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Effect of thymol in heating and recovery media on the isothermal and non-isothermal heat resistance of *Bacillus* spores

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ABSTRACT

Members of the genus *Bacillus* include important food-borne pathogen and spoilage microorganisms for food industry. Essential oils are natural products extracted from herbs and spices, which can be used as natural preservatives in many foods because of their antibacterial, antifungal, antioxidant and anti-carcinogenic properties. The aim of this research was to explore the effect of the addition of different concentrations of thymol to the heating and recovery media on the thermal resistance of spores of *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* at different temperatures. While the heat resistance was hardly reduced when thymol was present in the heating medium, the effect in the recovery medium was greater, reducing the $D_{100\,^{\circ}C}$ values down to one third for *B. subtilis* and *B. cereus* when 0.5 mM thymol was added. This effect was dose dependent and was also observed at other heating temperatures.

1. Introduction

Bacillus are Gram-positive and spore-forming bacteria. Bacterial spores are the main problem in many food industries not only for their ubiquitous nature, but also because of their ability to survive, in some instances, to the industrial sterilisation processes and, what is more, to proliferate under conditions generally presumed to prevent growth (such as low temperatures) (Scheldeman et al., 2006). Over-processing is often applied to the products that have a prolonged shelf life, being detrimental for their quality. Even though, in recent years, highly heat resistant spore-forming bacteria have increased challenges in industrial sterilisation processes to assure food safety and prevent spoilage (Oomes et al., 2007).

Combined processes have been developed in order to ensure microbial safety, affecting to a lesser extent the sensorial and nutritional properties. Different studies have shown the antimicrobial efficacy of essential oils, alone or in combination with other preservation methods, against spoilage and food-borne pathogens (Ultee et al., 2000; Tiwari et al., 2009; Esteban and Palop, 2011; Huertas et al., 2014).

Essential oils are natural products extracted from herbs and spices used as flavourings in the food industry. Nowadays their use as natural preservatives in many foods is gaining interest because of their antibacterial, antifungal, antioxidant and anti-carcinogenic properties. Thymol is a phenolic compound present in the essential oil fraction of *Oreganum* and *Thymus* plants. The addition of thymol to the foods that are going to be heat treated could reduce processing times and temperatures considerably. The thermal resistance of spores is strongly influenced by the heating and recovery conditions (González et al., 1996; Coroller et al., 2001; Lekogo et al., 2010; Esteban et al., 2013). A better knowledge of the effect of the preservation agents in the heating and recovery media on microorganisms would lead to a more rational design of thermal processes. In this regard, no research on the effect of the combination of preservation agents and heat under non-isothermal conditions has been conducted yet.

The aim of this study was double, on one hand to know and model the influence of different concentrations of thymol in the heating and in the recovery media on the heat resistance of *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* spores and, on the other hand, to explore the effect of thymol in both media under non-isothermal heating conditions on *B. subtilis* spores.

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2. Material and methods

2.1. Bacterial strains and culture conditions

The strains used in this study were *B. cereus* INRA ATZ421 (kindly supplied by Institut National de la Recherche Agronomique), *B. licheniformis* CECT 4525 and *B. subtilis* CECT 4071 (both supplied by the Spanish Type Culture Collection) and *Bacillus subtilis* AdHC1 (kindly supplied by Unilever Bestfoods Research Vlaardingen). All the strains were prepared on Petri dishes of plate count agar (PCA; Scharlau Chemie, Barcelona, Spain). The agar surface was inoculated with 0.2 mL of a 24 h culture grown in brain heart infusion broth (BHIB; Scharlau Chemie) at 37 °C. The agar plates were incubated at 37 °C for sporulation. For all microorganisms, once 90% sporulation was achieved, as determined by phase contrast microscopy (Leica, Wetzlar, Germany), spores were collected by flooding the agar plate with sterile distilled water, scratching the surface with a spatula. After harvesting, spores were washed four times by centrifugation at 3000 × g during 15 min at 4 °C and resuspension in sterile distilled water. The concentration of spores in the final suspension was adjusted at 10^9 spores mL^{-1} with sterile distilled water. The suspensions were stored at 4 °C for at least two weeks before being used to establish their heat resistance to allow spore maturation (Van Zuijlen et al., 2010). The spore suspensions were stored at 4 °C until use. One spore suspension was prepared for each strain.

2.2. Chemicals

Thymol (Sigma–Aldrich Chemie, Steinheim, Germany) stock solutions 0.5 M were made in 95% ethanol and stored at 4 °C.

2.3. Heat treatment

Heat resistance determinations were carried out in a thermoresistometer Mastia (Conesa et al., 2009). To perform each experiment 0.2 mL of the microbial suspension were inoculated into 400 mL of the heating medium in the vessel of the thermoresistometer. The D values were determined in BHIB at temperatures of 100, 105 and 110 °C for isothermal treatments. For non-isothermal treatments the thermoresistometer was programmed to perform a linear temperature profile, starting from an initial temperature of 95 °C at a constant heating rate of 1 °C min^{-1}. This is a common heating rate for static sterilization retorts. Also, previous studies (Esteban et al., 2013) showed no effect of this heating rate on the heat resistance of spores. Different concentrations of thymol (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM) were added to the heating medium, BHIB, and/or to the recovery medium, Brain Heat Infusion Agar (BHIA; Scharlau Chemie). Survival counting was based on duplicate counts, from appropriate dilutions in peptone water (Scharlau Chemie), plated in BHIA and incubated at 37 °C for 24 h. Preliminary experiments showed that longer incubation times did not modify plate counts. BHIB and BHIA were chosen as heating and recovery media respectively, in an attempt to have both media as similar as possible, to minimize the effect of other influencing factors on heat resistance.

2.4. Data analysis

Lineal survival curves were found for all microorganisms tested, with correlation coefficients higher than 0.96 in all cases. Hence, classical first order inactivation kinetics was chosen to fit survival curves. D values were estimated from the survival curves as the negative inverse of the slope. *z* values were estimated from the decimal reduction time curves (DRTCs) as the negative inverse of the slope. All resistance determinations for survival and recovery were performed at least twice in independent experiments at different days, using the only spore suspension prepared for each microorganism. Average D values and their associated standard deviation were calculated for each microorganism, thymol concentration and temperature. Differences between data were considered significant when $p \leq 0.05$.

The effect of the different concentrations of thymol in the recovery media on the D values at different temperatures was modelled by a secondary model based on the Bigelow model as proposed by Gaillard et al. (1998) and expanded by including the approach presented by Lekogo et al. (2010).

$$\log D = \log D^* - \frac{T - T^*}{z_T} - \frac{[thymol]}{z'_{thymol}} \quad (1)$$

where D^* is the D-value at 100 °C and without thymol in the recovery medium, z_T is the increase in temperature that leads to a 10-fold reduction in the D-value and z'_{thymol} is the increase of thymol concentration in the recovery medium that leads to a 10-fold reduction in the D-value.

For non-isothermal treatments, experimental data were confronted against survivor numbers predicted from D and *z* values obtained under isothermal conditions (Conesa et al., 2009). Predictions were based on the integration of heat treatment lethality, which was calculated applying the general method of process calculation (Stumbo, 1973; López et al., 2011).

The parameter values and their associated standard deviation were calculated by an appropriate statistical package (Matlab, The Math Works, Natick, USA). Significant differences between D-values were analyzed by ANOVA test at the 95% confidence level.

3. Results

The addition of thymol to the heating medium had a rather small effect on the thermal resistance of all the microorganisms tested (Fig. 1). The $D_{100\,°C}$ value decreased from 2.86 to 2.06 min in the case of *B. licheniformis* when 0.5 mM thymol was added to the heating medium. Similar or even lower decreases were found for the other microorganisms (Fig. 1). The presence of thymol in the recovery media had a greater effect on all the microorganisms, reducing their D_{100} values even down to one third for *B. cereus* and *B. subtilis* CECT 4071 when 0.5 mM thymol was added to the recovery medium (Fig. 1a and d respectively). When increasing concentrations of thymol were added to the recovery medium after the thermal treatment, increasing effects were shown for all microorganism, except for *B. subtilis* AdHC1, for which no significant differences were found among D values at the different thymol concentration tested but, even for this microorganism, a trend could be observed. Dose dependent effects were found at 100 °C for all the microorganisms through all the range of thymol concentrations tested (Table 1), with linear correlations between the log D values and the concentration of thymol in the recovery medium ($r^2 \geq 0.85$; Fig. 2).

The effect of the thymol concentration in the recovery medium was also observed at other heating temperatures for all microorganisms. Fig. 3 shows, as an example, the survival curves of *B. subtilis* AdHC1 at 105 °C.

The data on the effect of antimicrobials in the recovery medium at different temperatures were used to build mathematical models to predict the influence of thymol. These models were based on the Bigelow model, as proposed by Gaillard et al. (1998) and expanded by including the approach presented by Lekogo et al. (2010) (Eq. (1)). The z'_{thymol} values (Table 2) are the concentrations of thymol in the recovery medium necessary to reduce the D value to one tenth