Food Microbiology 50 (2015) 102–108

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Control of spoiler *Pseudomonas* spp. on fresh cut vegetables by neutral electrolyzed water

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ARTICLE INFO

Article history:
Received 8 November 2014
Received in revised form
1 April 2015
Accepted 8 April 2015
Available online 17 April 2015

Keywords:
Neutral electrolyzed water
Ready to eat vegetables
Water disinfection
Spoilage *Pseudomonas* spp.

ABSTRACT

In the present study, we evaluated the antimicrobial activity of neutral electrolyzed water (NEW) against 14 strains of spoilage *Pseudomonas* of fresh cut vegetables under cold storage. The NEW, produced from solutions of potassium and sodium chloride, and sodium bicarbonate developed up to 4000 mg/L of free chlorine, depending on the salt and relative concentration used. The antimicrobial effect of the NEW was evaluated against different bacterial strains at 10^5 cells/ml, with different combinations of free chlorine concentration/contact time; all concentrations above 100 mg/L, regardless of the salt used, were found to be bactericidal already after 2 min. When catalogna chicory and lettuce leaves were dipped for 5 min in diluted NEW, microbial loads of mesophilic bacteria and *Enterobacteriaceae* were reduced on average of 1.7 log cfu/g. In addition, when lettuce leaves were dipped in a cellular suspension of the spoiler *Pseudomonas chicorii* I3C strain, diluted NEW was able to reduce *Pseudomonas* population of about 1.0 log cfu/g. Thanks to its high antimicrobial activity against spoilage microorganisms, and low cost of operation, the application of cycles of electrolysis to the washing water looks as an effective tool in controlling fresh cut vegetable microbial spoilage contamination occurring during washing steps.

1. Introduction

Ready-to-eat (RTE) vegetables are fresh products with limited shelf-life that need to be stored under refrigeration conditions (D'Acunzo et al., 2012). *Erwinia* spp. and *Pseudomonas* spp. represent the main spoilage microbiota, which contribute to soft rot on RTE vegetables (Lund, 1983; Nguyen-the and Prunier, 1989), thanks to the their pectinolytic and proteolytic activities (Lee et al., 2013; Magnuson et al., 1990; Nguyen-the and Prunier, 1989) that can be also active at temperatures as low as 0–2 °C (Brocklehurst and Lund, 1981; Lund, 1983). High total psychrotrophic aerobic bacterial counts (TPACs) of vegetable crops allow quickly contamination of the process water, and subsequent TPACs do not change much throughout the production process (Holvoet et al., 2012). Disinfection is one of the most critical processing steps in fresh-cut vegetable production, affecting the quality, safety and shelf-life of the end product (Gil et al., 2009). Chemical methods of cleaning and sanitizing vegetable surfaces usually involve the application of mechanical washing in the presence of sanitizers, followed by rinsing with potable water (Artés and Allende, 2005). The most widespread sanitizer used in the washing water of the fresh cut vegetables is chlorine, at concentrations between 50 and 200 mg/L with contact times of 1–2 min (Parish et al., 2003). The advanced oxidation processes (AOPs) represent the newest development in sanitizing technology, where more oxidants are used simultaneously (Selma et al., 2008). Hadjok et al. (2008) have used UV and hydrogen peroxide (H_2O_2) for decontaminating several fresh produces, observing a greater reduction in *Pseudomonas fluorescens* and *Pectobacterium carotovora* populations than that obtained by calcium hypochlorite treatment (200 mg/L free chlorine). Gopal et al. (2010) proposed a combined treatment of fresh cut iceberg lettuce with electrochemically generated silver (5 mg/L) and hydrogen peroxide (0.4 mg/L); the treatment caused a significant reduction in the total plate count, *Pseudomonadaceae*, *Enterobactericeae* and yeasts and moulds immediately after washing, in comparison to water washed shredded lettuce. Also electrolysis of a diluted salt solution produces oxidant substances with antimicrobial activity such as free chlorine, ozone and free radicals. When anode and cathode are not separated by a membrane (Thorn et al., 2012) the resulting sanitizer is known as neutral electrolyzed water (NEW); NEW can be also obtained by mixing acidic and alkaline

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http://dx.doi.org/10.1016/j.fm.2015.04.003
0740-0020/

electrolyzed waters (Deza et al., 2003; Rico et al., 2008) or directly from the anodic side of the cell (Guentzel et al., 2008). Studies suggest that chlorine compounds when present in NEW can damage membranes of bacteria, but other modes of antimicrobial action (decarboxylation of amino acids, reactions with nucleic acids, oxidation of key enzymes) have been proposed as reviewed by Hricova et al., 2008. This technique has been suggested as a valuable disinfection tool for wash water sanitation in citrus processing (Fallanaj et al., 2011) and minimally processed vegetable industry (Guentzel et al., 2008; Ongeng et al., 2006). Abadias et al. (2008) found a reduction of 1.4 log cfu/g in *Erwinia carotovora* population inoculated in lettuce after treatment with NEW (89 mg/L free chlorine) or sodium hypochlorite containing 100 mg/L of free chlorine respect to samples treated with water. Ongeng et al. (2006) found a reduction of viable cell count from 9 log cfu/ml to undetectable level for *P. fluorescens*, *Rhanella acquatilis* and *Pantoea agglomerans* contaminating industrial water bodies when high current passed through the electrolytic cell. The treatment of fresh cut lettuce with NEW for 5 min showed a great reduction in psychrotrophic count respect to water dipping (Ongeng et al., 2006). Despite studies about the effect of NEW on spoilage bacteria populations of fresh cut vegetables (Abadias et al., 2008; Ongeng et al., 2006; Rico et al., 2008), to the best of our knowledge, no previous work has been carried out about the effect of NEW on spoilage *Pseudomonas* strains inoculated on fresh cut produces.

In this work, a preliminary *in vitro* evaluation of antimicrobial activity of NEW, obtained with different salts, was carried out simulating washing water contaminated by spoiler *Pseudomonas* strains; furthermore, the efficacy of NEW was studied on spoilage bacterial population contaminating fresh cut lettuce paying particular attention to the fate of spoilage pseudomonads. Changes in visual quality of lettuce leaves, stored in passive modified atmosphere, was recorded during cold storage.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. fluorescens L1A, L2A, L1B, L2B, L1C, I3B, I2B, *Pseudomonas simiae* I2A, *Pseudomonas putida* I1B, I2C, *Pseudomonas viridiflava* I1A, *Pseudomonas jessenii* I3A, I1C, *P. chicorii* I3C, *Pseudomonas koreensis* I4C strains, isolated from refrigerated ready-to-eat (RTE) curly endive (*Chicorium endivia* L. var. *crispum* Lam.) and head lettuce (*Lactuca sativa* L. var. *capitata* L.) trocadero type and identified as recently reported (Baruzzi et al., submitted for publication), as well as *Pseudomonas aeruginosa* DSM939 were stored in the Institute of Sciences of Food Productions bacterial collection. *P. fluorescens* NCPPB 1964^{t} the type strain of this species and *Pseudomonas marginalis* NCPPB 667, isolated from catalogna chicory (*Cichorium intybus* L.), come from the National Collection of Plant Pathogenic Bacteria and *Pseudomonas syringae* PS1 from Romain lettuce (*L. sativa* L. var. *longifolia*) were included in the microbial collection of the Department of Soil, Plant and Food Sciences (Bari, Italy). Strains were usually grown in 10 ml of mPlate Count Broth (mPCB, Becton Dickinson Italia, Milan, Italy), incubated for 24 h at 30 °C in mild stirring (90 rpm) conditions. These cultures were maintained on Plate Count Agar plates (PCA, Biolife, Milan, Italy) until their use in antimicrobial assay.

2.2. Production of NEW and measurement of chlorine concentration

Electrolyzed water was produced using the Eva System apparatus provided with Dimensionally Stable Anodes electrodes (De Nora Next, Milan, Italy) with NaCl and $NaHCO_3$ as electrolyte at the concentration of 1 g/L or KCl solution (15 g/L) and from tap water without any inorganic salt added. Electrolyzed water was collected after 30, 60 and 120 min of electrolysis. Amperage between electrodes was fixed at 4A. For all experiments water was circulated through the electrolytic cell with a pump delivering a volumetric flow rate of 300 L/h/cell.

The free chlorine concentration (mg/l) was determined by N, N-diethyl-p phenylenediamine (DPD1) colorimetric method, using a digital chlorine kit (SWAN Chematest 20, Analytic Instrument, Switzerland). The measurement of pH and ORP was determined by a pH meter (Model pH50 Lab pH Meter XS-Instrument, Concordia, Italy).

In order to draw the curve of free chlorine concentration versus time, leaves of red chicory (*Chicorium intybus* L.), head lettuce trocadero type, iceberg lettuce and chard (*Beta vulgaris* L. var. *cicla* L.) were roughly cut and mixed together. A sample of this vegetable mixture (25 g) was dipped in 2 L of NEW (obtained by using KCl as electrolyte) diluted with autoclaved tap water to 300 ± 5 mg/L of free chlorine, measuring residual chlorine concentration, after 5, 30, 60, 90, 120 and 180 min, as described above.

2.3. Antimicrobial activity of NEW in vitro

2.3.1. In vitro assays

In the first assay was used NEW obtained from 0.1% w/v of sodium chloride or sodium bicarbonate solutions in tap water against *P. fluorescens* NCPPB 1964, *P. marginalis* 667, and *P. syringae* PS1 as indicator strains; electrolyzed tap water without salts was used as a control. Each sample of NEW, 9.9 ml, was contaminated with 0.1 ml of bacterial suspension having OD_{600} = 0.3 ± 0.05 (about at 8 log cfu/ml). Contaminated NEW samples were incubated at 4 °C for 2 and 5 min afterwards 0.1 ml was diluted in 9.9 ml of sterile saline solution to dilute free chlorine potentially still having antimicrobial activity. Surviving bacteria were enumerated after incubation (24 h at 30 °C) of PCA plates seeded with decimal dilution, in sterile saline, of 1:100 NEW samples. Each strain was assayed three times, independently (N = 3). Based on results, 14 spoilage *Pseudomonas* strains, as well as, *P. aeruginosa* DSM939, were used for an additional *in vitro* assay by using the most probable number (MPN) method for enumeration of viable microbial cells. Three samples of NEW, produced from potassium chloride as electrolyte, were prepared after 2 h of electrolysis (4000 mg/L free chlorine, pH = 8.2, ORP = 846 mV). The NEW, diluted to 400, 200, and 100 mg/L of free chlorine, was inoculated as previously reported and incubated at 4 °C for 2 min. Inoculated NEW samples were decimally diluted four times in mPCB. Occurrence of visible microbial growth was recorded after 24 h at 25 °C; un-treated bacterial suspensions were used as a positive control. Absorbance value of test tubes without visible growth was recorded at 600 nm and compared with un-inoculated (blank) tubes. Test tubes were considered positive when OD_{600} readings were 0.050 higher than blank tubes. Calculation of MPN was obtained comparing the results with MPN tables (Man, 1983). In order to avoid false negative results deriving from chlorine stressed cells, test tubes were incubated for additional 24 h at 30 °C. In this case the assay was performed a single time with three repetition for each strain (N = 3).

2.3.2. Antimicrobial activity of NEW against natural microbial populations contaminating fresh cut vegetables

Fresh heads of head lettuce trocadero type and catalogna chicory purchased from local farmers were stripped of outer leaves, washed with tap water to remove impurities and cut into small pieces or at the base of the heart, respectively. A portion of 200 g of each vegetable was washed for 2 min into 4 L of sterile tap water and then dipped in 4 L of NEW (200 mg/L of free chlorine from KCl) at 4 °C for 5 min. A different sample of each vegetable was washed