears depletion (Luo *et al.* 2011). In this study, the dynamic changes of chlorine concentration during repeated wash of cut lettuce in the same chlorine solution, with periodic replenishment of free chlorine, were simulated. The effect of T128 on the survival of *E. coli* O157:H7 was assessed. As shown in Fig. 1, the free chlorine quickly decreased as shredded lettuce was washed, releasing exudates into the solution. The free chlorine concentration dropped from originally 35 mg/L to near zero after only four washes (8 lb shredded lettuce). Replenishing the solutions with the same amount of 6% NaClO as originally used (20 mL) after 7 and 14 washes restored free chlorine to 17 and 7.2 mg/L, respectively. However, these free chlorine concentrations were rapidly depleted following additional washes. This sequence of events and conditions highlights the futility of replenishing wash solutions with additional free chlorine-producing chemicals once the organic load reaches critical levels. In the presence of high organic load, significantly ($P < 000.1$) greater *E. coli* survival occurred in the industry standard pH 6.5 control (using citric acid) than in the T128 test solution. Survival of inoculated *E. coli* O157:H7 in the pH 6.5 control solution was observed after five washes, and was rapidly augmented as more lettuce was washed in the same solution. Low survival of *E. coli* O157:H7 in the test solution was detected after seven washes. A similar pattern of survival by *E. coli* O157:H7 was observed after free chlorine was replenished and additional lettuce was washed. Taken together, these results indicate that the test solution maintained higher bactericidal activity than the control as the organic loads increased and free chlorine neared depletion.

TABLE 1. SUSCEPTIBILITY OF *E. COLI* O157:H7 CELLS TO LOW LEVELS OF FREE CHLORINE IN DIFFERENT WASH SOLUTIONS

Free chlorine	Survival of *E. coli* O157:H7 (log cfu/mL)				
(mg/L)	Citric acid pH 6.5	Citric acid pH 3.0	T128 pH 3.0	Citric acid pH 5.0	T128 pH 5.0
0.8	ND	ND	ND	ND	ND
0.4	ND	ND	ND	ND	ND
0.2	–0.07 ± 0.44	–0.31 ± 0.23	ND	–0.20 ± 0.35	ND
0.1	1.19 ± 0.243	1.00 ± 0.20	ND	1.31 ± 0.15	ND
0.05	1.93 ± 0.09	1.71 ± 0.09	ND	1.96 ± 0.21	ND
0.025	3.75 ± 0.20	3.34 ± 0.12	1.18 ± 0.18	3.67 ± 0.14	1.81 ± 0.15
0	5.15 ± 0.12	5.11 ± 0.12	5.11 ± 0.12	5.18 ± 0.12	5.18 ± 0.12

Data represent the average of three replications ± the standard deviation.
ND, not detectable at the detection limit of –0.42 log cfu/mL.

Effect of T128 on *E. coli* O157:H7 Inactivation in Wash Solutions Containing LE

Since the aforementioned study used two different pH values (6.5 for control versus 3.0 for T128), additional experiments were conducted to compare *E. coli* O157:H7 survival in chlorine solutions with pH adjusted to the same levels using either T128 or citric acid. The LE was used in this study to provide better control of the organic load dosing than that provided by cut lettuce shreds in the wash solutions.

pH 3.0 Solutions At pH 3.0 and 3.0% LE, greater than 10 bacterial cells survived in the control solution when the free chlorine concentration approached depletion; and increased survival of *E. coli* O157:H7 occurred at LE concentration of 4.0% (Fig. 1A). In contrast, in the pH 3.0 T128 solution, no bacterial survival was observed at ≤3.5% LE; and ≤1 cfu/mL of inoculated bacteria was detected at 4.0% LE concentration. The significantly ($P < 0.0001$) reduced bacterial survival of *E. coli* O157:H7 in the presence of T128 at the same pH as the control suggests that the reduced pathogen survival in the wash solution is not caused by the low pH of T128.

pH 5.0 Solutions Significant bacterial survival was observed in the control solution when the LE concentration reached 2.0%, and large populations (10,000 cfu/mL) of *E. coli* O157:H7 survived when LE concentration reached 4.0% (Fig. 2B). However, no bacterial survival was detected in the 3.0% LE pH 5 test solution with T128, and survival of *E. coli* O157:H7 was approximately 100-fold less than for the citric acid control at 4.0% LE.

Overall Concentration of LE significantly affected survival for all treatments ($P < 0.0001$). Survival of *E. coli* O157:H7 in the presence of T128 was substantially reduced compared to the controls in each experiment. Under conditions of high organic load, the presence of T128 significantly enhanced the efficacy of the wash solution to inactivate bacteria relative to that of the control solutions ($P < 0.0001$) (Fig. 2). Without T128 in the wash solutions, regardless of pH, bacterial survival was enhanced, as soon as the free chlorine in solution was reduced to background levels by the introduction of high organic load. In contrast, bacterial survival was not observed under these conditions in the presence of T128, at either pH 3.0 or pH 5.0, even though the measured free chlorine content was reduced as much as in the controls; while only very low numbers of *E. coli* O157:H7 survived even in the presence of very high levels of organic matter.

Effect of T128 on Bacterial Survival in Low Chlorine Solutions without Organic Load

This study evaluated the effect of T128 on pathogen survival in wash solutions with free chlorine concentrations precisely controlled (via twofold serial dilutions rather than by reacting with organic materials). Side-by-side comparison of T128 and citric acid at pH 3.0 and 5.0 plus the pH 6.5 industry standard were performed. Survival of *E. coli* O157:H7 was significantly affected by chlorine concentration, pH and T128 application and their interactions (Table 1). No pathogen survival was detected when the free chlorine was ≥0.4 mg/L regardless of T128 application and pH (Table 1). However, when free chlorine was decreased to 0.2 mg/L, *E. coli* O157:H7 survived in the absence but not in the presence of T128 at all of the pH values tested ($P < 0.0001$). Additional decreases in the free chlorine concentration significantly increased the populations of *E. coli* O157:H7 surviving in the wash solutions in the absence of T128. However, in the presence of T128, no *E. coli* O157:H7 survival was detected until the free chlorine concentrations decreased to 0.025 mg/L in wash solutions. However, in the absence of any free chlorine in the wash solution, there was no difference in pathogen survival between T128 and the control at all pH levels tested.

Our previous experiments showed that T128 alone has a weak antimicrobial capacity. With high concentration (2.5%) and for extended reaction time (over 2 min), T128 can reduce microbial counts (Nou *et al.* 2011). However, the 30-s exposure time and the 0.05% T128 used in this study are far below the effective bactericidal T128 concentration and exposure time. Since some chemical treatments can interact with bacterial cells and increase cell susceptibility to extremely low levels of free chlorine in wash solutions, this possibility was further examined by pretreating *E. coli* O157:H7 cells with T128 before exposure to free chlorine. Results indicate that cell sensitivity to low levels of chlorine did not differ between bacteria pretreated with saline solution, with or without T128 (data not shown). This result suggests that T128 is unlikely to interact with bacterial cells independently of free chlorine in wash solutions. Thus, the observed enhanced antimicrobial activity of T128 at low levels of free chlorine may be attributed to the synergistic effect between T128 and low levels of free chlorine.

Maintaining effective free chlorine levels in a dynamic wash system during fresh-cut produce processing is a complicated procedure that requires continuous monitoring and interventions. Overapplication of free chlorine-generating chemicals is strongly discouraged because of the potential for formation of toxic chlorinated by-products and chlorine off-gas, both of which are hazardous to human health. However, frequent surges in organic load can result in rapid depletion of free chlorine in wash solutions, resulting in process control failure. In this study, we demonstrated that, in the presence of wash solutions containing T128, the potential for bacterial pathogen survival was reduced, and the mode of action is likely due to the synergistic effect between T128 and low levels of chlorine.

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