academicJournals

Vol. 7(22), pp. 2736-2740, 28 May, 2013
DOI: 10.5897/AJMR12.1789
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Thermal inactivation of *Alicyclobacillus acidoterrestris* spores isolated from a fruit processing plant and grape juice concentrate in South Africa

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Accepted 29 May, 2013

Thermal inactivation at 95°C for two strains of *Alicyclobacillus acidoterrestris* isolated from contaminated fruit juice concentrates were investigated in a 0.1% (m/v) peptone buffer solution (pH 7.04) and grape juice (pH 4.02, 15.5 °Brix). The thermal inactivation of *A. acidoterrestris* spores followed first-order kinetics, suggesting that as the microbial population is exposed to a specific high temperature, the spores were inactivated at a constant rate. D-values determined in the buffer solution were calculated to be 1.92 ± 0.39 and 2.29 ± 0.50 min, while in grape juice D-values were found to be 2.25 ± 0.53 and 2.58 ± 0.32 min for the two strains tested.

Key words: *Alicyclobacillus acidoterrestris*, endospores, thermal inactivation, heat resistance, spoilage, fruit juice concentrate, D-value.

INTRODUCTION

Fruit concentrate has traditionally been regarded to be resistant to spoilage by deteriogenic micro-organisms due its physical and chemical characteristics. These characteristics include a low pH of between 3.5 to 4.0, low water activity, high sugar concentration (typically around 66 °Brix), and reduced aeration capacity and dissolved oxygen (Palop et al., 2000). The addition of a hot-fill and hold pasteurisation process as used in the fruit beverage industry, where the product is held at 86 to 95°C for approximately 2 min, is also sufficient to destroy most non-spore forming micro-organisms (Palop et al., 2000; Chang and Kang, 2004).

Spoilage of commercially available pasteurised fruit juice was first reported by Cerny et al. (1984) who found shelf-stable, aseptically packaged apple juice to have an off-flavour. Following this report, an increasing number of spoilage incidents arose and almost all of these were caused by the spore-forming, thermo-acidophilic bacteria *Alicyclobacillus acidoterrestris*. Spoilage has been to date reported in apple, pear, orange, peach, passion, mango and white grape juice, with shelf-stable apple juice most frequently being spoiled (Borlinghaus and Engel, 1997; Chang and Kang, 2004; Walker and Phillips, 2008; McKnight et al., 2010). More diverse products such as shelf-stable iced tea containing berry juice, the ingredients of rose hip and hibiscus teas (Duong and Jensen,

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2000), a carbonated fruit drink (Pettipher, 2000) and diced canned tomatoes (Chang and Kang, 2004) have also had incidences of spoilage caused by *A. acidoterrestris*. The fruit juice industry now recognizes *A. acidoterrestris* as a major quality control target for pasteurisation (Yamazaki et al., 1996; Pettipher et al., 1997; Silva and Gibbs, 2004; Walker and Phillips, 2008; Bevilacqua et al., 2008).

Spoilage caused by this bacterium is difficult to detect visually. The spoiled juice appears normal, or might have light sediment with no gas formation. Often, the only evidence of spoilage is apparent as a medicinal or phenolic off-flavour (Walls and Chuyate, 1998; Jensen, 1999). The chemicals responsible for this off-odour were identified as guaiacol (2-methoxyphenol) and other halophenols such as 2,6-dichlorophenol (2,6-DCP) and 2,6-dibromophenol (2,6-DBP). Guaiacol can be detected by smell in fruit juices at 2 ppb and was detected in orange and apple juices in the presence of around 5 log cfu.ml^{-1} of *A. acidoterrestris* cells (Gocmen et al., 2005).

A wide range of D-values have been reported by researchers for the heat resistance of *A. acidoterrestris* spores, as the experimental conditions and protocols vary and the taxonomy of this group is still unclear. The $D_{95°C}$ ranged from 1 min in berry juice to 9.98 min in non-clarified lemon juice, suggesting that spores survive the typical juice pasteurisation process applied during fruit juice and concentrate production and in fact provide a heat-shock treatment that may stimulate spore germination and outgrowth (Splittstoesser et al., 1994; Eiroa et al., 1999; Orr and Beuchat, 2000; Maldonado et al., 2008; Spinelli et al., 2009). Differences between the D-values reported in literature may be attributed to differences in strains, sporulation temperature, nutrient composition and pH of the heating medium, water activity, presence or absence of divalent cations and antimicrobial compounds (Bahçeci and Acar, 2007). Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius et al., 1998; Orr and Beuchat, 2000; McIntyre et al., 1995; Groenewald et al., 2008). The objective of this research was to determine the D-values in buffered water and single strength grape juice of spores of *A. acidoterrestris* strains isolated from a South African fruit concentrate processing environment. Strains were tested at 95°C, which in the region, is the highest temperature used during flash pasteurisation, to determine their ability to survive commercial pasteurisation regimes.

MATERIALS AND METHODS

Bacterial strains

A. acidoterrestris K47 (Witthuhn et al., 2007), a strain isolated from grape juice concentrate and *A. acidoterrestris* FB2 (Groenewald et al., 2008) isolated from pear juice concentrate were used in this study. Potato dextrose agar (PDA) (Biolab, Biolab Diagnostics, Midrand, SA) adjusted with tartaric acid (1N) (Saarchem, Krugersdorp, South Africa) to a final pH of 4 was used as a culture medium (Witthuhn et al., 2007).

A. acidoterrestris spore suspension

Spores were produced on PDA (Biolab) incubated at 45°C for 5 to 7 days until approximately 70% of cells sporulated, as determined by microscopic examination. Spores were removed by gently agitating each plate using a glass spreader after adding 5 ml of sterile distilled water. The spore suspension was centrifuged at 5 000 x*g* (Beckman Coulter TJ-25 Centrifuge, Beckman Coulter Inc., USA) for 15 min after which the supernatant was discarded and the pellet was resuspended. Spores were cleaned by washing of the pellets with sterile distilled water, followed by centrifugation and this was repeated five times. Pellets were then resuspended in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck, Halfway House, Gauteng, SA). The spore suspension was heated at 80°C for 10 min to eliminate vegetative cells and stored at 4°C.

Thermal inactivation and enumeration

A Colworth House submerged-coil heating apparatus (Protrol Limited, Surrey, United Kingdom) was used for the investigation of the thermal inactivation of *A. acidoterrestris* spores. The apparatus has a narrow bore stainless steel coil (9.5 ml total volume, 3.175 mm outer diameter, 0.5 mm thickness), fully submerged in thermostatically controlled water bath and a automatic sampler with the sampling frequency controlled by a DOS based computer program. The time for the sample to reach the water bath temperature was 1 s. Cleaning was performed by first injecting industrial alcohol and then sterile water. The water bath was set at a temperature of 95°C and ten sampling times at 1 min intervals were programmed. A temperature of 95°C was chosen as being representative of the highest temperature used in commercial pasteurisation regimes. Ten milliliters of either inoculated 0.1% (m/v) peptone (Biolab) buffer solution (pH 7.04) or 15.5 °Brix single strength grape (pH 4.02) were injected into the submerged coil, followed by immediate initiation of the timing sequence. The remaining peptone buffer solution (Biolab) or grape juice were used for the time zero determination of the viable spore count. Times were selected in order to cover spore inactivation until approximately 10^2 cfu.ml^{-1} of *A. acidoterrestris* spores remained. For each sampling time, 500 µl of the heated spores were collected and promptly cooled by dilution with 5 ml SSS at room temperature. The samples were left at ambient temperature for approximately 4 h to allow further cooling and recovery of the heat-shocked spores.

Serial dilutions of the samples were then prepared (10^{-1} to 10^{-6}) and 100 µl of each of the different sample dilutions were plated in triplicate onto PDA (Biolab) (pH 4). Plates were incubated aerobically at 45°C and examined for growth after 96 h. The results obtained were expressed as colony forming units per milliliter (cfu.ml^{-1}). The efficacy of thermal treatments in terms of eliminating *A. acidoterrestris* spore was measured by their decimal reduction time (D) which, for this study, was defined as the time (min) of a given treatment for the number of survivors to be reduced by one log cycle. In this study, the D-value at 95°C was abbreviated as D_{95}. D-values were calculated from the slope of the regression line when time (x-axis) was plotted against cell counts (cfu.ml^{-1}) (y-axis). Experiments were performed in triplicate. Descriptive statistical calculations were applied to the data in order to determine the standard error.

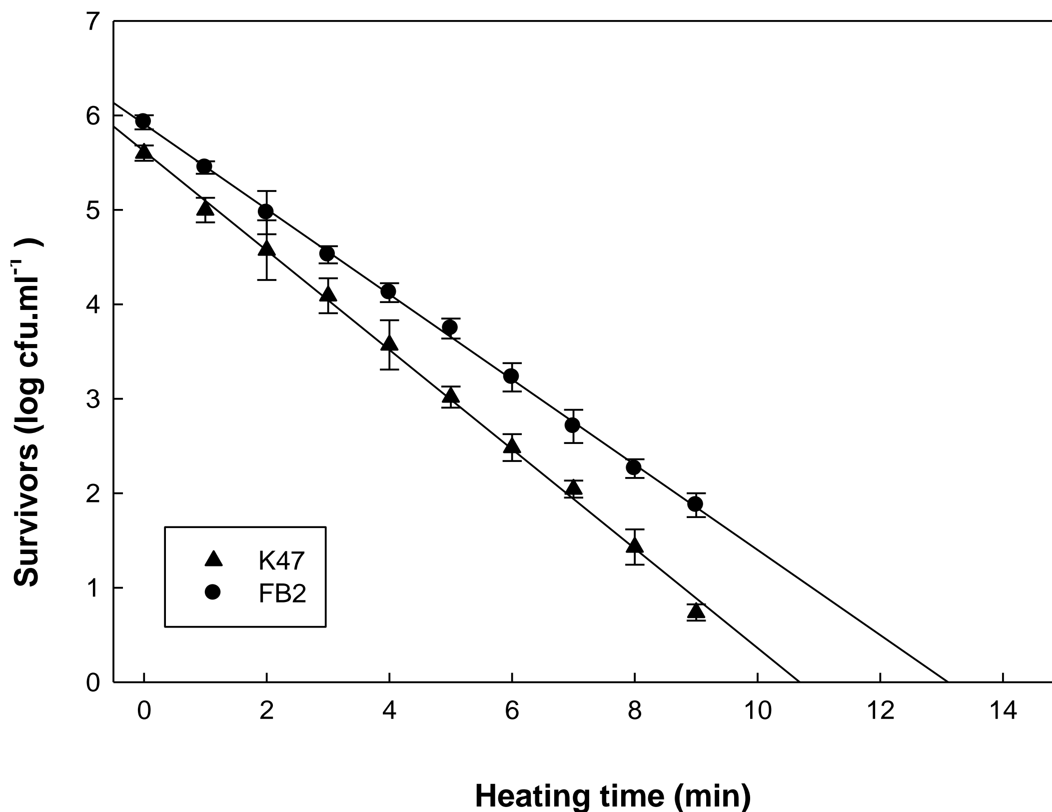

Figure 1. Impact of temperature at 95°C on *A. acidoterrestris* strains K47 and FB2 at a starting concentration of 6 x 10^5 cfu.ml^{-1} in a peptone buffer solution (each data point represents triplicate values. The standard deviation was used as the error-bar).

RESULTS AND DISCUSSION

A linear-log relation was observed between the endospore concentration and time (Figures 1 and 2) suggesting that as the microbial population was heated at a specific temperature, the spores were inactivated at a constant rate. A similar first-order kinetic relationship was observed by other authors (Pontius et al., 1998; Silva et al., 1999). In this study, D_{95}-values were calculated to be 1.92 ± 0.39 and 2.29 ± 0.50 min for *A. acidoterrestris* strains K47 and FB2, respectively in a 0.1% (m/v) peptone buffer solution (Biolab) (pH 7) (Figure 1), and 2.25 ± 0.53 and 2.58 ± 0.32 min in grape juice (pH 4.05, °Brix 15.5) (Figure 2).

Splittstoeser et al. (1999) reported similar D_{95}-values in Concord grape juice (pH 3.5, 16 °Brix) for *A. acidoterrestris* strain WAC spores of 1.9 min and a value of 2.4 min for the same strain in grape juice (pH 3.3, 15.8 °Brix). However, D-values amongst strains of *A. acidoterrestris* varied greatly when tested in different fruit juices with similar levels of acidity and concentration of dissolved sugars. Maldonado et al. (2008), reported D_{95}-values in non clarified lemon juice (pH 2.45, 9.8 °Brix) of 9.98 min, while McIntyre et al. (1995) found *A. acidoterrestris* spores to have a D_{95}-value of only 1.0 min in berry juice. These differences could be explained due to differing compositions of fruit products, including soluble solids, which might increase the heat resistance of spores (Maldonado et al., 2008).

Heat resistance between strains of *A. acidoterrestris* also varies greatly (Pontius et al., 1998; Eiora et al., 1999; Bahçeci and Acar, 2007). Confirmation of strain defences is provided in this study with *A. acidoterrestris* FB2 showing more thermal resistance than *A. acidoterrestris* K47 in a peptone buffer solution (Biolab), as well as grape juice (D_{95}-values of 1.92 ± 0.39 and 2.29 ± 0.50 min and 2.25 ± 0.53 and 2.58 ± 0.32 min, respectively). The steeper slope of the regression line for the 6 x 10^5 cfu.ml^{-1} *A. acidoterrestris* inoculum in peptone buffer solution (Biolab) (Figure 1) as opposed to the grape juice, and the subsequent lower D_{95}-values of 1.92 ± 0.39 and 2.29 ± 0.50 min for *A. acidoterrestris* K47 and FB2, respectively can be ascribed to the lower pH and higher percentage of soluble solids (°Brix) in the grape juice.

Results from this study indicated that the spores of *A. acidoterrestris* may survive in fruit juices after pasteurisation treatment commonly applied in the food industry, at least under the conditions described in the current study.

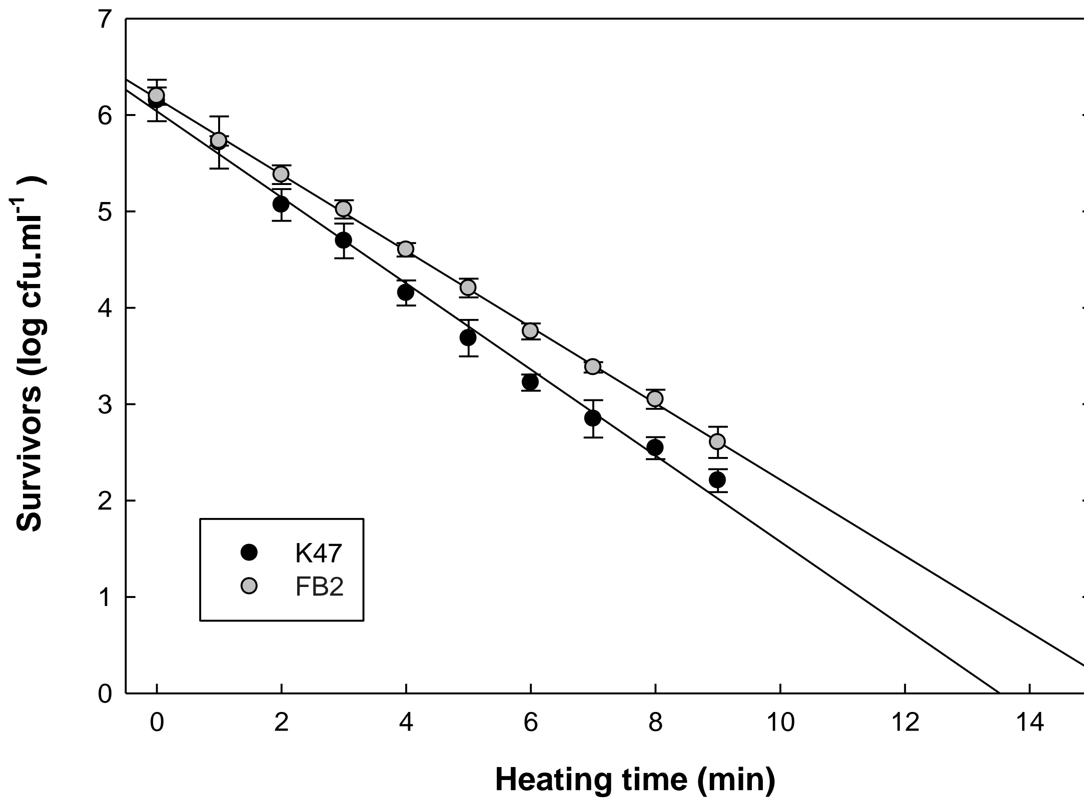

Figure 2. Impact of temperature at 95°C on *A. acidoterrestris* strains K47 and FB2 at a starting concentration of 6 x 10^5 cfu.ml^{-1} in a 15.5 °Brix single strength grape juice (pH 4.02) (each data point represents triplicate values. The standard deviation was used as the error-bar).

Since the implementation of a more severe heat process required to inactivate spores of *A. acidoterrestris* will also produce unacceptable organoleptical changes in the product, and the fact that no species of *Alicyclobacillus* have shown any pathogenic potential, it would serve little purpose to set pasteurisation temperatures to target *A. acidoterrestris.* It is important to note that incidence of *A. acidoterrestris* in fruit juice is not directly associated with deterioration. Detection of *A. acidoterrestris* in non-deteriorated fruit juices (Previdi et al., 1997; Cerny et al., 1999; Bahceci et al., 2005; Walker and Phillips, 2008) suggests deterioration to be incidental, requiring adequate conditions for its development. The susceptibility of fruit juice to spoilage is dependant on initial levels of contamination and the conditions of storage of the fruit juice. Manufacturers of fruit juice concentrate should minimised the risk of spoilage by *A. acidoterrestris* through the use of good manufacturing practices during fruit processing and the implementation of HACCP procedures, substituting Food Safety Hazards, normally associated with HACCP studies, with the risk of spoilage by *A. acidoterrestris* in the final product. Storage of pasteurised fruit products below 20°C would also prevent spoilage since the growth of this bacterium is suppressed at these temperatures.

ACKNOWLEDGEMENTS

We gratefully acknowledge Nampak Group RandD (17 Benbow Avenue, Cape Town, SA) for technical support and the use of the D-value apparatus, and the National Research Foundation (RSA) for their financial support.

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