ORIGINAL RESEARCH

Kinetic studies on combined high pressure and temperature inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice

Adriano Ardia,[1] Dietrich Knorr,[1] Giovanna Ferrari,[2] Volker Heinz[1]

[1]Department of Food Biotechnology and Food Process Engineering, Berlin University of Technology, Berlin, Germany;
[2]Department of Chemical Engineering, University of Salerno, Fisciano, Italy

Abstract: *Alicyclobacillus acidoterrestris* spores were inactivated in orange juice by high-pressure treatment combined with elevated temperatures. The treatment time was shorter in comparison with the holding time required by thermal processing. To reach spore inactivation of at least five log cycles, hydrostatic pressures and initial process temperatures were applied in a range of 100–700 MPa and 80–95 °C. The impact of the combined action of pressure and temperature on spore reduction was also predicted by a mathematical model derived from the experimental survival curves and by making use of empirical equations and theoretical considerations.
Keywords: *Alicyclobacillus acidoterrestris*, spores, pressure, temperature, orange juice

Introduction

Bacterial spores themselves are not a hazard to the food industry. It is the eventual germination, outgrowth and proliferation of the microorganism that results in spoilage, sometimes causing problems of 'off' flavour and browning of the product. Sporulation is an irreversible process that occurs normally after the exponential growth phase when the generation time increases.

Factors affecting endospore formation include temperature of growth, pH of the medium, aeration, the presence of minerals (especially Mn^{2+} and Ca^{2+}), the presence of certain carbon or nitrogen compounds and their concentrations (Bergère 1991). The acidification of foods is often used to prevent the growth of some heat-resistant microorganisms, because the low pH can reduce the thermal resistance of spores and because many spore-forming bacteria cannot germinate below a certain pH value. Most of the considerations of how spores resist thermal inactivation are closely linked to the spore ultra-structure (Tipper and Gauthier 1972; Warth 1978).

The high resistance of endospores is of practical concern to those seeking to manufacture shelf-stable food products. In recent decades, several thermoacidophilic spore-forming bacteria have been isolated from spoiled fruit juices (Cerny et al 1984; Splittstoesser et al 1994; McIntyre et al 1995) and implicated in acidic beverage and fruit juice spoilage incidents in the USA, UK, Japan and elsewhere. These bacteria were identified as *Alicyclobacillus acidoterrestris* (Yamazaki et al 1996). *Alicyclobacillus acidoterrestris* is considered to be one of the important target microorganisms in quality control of acidic canned foods.

Due to its thermoacidophilic nature, the spores of this bacterium cannot be destroyed by classical pasteurisation treatments usually applied in the juice industry (Murakami et al 1998; Pontius et al 1998). Therefore, there is a need for an alternative technology to replace conventional pasteurisation and overcome the problems related to the spores of *Alicyclobacillus acidoterrestris*.

High hydrostatic-pressure processing is capable of inactivating bacterial spores more effectively when combined with elevated temperatures (Sale et al 1970; Hayakawa et al 1994; Ananta et al 2001). High-pressure processing is a tool that has recently reached industrial relevance, with industrial-sized pressure units available up to 800 MPa. High-pressure treatment has been used for sterilisation or pasteurisation of foods, although sterilisation has been limited to small-scale processing. Even though the temperature used in combination with pressure is relatively low (below 50 °C), the death rate changes considerably with temperature and the inactivation behaviour of spores by pressurising is different from that of spores by heating (Hayakawa et al 1994; Okazaky et al 1994). It is therefore important to clarify kinetically the inactivation behaviour of various kinds of bacterial spores as a function of temperature and pressure. Only few published reports were

Correspondence: Volker Heinz, Department of Food Biotechnology and Food Process Engineering, Berlin University of Technology, Königin-Luise Str 22, D-14195 Berlin, Germany; tel +49 30 314 71441; fax +49 30 832 7663; email volker.heinz@tu-berlin.de

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found regarding the thermal resistance of *A. acidoterrestris* and no reports regarding its pressure resistance (Murakami et al 1998).

The aim of this work was to investigate the combined thermal–pressure inactivation of *Alicyclobacillus acidoterrestris* spores in an orange juice medium by modelling the survival curves using empirical equations, derived from *n*th-order kinetics, and theoretical considerations. Kinetics data for the inactivation of spores, applying high pressure ranging between 100 and 700 MPa at starting temperatures of 80, 85, 90 and 95 °C, were analysed to derive a mathematical model to predict spore reduction by the combined action.

Materials and methods

Spore production

Alicyclobacillus acidoterrestris DMS 2498 spore suspensions (provided by ECKES-GRANINI, Nieder-Olm, Germany) with initial counts of 4×10^9 to 8×10^9 CFU(colony-forming unit)/mL were incubated for 24 h in 20 mL standard 1 nutrient broth (Merck, Darmstadt, Germany) at 55 °C. After incubation, 3 mL of the suspension was spread-plated in petri dishes on selected agar (DSMZ (Deutsche Sammlung Von Mikroorgaiesmen Und Zellkulturen) nr 402) supplemented with 10 mg/L $MnSO_4 \cdot xH_2O$.

After 4–5 days incubation at 55 °C, the percentage of sporulated cells was assessed by phase contrast microscopy. When 90% of the cells were sporulated, the incubation was terminated. Spore and vegetative cells were separated by centrifugation at 4923 *g* for 20 min, followed by a treatment with 70% EtOH and subsequent centrifugation. Finally, the pellet was re-suspended in sterile distilled water, placed in cryogenic vials (Nalgene, Rochester, NY, USA) with a total volume of 2 mL and subsequently frozen at −20 °C. For the dilution phase, Ringer solution was used. Colonies were counted after incubation on DSMZ 402 agar at 55 °C for 2 days. For the thermal and also combined thermal–pressure treatments, the spores were inoculated directly in commercially available orange juice at a ratio of 1:10. For the thermal treatments, the samples were put in special glass containers, hermetically closed, to prevent the evaporation of the solution due to the long processing time at high temperature. For the combined temperature–pressure treatments, the samples were put in special containers (Nunc Cryo Tubes nr 375299, Nunc A/S, Roskilde, Denmark) with a volume of 1.8 mL.

Spore inactivation by thermal treatment was investigated using a thermostatic bath filled with silicon oil at starting temperatures of 80, 85, 90 and 95 °C, while combined temperature- pressure treatments were carried out by a high-pressure unit at 100, 200, 300, 500, 600 and 700 MPa and the same set of temperatures.

High-pressure equipment

The high-pressure multivessel (model U111; Unipress, Warsaw, Poland), schematically represented in Figure 1, consisted of five equal vessels in a parallel circuit, completely immersed in a thermostatic bath filled with silicon oil and connected to a pressure intensifier through capillary tubes (HP Standard Tubing 1/4, Citec, Switzerland). This design allowed for the simultaneous treatment of five different samples in one pressure build-up step at close to isothermal conditions. Each chamber was equipped with a K-type thermocouple and a pressure sensor to monitor the temperature and pressure history of each sample during the treatment cycle.

Through the radial pump (Mannesman Rexroth Polska, Warsaw, Poland) it was possible to reach a pressure of 70 MPa in the low-pressure part of the intensifier while, due to the section reduction (multiplying factor 10), it was possible to reach a maximum pressure level of 700 MPa in the upper part of the same.

The occurring heat of compression (in aqueous media approximately 3 °C per 100 MPa) was taken into account by

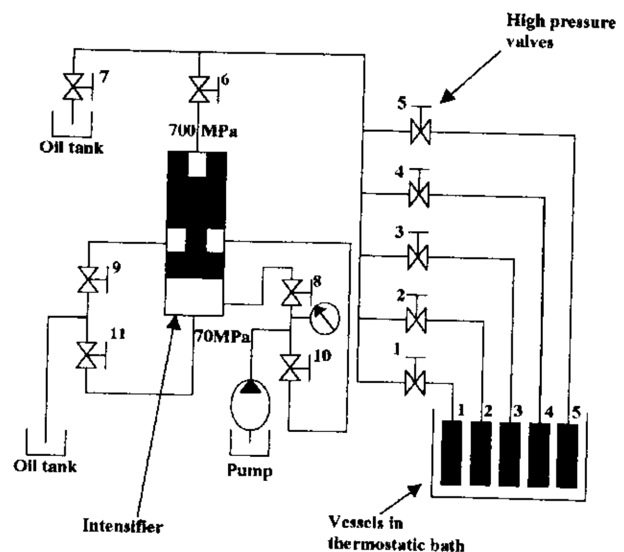

Figure 1 Schematic hydraulic diagram of multivessel high-pressure apparatus U111. The intensifier is connected with the pressure vessels through high-pressure valves (1–5). The multiplication factor (≈11) of the intensifier leads to a maximum pressure of 700 MPa. Valves 6–11 are used for loading and unloading the pressure medium (oil).

keeping the sample at a lower temperature level prior to pressurisation. The timer for measuring the reaction time was started after having reached isobaric and isothermal conditions (typically 30 s after the onset of compression).

Regression analysis

The experimental spore reduction levels (N/N_0) were studied by fitting an nth-order decay reaction:

$$\frac{dN}{dt} = -k \cdot N^n \quad (1)$$

or upon integration:

$$\left(\frac{N}{N_0}\right) = (1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1))^{\frac{1}{1-n}} \quad (2)$$

The second equation was used in its logarithmic form:

$$\log \frac{N}{N_0} = \log(1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1))^{\frac{1}{1-n}} \quad (3)$$

The inactivation rate constant k was obtained from regression analysis of the experimental kinetic data at constant pressure and temperature. A common reaction nth-order, which could fit the inactivation data of all pressure–temperature settings investigated, was obtained by minimising the accumulated residual standard error. Regression analysis was performed using TableCurve 2D-3D (SPSS, Chicago, IL, USA).

Results and discussion

In Figures 2, 3, 4 and 5, the thermal and the combined pressure–temperature inactivation of the spores, at starting

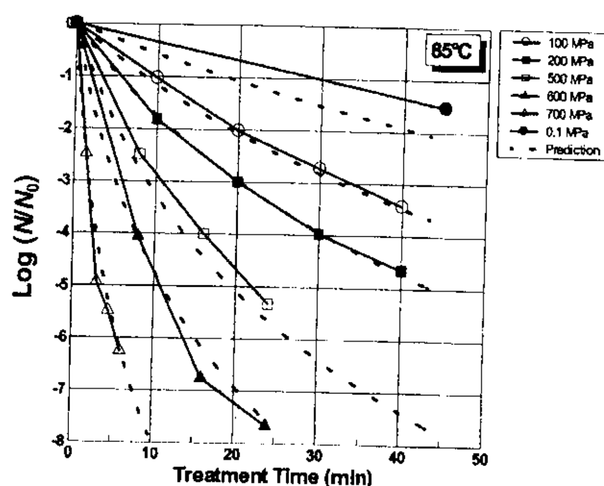

Figure 3 Temperature and combined temperature–pressure inactivation of spores. Starting temperature 85 °C. Experimental data (solid lines) and predicted data from the model equations (2) to (4) (dotted lines).

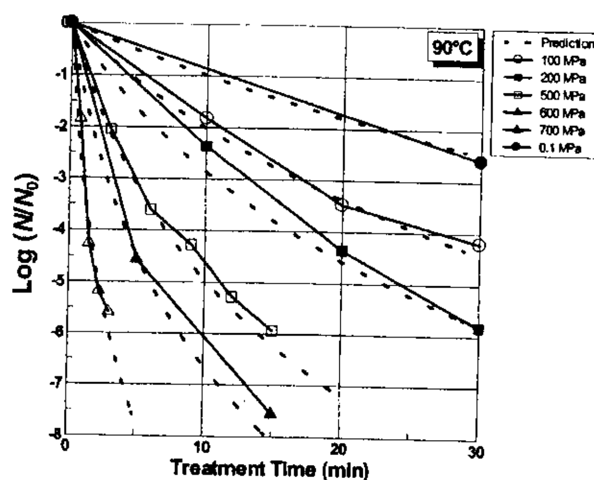

Figure 4 Temperature and combined temperature–pressure inactivation of spores. Starting temperature 90 °C. Experimental data (solid lines) and predicted data from the model equations (2) to (4) (dotted lines).

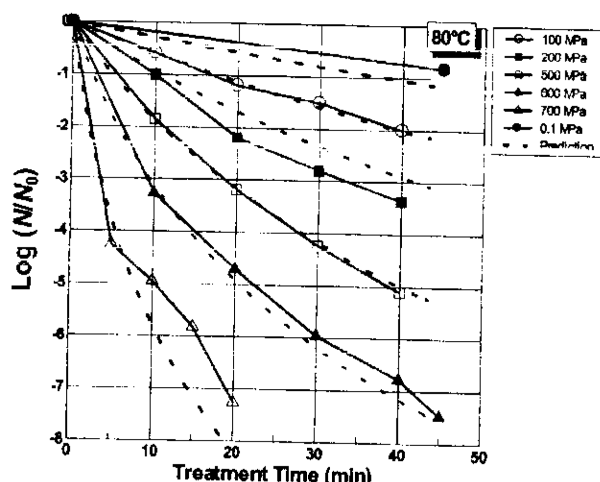

Figure 2 Temperature and combined temperature–pressure inactivation of spores. Starting temperature 80 °C. Experimental data (solid lines) and predicted data from the model equations (2) to (4) (dotted lines).

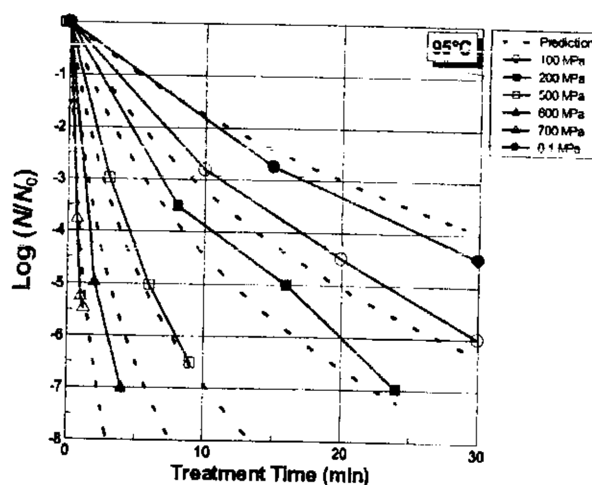

Figure 5 Temperature and combined temperature–pressure inactivation of spores. Starting temperature 95 °C. Experimental data (solid lines) and predicted data from the model equations (2) to (4) (dotted lines).

temperatures of 80, 85, 90 and 95 °C, are represented. In order to present more comprehensive results, the 300 MPa kinetics are not shown in the figures.

Thermal kinetics followed substantially a linear behaviour, while combined treatments seemed to produce 'quasi-linear' kinetics. For thermal kinetics, only the first inactivation point was considered in the graphs, to simplify the view of the whole inactivation process. The sole thermal treatments were applied for 60, 165, 300 and 385 minutes at starting temperatures of 95, 90, 85 and 80 °C, respectively. The solid lines represent the experimental data, while the dashed lines show the result of the regression analysis using a reaction order of $n = 1.1$.

In accordance with results from other authors (Lee et al 2002), high-temperature treatments alone did not result in a significant reduction of *A. acidoterrestris* spores, while a combination of high pressure and heat strongly reduced the numbers of survivors.

The reaction order has been derived from the accumulated standard error of all experimental inactivation kinetics investigated. The standard error function passes through a minimum at a reaction order of $n = 1.1$ (see inset of Figure 6). To describe the correlation between rate constant, pressure and temperature, the following equation was used (Eyring 1935):

$$\ln(k) = k' + \frac{(-\Delta V^{*})}{R \cdot T} \cdot p \qquad (4)$$

Equation (4), derived from the transition state theory, introduces the activation volume ΔV^{*} as the characteristic parameter of the pressure dependence of the rate constant k. In this equation, k' denotes the natural logarithm $\ln(k_0)$ at the reference state $p = 0.1$ MPa. To provide an overall model of the inactivation reaction rate, it is essential to find a function relating k' with the temperature level of the pressure treatment. In this case an Arrhenius-type equation was used:

$$k' = A_0 + \frac{E_a}{R \cdot T} \qquad (5)$$

Equation (4) is applicable to isothermal situations. R denotes the universal gas constant ($R = 8.314$ J/mol^{-1}K^{-1}). For the activation volume (ΔV^{*}), a second-order polynomial equation for pressure was applied to yield a satisfying fit of the experimental data:

$$(-\Delta V^{*}) = A_1 + A_2 \cdot p + A_3 \cdot p^2 \qquad (6)$$

From regression analysis, the polynomial parameters were determined as follows: $A_0 = 44.7 \pm 2.1$; $E_a = -145070 \pm 6340$ kJkg^{-1}; $A_1 = -32.3 \pm 3.3$ kJkg^{-1}MPa^{-1}; $A_2 = 0.079 \pm 0.012$ kJkg^{-1}MPa^{-2}; $A_3 = -7.9 \cdot 10^{-5} \pm 1.1 \cdot 10^{-5}$ kJkg^{-1}MPa^{-3}.

Combining equations (2) and (4) and solving for the pressure level, once time t, temperature T and inactivation level $\log(N/N_0)$ have been fixed, it was possible to identify all the p–T conditions resulting in the same inactivation level

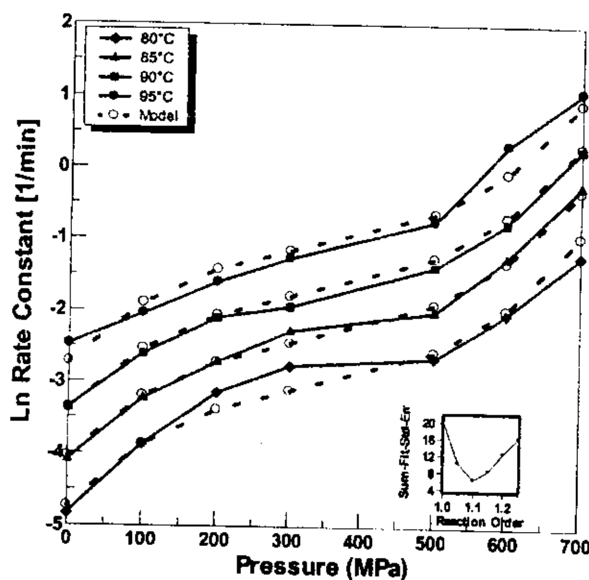

Figure 6 Rate constants of 1.1-order inactivation reaction plotted logarithmically versus pressure. Solid lines show the experimental data. Dotted lines are from the model fitting the original data by equation (4). The inset shows the overall standard error from the kinetic analysis.

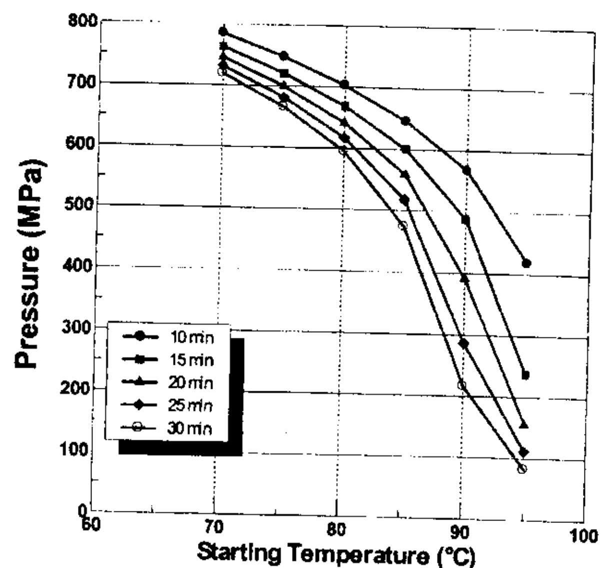

Figure 7 Pressure and temperature combinations for 6 log cycles spore reduction at different treatment times (from 10 to 30 minutes). Data are calculated combining equations (2) and (4) and solving for pressure, after having fixed the starting temperature, treatment time and log reduction.

for different treatment times (see Figure 7). At higher temperatures, a lower pressure level was needed to reach 6 log cycles inactivation, and vice versa.

From the model, it was predicted that a pressure of 780 MPa was needed to reach 6 log cycles inactivation at 95 °C with a treatment time of 30 seconds. As anticipated in equation (2), when increasing the initial concentration of spores a lower pressure level was necessary to achieve the same inactivation ratio (N/N_0) at the same treatment temperature.

Conclusions

The aim of this work was to describe the combined thermal and pressure inactivation of spores in orange juice. A linear behaviour of thermal inactivation was found, while minor deviations from linearity were found for the combined pressure–temperature treatment. Using the final model, it was possible to reproduce the inactivation behaviour of spores of *Alicyclobacillus acidoterrestris*. Temperature-pressure conditions required to achieve identical inactivation levels could be calculated. As shown in Figure 7, a longer treatment time was needed to reach the same level of inactivation using lower pressures or temperatures. In particular, while a 10 minute treatment time was needed when using 600 MPa and 90 °C initial temperature, starting from the same temperature but using a lower pressure, such as 300 MPa, required a 30 minute treatment time.

It is interesting to note that by coupling pressure and temperature it was possible to reduce the temperature level and the holding time of the inactivation process. Comparing the thermal effect of 80 °C starting temperature with the combined effect of 80 °C and 700 MPa, is possible to see a substantial difference in the inactivation level and the required treatment time: 45 minutes to reach less than 1 log cycle inactivation at 80 °C, and 20 minutes to inactivate more than 7 log cycles at 80 °C and 700 MPa.

In this way it was possible to find the optimum combination among the operative conditions in order to reduce the quality damage due to thermal treatments and to find the high-pressure level needed for spore sterilisation in food products.

Since pressurisation is a 'quasi-adiabatic' transformation, a temperature profile is present in the vessel and in the product itself. An increase of temperature is consequently applied during the pressure build-up phase only. Once the operating pressure is reached, the temperature of the product slowly decreases to its starting value because of the equilibration with the temperature of the pressure medium and the temperature of the vessel.

For industrial applications of high-pressure technology, it is not necessary to warm-up the product prior to the compression. Calculating the increase in temperature due to the adiabatic heating, it is possible to start the process with a lower product temperature, resulting in higher product quality and an economical, optimised sterilisation process.

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References

Ananta E, Heinz V, Schlüter O, Knorr D. 2001. Kinetic studies on high-pressure inactivation of *Bacillus stearothermophilus* spores suspended in food matrices. *Innovative Food Sci Emerg Technol*, 2:261–72.

Bergère JL. 1991. Spores formation and germination of *Bacillus cereus*: the spore cycle. *Bull IDF*, 275:9–14.

Cerny G, Hennlich W, Poralla K. 1984. Spoilage of fruit juice by bacilli: isolation and characterization of the spoilage organisms. *Eur Food Res Technol (Zeitschrift für Lebensmittel- Untersuchung und -Forschung)*, 179: 224–7.

Eyring H. 1935. The activated complex in chemical reactions. *J Chem Phys*, 3:107–15.

Hayakawa I, Kanno T, Yoshiyama K, Fujio Y. 1994. Oscillatory compared with continuous high pressure sterilization on *Bacillus stearothermophilus* spores. *J Food Sci*, 59:164–76.

Lee SY, Dougherty RH, Kang DH. 2002. Effect of high pressure combined with heat on reducing populations of *Alicyclobacillus acidoterrestris* in apple juice. In: Food microbiology: fruit and vegetables. Section 15D–19. 2002 Annual Meeting and Food Expo. 2002 Jun 16: Anaheim, CA, USA.

McIntyre S, Ikawa JY, Parkinson N, Haglund J, Lee J. 1995. Characteristics of an acidophilic Bacillus strain isolated from shelf-stable juices. *J Food Prot*, 58:319–21.

Murakami M, Tedzuka H, Yamazaki K. 1998. Thermal resistance of *Alicyclobacillus acidoterrestris* spores in different buffers and pH. *Food Microbiol*, 15:577–82.

Okazaky T, Yoneda T, Suzuki K. 1994. Combined effects of temperature and pressure on sterilization of *Bacillus subtilis* spores. *Nippon Shokulun Kogyo Gakkaishi*, 41:536–41.

Pontius AJ, Rushing JE, Foegeding PM. 1998. Heat resistance of *Alicyclobacillus acidoterrestris* spores as affected by various pH values and organic acids. *J Food Prot*, 61:41–6.

Sale AJH, Gould GJW, Hamilton WA. 1970. Inactivation of bacterial spores by hydrostatic pressure. *J Bacteriol*, 121:664.

Splittstoesser DF, Churey JJ, Lee CY. 1994. Growth characteristics of aciduric sporeforming bacilli isolated from fruit juice. *J Food Prot*, 57:1080–3.

Tipper DJ, Gauthier JJ. 1972. Structure of the bacterial endospore. In Halvorson HO, Hanson R, Campbell LL, eds. Spores V. Washington, DC: American Society for Microbiology. p 3–12.

Warth AD. 1978. Molecular structure of the bacterial spore. *Adv Microb Physiol*, 17:1–45.

Yamazaki K, Teduka H, Shinano H. 1996. Isolation and identification of *Alicyclobacillus acidoterrestris* from acidic beverages. *Biosci Biotechnol Biochem*, 60:543–5.