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Thermobacteriological characterization of *Enterobacter sakazakii*

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ABSTRACT

In the present study the influence of various environmental and physiological factors on the heat resistance of *Enterobacter sakazakii* (*Cronobacter*) have been investigated. Our results demonstrated that the heat resistance of *E. sakazakii* depended on the strain studied, the growth conditions — phase and temperature — the characteristics of treatment medium and the recovery conditions. The strain STCC 858 (ATCC type strain 29544) showed maximum heat resistance among the strains tested and it was selected for the further study. Stationary-phase cells grown between 20 and 37 °C (mean $D_{60}=0.9$ min) resulted to be more resistant than cells grown at 10 °C ($D_{60}=0.2$ min). Resistance decreased when the treatment medium pH was lower than pH 6.0, and it increased with decreasing water activity of the treatment medium, with a 32-fold increase in resistance when lowering water activity to 0.96. z value at pH 4.0 ($z=4.79$ °C) was significantly higher than at pH 7.0 ($z=4.06$ °C), although *E. sakazakii* cells were approximately 10 times more heat resistant at pH 7.0 than at pH 4.0 within the range of temperatures tested. Contrary to pH, the magnitude of the influence of a_w on heat resistance did not significantly change with treatment temperature. The proportion of sublethally damaged cells was similar regardless of the treatment medium pH, but it decreased when lowering the water activity. Nevertheless, increasing treatment temperature would not result in a decreased proportion of sublethally injured *E. sakazakii* cells within the surviving population. Thus, the design of a theoretical combined process that could take advantage of the occurrence of sublethally injured cells would be similarly effective at low and high temperatures. *E. sakazakii* proved to be more heat resistant in four different liquid food matrixes than in buffers at the same pH, and this disagreement was especially higher in orange juice, which resulted to be the product that induced a greater protective effect in *E. sakazakii* cells against heat.

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1. Introduction

Currently, thermal processing is the most commonly used method for food preservation. It is an efficient and reliable process reducing the risks associated with foodborne pathogens and diminishing the activity of several enzymes, and it is also an economical technology for the food industry in terms of energy. Nevertheless, excessive heat causes unwanted side effects in the organoleptic, nutritional and functional properties of foods (Barbosa-Cánovas et al., 1999). This limitation, together with current challenge to satisfy the consumers' demand for fresher products has led a great concern for the food industry. In this way, new production systems are intended to offer best sensory quality products by reducing the intensity of heat treatments, which can be questioned in terms of food safety. Since 1977, new or newly characterized foodborne pathogens have been recognized at the rate of approximately one every 2 years. They tend to cause infection at relatively low doses in humans and to appear in food vehicles that are not subjected to cooking conditions that kill them (Tauxe, 2002). Therefore, the optimization of the heating step by defining the target pathogen's heat resistance and bringing accurate predictions of thermal death rates is necessary to achieve desired safety margins whilst minimizing the processing.

The heat resistance of several well-known species responsible for food poisoning outbreaks, such as *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157:H7, is well referenced in the literature (Doyle et al., 2001; Doyle and Mazzotta, 2000; Stringer et al., 2000), whereas the heat lethal effect on other potential pathogenic microorganisms, such as *Enterobacter sakazakii* (*Cronobacter*), has scarcely been investigated. The fact that epidemiologic investigations have implicated rehydrated powdered infant formula as a source of *E. sakazakii* and responsible for outbreaks and several cases of neonatal infection (Muytjens et al., 1983; Van Acker et al., 2001) has led researchers to limit their studies on the heat resistance of *E. sakazakii* to this product (Al-Holy et al., 2009; Breweer et al., 2003; Edelson-Mammel and Buchanan, 2004; Iversen et al., 2004; Nazarowec-White and Farber, 1997; Osaili et al., 2009; Shaker et al., 2008). However, recent reviews (Friedemann, 2007; Gurtler et al., 2005) have summarized some reports on the presence of *E. sakazakii* in a wide variety of food and food ingredients of animal and vegetable origin.

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Moreover, it is reported that the kind of processing of *E. sakazakii*-contaminated food is not restricted to dry products: fresh, frozen, ready-to-eat, fermented and cooked food products, as well as beverages and water suitable for the preparation of food, have been found to be contaminated by *E. sakazakii* (Friedemann, 2007). It should also be taken into account that the population susceptible to *E. sakazakii* infection is not only restricted to neonates, but also immunocompromised adults and elderly patients may also be under risk, too (Lai, 2001). Therefore, *E. sakazakii* might be regarded as a potential risk for food safety and, by extension, for human health, especially in minimally or mildly processed foods.

As previously pointed out, an effective use of thermal treatments for the inactivation of *E. sakazakii* requires accurate information about its heat resistance. It is well known that microbial heat resistance is influenced by several environmental factors (Hansen and Riemann, 1963; Tomlins and Ordal, 1976). The most general classification organizes these factors as prior to heat treatment, simultaneously with, and subsequent to heat treatment. Factors that act before the heat treatment are those related to growth conditions and exposure to sublethal stresses of different natures. Factors acting during the treatment are the composition and some characteristics, such as pH and water activity, of the heating media. Finally, those factors with influence after the heat treatment are the recovery conditions that can affect the detection of survivors. Although there are some published data on the heat resistance of *E. sakazakii* in infant formula milk (IFM), as previously mentioned, there is little information about the influence of these environmental factors on its heat resistance. The occurrence of sublethal injury during treatment and the possible development of resistance to heat, which can determine the effectiveness of the treatments, have not been described either.

This investigation was carried out (i) to determine the influence of growth phase, growth temperature, heat shock, pH and water activity of treatment medium on the heat resistance of *E. sakazakii*, (ii) to evaluate the occurrence of sublethal injury in the cytoplasmic membrane of *E. sakazakii* heat-treated cells, and (iii) to provide a first approach to the heat resistance of *E. sakazakii* in four real foods.

2. Materials and methods

2.1. Micro-organisms and growth conditions

E. sakazakii STCC 858 (ATCC type strain 29544) was provided by the Spanish Type Culture Collection (Burjassot, Valencia, Spain), *E. sakazakii* NCTC 8155, NCTC 9238 and NCTC 9529 were provided by the National Collection of Type Cultures (NCTC, HPA, Salisbury, UK). Two of these strains are clinical isolates (STCC 858 and NCTC 8155) and the remaining two strains are food isolates (NCTC 9238 and NCTC 9529). During this investigation, the cultures were maintained frozen at −80 °C in cryovials. Micro-organisms were recovered from each cryovial by surface spreading on plates of OK Agar (Vitaltech Ibérica S. L., Barcelona, Spain) and incubated for 24 h at 37 °C (Oh and Kang, 2004). Those colonies that produced fluorescence under long wavelength UV light (365 nm) were identified as *E. sakazakii*. A broth subculture was prepared by inoculating, with one single colony from a plate of OK Agar, a flask containing 10 mL of sterile Tryptone Soya Broth (Biolife, Milan, Italy) supplemented with 0.6 % of yeast extract (Biolife) (TSBYE). After inoculation, the flask was incubated at 30 °C overnight in a rotary shaker (Selecta, mod. Rotabit, Barcelona, Spain) at 150 rpm.

Flasks containing 50 mL of sterile TSBYE were inoculated with the overnight subculture to a concentration of $5 \cdot 10^4$ cells/mL, and then incubated under agitation for 150, 50, 24 and 24 h at 10, 20, 30 and 37 °C, respectively, to reach the stationary growth phase with a final concentration of approximately $5 \cdot 10^9$ CFU/mL. Exponential-phase cells were obtained following the same procedure, but incubating for 8 h at 30 °C, to reach an approximately concentration of $5 \cdot 10^8$ CFU/mL.

2.2. Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC, as previously described by Condón et al. (1993). Once the preset temperature had attained stability ($T \pm 0.05$ °C), 0.2 mL of an adequately diluted microbial cell suspension (approximately 10^8 cells/mL) were inoculated into the corresponding treatment medium (350 mL). After inoculation, 0.2 mL samples were collected at different heating times and immediately pour plated. Heat treatments were conducted at 54, 58, 60, 62, 64, 66 and 68 °C. All experiments were performed at least by triplicate in independent working days.

McIlvaine citrate-phosphate buffers (Dawson et al., 1974) of pH 4.0, 5.0, 6.0 and 7.0 were used as heating media. Sucrose (Azucarera Ebro, Madrid, Spain) was added to McIlvaine buffer of pH 7.0 at different concentration to obtain media of different water activity (0.98 and 0.96). Water activity was measured at 25 °C by the dew point method (model CX-1; Decagon Devices, Pullman, Washington, USA).

Orange juice (García Carrión S.A., Spain), vegetable soup (Interal, S.A., Spain), chicken soup (Interal, S.A., Spain) and milk powder (Sveltesse Calcium Plus, Nestlé Spain S.A.) were purchased from local supermarkets in Zaragoza, Spain. When orange juice, vegetable soup and chicken soup were used as treatment media, they were directly introduced in the treatment vessel. Milk powder was rehydrated in sterile distilled water following the manufacturer's instructions (100 g/L) and then introduced in the treatment vessel.

2.3. Heat shock treatments

Heat shock treatments were carried out in a test tube containing 5 mL of sterile TSBYE preheated by immersion in a thermostated bath (mod. Digiterm, Selecta, Barcelona, Spain). Once the contents of the tube had stabilized at the selected heat shock temperature, the medium was inoculated with 0.2 mL of the stationary phase cell suspensions. After 1-h incubation, 0.2 mL of suspension were extracted and injected in the thermoresistometer. No growth or inactivation was detected ($p > 0.05$) after heat shock treatments (data not shown).

2.4. Incubation of treated samples and survival counting

Triptone Soya Agar (Biolife, Milan, Italy) supplemented with 0.6 % of yeast extract (TSAYE) used as non-selective medium was added to heat-treated samples placed onto Petri dishes, and then incubated at 37 °C for 24 h. In order to evaluate the occurrence of sublethal damage, samples were also plated onto TSAYE with 5 % of NaCl (TSAYE-SC) as selective recovery media. That was the maximum non inhibitory sodium chloride concentration for native cells (data not shown). Samples recovered in selective media were incubated for 48 h. Previous experiments demonstrated that longer incubation times did not influence survival curves. After incubation, CFUs were counted with an Improved Image Analyzer Automatic Colony Counter (Protos, Synoptics, Cambridge, UK) as previously described by Condón et al. (1996).

The proportion of sublethally injured cells was estimated by comparing the number of $\log_{10}$ cycles of inactivation obtained after plating heat-treated cells onto the non-selective (TSAYE) and selective (TSAYE-SC) media.

2.5. Heat resistance parameters

The traditional model based on the first-order inactivation kinetics was used to describe survival curves obtained under isothermic conditions. D_T value or decimal reduction time (min) is mathematically the negative inverse of the slope of the regression line of the survival curves at a constant treatment temperature *T*. Survival curves were obtained by plotting the $\log_{10}$ number of survivors *vs.* the treatment time (min).

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