Food Control 22 (2011) 1456–1460

Contents lists available at ScienceDirect

Food Control

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

Effects of culture conditions on the subsequent heat inactivation of *E. coli* O157:H7 in apple juice

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A R T I C L E I N F O

Article history:
Received 20 August 2010
Received in revised form
25 February 2011
Accepted 1 March 2011

Keywords:
Acid adaptation
Acid stress
E. coli O157:H7
Thermal adaptation
Thermal resistance

A B S T R A C T

Escherichia coli O157:H7 cells were grown in various acidic growth media including an acidogenic nutrient broth (NB) supplemented with 1% glucose (NBG) and NB acidified to pH 4.5 with 0.25% citric or malic acids. The pH in NBG continuously decreased, reaching a final pH of 4.46 after 28 h. The pH in the control culture (NB) did not significantly change throughout the incubation. When heated in apple juice at 55 °C, cells grown in NBG had the significantly highest D_{55} value (250 ± 39.67 s). Thermal inactivation rates established for cells grown in NB, citric acid- and malic acid-acidified NBs were 51.39 ± 1.34, 25.46 ± 1.21, and 45.42 ± 0.36 s, respectively. When compared with the D_{55} values of cells previously exposed to combinations of environmental stress factors, cells grown in NBG were also found to be significantly heat resistant and were deemed appropriate to be used in heat challenge studies.

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1. Introduction

Outbreaks of infections caused by *Escherichiacoli* O157:H7 due to consumption of unpasteurized fruit juices have once puzzled food safety analysts and manufacturers since the inherent acidity of such products have been thought effective in controlling pathogens (Keller & Miller, 2006; Mazzotta, 2001). In 1991, *E. coli* O157:H7 was for the first time confirmed cause of an outbreak that involved consumption of freshly pressed apple juice (Besser et al., 1993). Vojdani, Beuchat and Tauxe (2008) summarized that from 1995 to 2005, 21 juice-associated outbreaks were reported to the US Centers for Disease Control and Prevention (CDC), resulting in 1366 illnesses. Most of these outbreaks involved the consumption of apple and orange juice products. Among pathogens reported to be of significance in fruit juice processing, *E. coli* O157:H7 has been given more attention due to its low infectious dose, disease severity and survival characteristics (Doyle, 1997). In reported outbreaks of diseases involving fruit juices, *E. coli* O157:H7 and enterotoxigenic *E. coli* strains were reported to be most common causative agent, followed closely by *Salmonella* spp. (Federal Register, 2001; Harris et al., 2003; Vojdani et al., 2008). *E. coli* O157:H7 has been reported to have an infective dose of as low as 2–2000 cells (Doyle & Buchanan, 1997), and causes hemorrhagic colitis that may eventually lead to complications like hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Padhye & Doyle, 1992). Aside from fruit juices, the organism was also associated with outbreaks involving other acidic foods such as fermented sausages (CDC, 1995), mayonnaise (Weagent et al., 1994) and yogurt (Morgan et al., 1993).

Studies have shown that pathogens like *E. coli* O157:H7 and *Salmonella* spp. are capable of developing adaptive mechanisms by undergoing genetic and physiologic changes that allow the cells to stay viable in acidic food environments (Foster & Hall, 1990; Goodson & Rowbury, 1989; Linton et al., 1997; Moat et al., 2002). A number of studies have also shown that adaptation of *E. coli* O157:H7 to acidic conditions by exposure to gradually decreasing pH led to cross protection against thermal inactivation in fruit juices, milk and chicken broth (Buchanan & Edelson, 1999; Mazzotta, 2001; Ryu & Beuchat, 1998; Sharma et al., 2005). Furthermore, induction of acid shock by exposing cells from high to low pH prior to heat inactivation was also shown to result in cells with thermal resistance greater than non-shocked counterparts (Ryu & Beuchat, 1998). Since acidification and heating are common means of controlling microorganisms in foods (Brown & Booth, 1991; Buchanan & Edelson, 1999), the ability of the pathogen to develop increased resistance to these control factors raises safety concerns to products including fresh squeezed (unpasteurized) and pasteurized fruit juices. This study was conducted to determine and compare the heat resistance characteristics of *E. coli*

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doi:10.1016/j.foodcont.2011.03.011

O157:H7 after prior subjection to various acidic growth conditions; including exposures to gradual acidification due to acids produced by the bacterium from glucose metabolism, and abrupt acidifications with organic fruit acids. The thermal resistances of the pathogen established in this study were also compared to those of cells subjected to various combinations of pH, a_w and temperature stresses commonly encountered in food and food processing ecologies.

2. Materials & methods

2.1. E. coli O157:H7 propagation and inoculum preparation

The organism is a clinical isolate obtained from the Hiroshima City Institute of Public Health (HCIPH), Hiroshima, Japan. When suspended in apple juice, the test *E. coli* O157:H7 (HCIPH 96055) was previously reported to be significantly more resistant to heating compared to the non-pathogenic *E. coli* K-12 (Institute of Fermentation, Osaka [IFO] 3301), *Salmonella enteritidis* (HCIPH B11), *Salmonella typhimurium* (American Type Culture Collection [ATCC] 12529), *Listeria monocytogenes* 4b (HCIPH AS-1), and *L. monocytogenes* 1/2c (HCIPH M24-1) (Gabriel & Nakano, 2009). Culture maintenance was conducted by weekly subculture on nutrient agar (NA) slant.

Prior to experimentations, the organism was loop-inoculated in nutrient broth (pH 7.0) (NB, Eiken Chemical Co. Ltd., Tokyo, Japan) and incubated at 35 °C for 18 h. Cells were then harvested from the broth cultures using a high speed refrigerated centrifuge (Kubota 6500, Kubota Corp., Tokyo, Japan), and then appropriately diluted with phosphate-buffered saline (PBS) prior to thermal inactivation studies. The PBS was compounded based on the formulation recommended by the Japanese Food Hygiene Association (JFHA) (2004). Briefly, a phosphate buffer solution was prepared by mixing 500 ml 0.5 M KH_2PO_4 and 175 ml 1 N NaOH solutions prior to bulking to 1 L with distilled water (DW) and pH adjustment to 7.2. The PBS was prepared by mixing 8.5 g NaCl, 1.25 of the prepared buffer; and bulking the mixture with DW to make 1 L. All reagents used in PBS preparation were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Acidic growth conditions

Prior to thermal resistance determinations, *E. coli* O157:H7 was exposed to various acidic conditions by inoculating in a number of growth media. Cells were exposed to gradual acidification by inoculating 3 ml aliquot of *E. coli* O157:H7 suspension pre-cultured in NB at 35 °C for 18–24 h into 300 ml acidogenic broth medium made from 1% glucose-supplemented NB (NBG, initial pH = 6.7). Cells were also exposed to abrupt acidification by similarly inoculating pre-cultured cells into NB acidified to pH 4.5 with 0.25% malic or citric acid; and statically incubating at 35 °C for 18–24 h. For control, cells were also inoculated and grown in a typical NB medium (pH 7.0).

Changes in the microbial population and growth medium pH were determined in NBG and control NB until late stationary growth phase (28 h). Cell counts were determined by serially diluting 1 ml aliquot of sampled growth media with 9 ml PBS, surface-plating appropriate dilutions onto NA plates, and incubating at 35 °C for 24 h. Determinations of NB and NBG pH were conducted by obtaining 20 ml aliquots from the growth media, filter sterilizing by passing through 0.22 μm syringe-driven filter units (Millex, Millipore Corp., Bedford, MA, USA), and pH measurement using a Horiba Navi pH meter (F-52, Horiba Ltd., Kyoto, Japan) calibrated with pH 7.0 and 4.0 standard solutions (Horiba Ltd., Kyoto, Japan).

2.3. Thermal resistance determinations

Cells from each of the growth media were harvested by centrifuging 1.0 ml of the NB suspensions on a bench top centrifuge (Kubota, KM-15200, Tokyo, Japan) at 8000 rpm for 5 min. Following supernatant liquid decantation, the cells were re-suspended in PBS for not longer than 20 min prior to thermal inactivation. For the thermal resistance determinations, 9.9 ml apple juice (Kurashimoa 100% Apple Juice, Tokyo Meiraku, Chiba, Japan, pH 3.78, 11.15 °Brix) in glass test tubes (24 mm i.d.) were heated to 55 °C on a water bath (Yamato Thermo Mate, Yamato Scientific Co., Ltd., Tokyo, Japan). The juice temperature was measured by inserting a thermometer through the cold point of a control tube. When the cold point temperature reached 55 °C, 0.1 ml of PBS cell suspension was pipetted into each of the tubes. The inoculated tubes were constantly, manually agitated throughout the heating period that ranged from 0 to 80 s. After heat treatments, tubes were immediately immersed into and kept in an ice bath until survivor enumerations.

Survivor enumerations were conducted by serially diluting 1 ml aliquots of heated juice with 9.0 ml PBS and surface-plating appropriate dilutions onto NA. Plates were incubated at 35 °C for 24–48 h before colony enumeration. The populations enumerated were expressed as log CFU/ml. The death kinetics was characterized using the freeware DMFit (Version 2.1) of the Institute of Food Research, Norwich, UK. The data from the enumerated populations were fitted into the model of Baranyi and Roberts (1994) to calculate for the lag times and death rates (k_D). The death rates at 55 °C were then expressed in terms of the decimal reduction times (D_{55} values) by calculating for the reciprocal of the k_D values; graphically, the negative inverse of the slope of the log-linear inactivation curve of the organism. The D_{55} values are equivalent to the number of unit time necessary to reduce the initial populations of *E. coli* O157:H7 in the apple juice heated at 55 °C by 90% (Gabriel and Nakano, 2009). If a survival curve was characterized with a lag time prior to log-linear inactivation, the time to 90% reduction was determined as the sum of the lag time and the D_{55} value calculated from the succeeding log-linear inactivation curve.

2.4. Statistical analyses

To test for differences (95% level of significance) between the measured values per treatment, the data gathered were subjected to the general linear model procedure (PROC GLM); followed by the Duncan Multiple Range Test using the SAS statistical software package version 8.0 (SAS Institute, Cary, NC).

3. Results & discussion

3.1. E. coli O157:H7 growth in NB and NBG

The changes in the population of the test pathogen in NB and NBG, as well as the pH values of the growth media throughout the incubation period are presented in Fig. 1. Results showed that supplementation of NB with 1% glucose significantly reduced ($P < 0.05$) the initial pH to 6.8 after sterilization at 121 °C for 15 min. The difference in the initial pH however, did not have an effect on the growth of the inoculated *E. coli* O157:H7. In the summary of growth parameter kinetics presented in Table 1, the lag times of the pathogen in NB and NBG were not significantly different ($P > 0.05$). In both culture media, significant reductions in pH were observed 6 h post inoculation, which coincided with the logarithmic phase of microbial growth. Results also showed that the growth rates of the pathogen in both media were not significantly different. The pH of NB slightly increased after 8 h and did not considerably change throughout the incubation period. The final pH of NB was recorded at 7.09 ± 0.36. On the other hand, the pH of NBG continuously decreased during incubation, resulting in a final pH value of 4.47 ± 0.04. Furthermore, injury rates in both NB and NBG were measured after 24 h by determining the differences in enumerated

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