

Influence of high-pressure–low-temperature treatment on the inactivation of *Bacillus subtilis* cells

T. Shen^{a,*}, G. Urrutia Benet^{b,1}, S. Brul^{a,2}, D. Knorr^{b,1}

^aUnilever Research and Development Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

^bDepartment of Food Biotechnology and Food Processing Engineering, Berlin University of Technology, Königin-Luise-Str. 22, D-14195 Berlin, Germany

Received 18 October 2004; received in revised form 3 March 2005; accepted 17 March 2005

Abstract

High pressure inactivation processes, especially at subzero temperatures, were performed on *Bacillus subtilis* vegetative cells at various pressure, temperature and time combinations. Whilst atmospheric pressure, lowering the temperature for various periods to as low as $-45\text{ }^{\circ}\text{C}$ was found to have minor anti-microbial effects. Upon application of high pressure various phase transitions of ice occurred in the microbial suspensions under study. After pressure treatment at 150–450 MPa, cells were plated under optimal conditions to assess cell viability. Treatments of cells between 250 and 350 MPa at $-25\text{ }^{\circ}\text{C}$ were the most effective in inactivation. For these conditions, a double effect of extracellular solid–solid (Ice I–III) phase transition and possible intracellular solid–liquid phase transition is suggested to be the key in mediating the observed drop in viability.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: High-pressure–low-temperature (HPLT); Microbial inactivation; *Bacillus* inactivation

Industrial relevance: The possibility to inactivate pathogenic microorganisms at frozen storage temperatures via solid–solid phase transitions of different types of ice (ice I, III, V) under moderate pressures (250 to 350 MPa) offers an attractive alternative as low temperature decontamination process for foods. The data indicate up to 5 log cycle reductions in model systems.

1. Introduction

High hydrostatic pressure has attracted much interest as an alternative to heat as a means of inactivating microbes in food (Knorr, 1995). To ensure that pressure-treated foods are microbiologically safe, it is necessary to define pressure treatments that will ensure destruction of the pathogens of concern in different foods (Mackey, Forestière, & Isaacs, 1995). Although the use of high hydrostatic pressure technology as a pasteurization treatment is increasing (Schlüter, Urrutia Benet, Heinz, & Knorr, 2004), knowledge

of the underlying mechanism of inactivation is limited (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989).

A vast amount of data exists on the kinetics of pressure inactivation of vegetative microorganisms (Cheftel, 1995) at ambient and elevated temperatures (Heinz & Knorr, 1996; Heinz, Knorr, & Schlüter, 1998). In contrast, limited information has been gathered so far concerning microbial inactivation by means of high pressure in the low temperature range. Empirical data have shown that high pressure processing at low to ambient temperatures yielded improved microbial inactivation and better sensorial characteristics of vegetables (Brul, Rommens, & Verrips, 2000; George, 2000; Smelt, Hellemons, & Brul, 2001). Preliminary data on selected pathogenic organisms suggest more effective inactivation under pressure at low (-10 – $-5\text{ }^{\circ}\text{C}$) temperatures as compared to ambient (20 – $40\text{ }^{\circ}\text{C}$) ones (George, 2000).

* Corresponding author. Tel.: +31 10 4605161; fax: +31 10 4605188.

E-mail addresses: Tong.Shen@Unilever.com (T. Shen),

Gabriel.Urrutia@tu-berlin.de (G. Urrutia Benet).

¹ Tel.: +49 30 314 71248; fax: +49 30 832 7663.

² Tel.: +31 10 4605161; fax: +31 10 4605188.

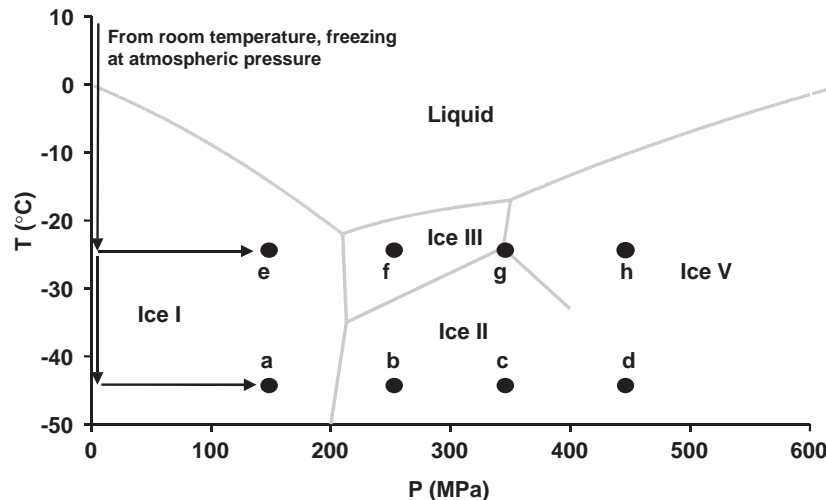


Fig. 1. P/T conditions of experiments: (a) $-45\text{ }^{\circ}\text{C}/150\text{ MPa}$; (b) $-45\text{ }^{\circ}\text{C}/250\text{ MPa}$; (c) $-45\text{ }^{\circ}\text{C}/350\text{ MPa}$; (d) $-45\text{ }^{\circ}\text{C}/450\text{ MPa}$; (e) $-25\text{ }^{\circ}\text{C}/150\text{ MPa}$; (f) $-25\text{ }^{\circ}\text{C}/250\text{ MPa}$; (g) $-25\text{ }^{\circ}\text{C}/350\text{ MPa}$; (h) $-25\text{ }^{\circ}\text{C}/450\text{ MPa}$.

The different possibilities of processing food products in the high-pressure–(subzero) low-temperature (HPLT) range described by Urrutia Bennet, Schlüter, and Knorr (2004) include ice phase transitions that should affect the viability of unwanted micro-organisms when present in treated products. A recent study from Luscher, Balasa, Fröhling, Ananta, and Knorr (2004) indicated that inactivation of the gram-positive bacterium *Listeria innocua* is more effective after it had undergone Ice I–III solid–solid phase transition. In the current paper, *Bacillus subtilis* PS832 vegetative cells were selected as a model for spore-forming gram-positive bacteria. We chose this model, as we wanted to assess whether the HPLT treatment is also effective against vegetative cells of a spore-forming microbe. These microorganisms are a challenge to the food industry since they can produce highly thermal resistant bacterial survival structures. Upon germination, these spores may give rise to biofilm formation for instance on piping of food manufacturing factories (den Aantrekker et al., 2003). Such spores may survive the preservation treatment and can subsequently germinate in the product when distributed through the rest of the food chain. This causes economical loss and in some cases public health concern.

Here, the inactivation of *B. subtilis* strain PS832, grown in TSB as culture medium, was studied for different pressure, temperature and time combinations. Some of these combinations lead to solid–solid phase transitions after freezing at atmospheric pressure and subsequent pressurisation. Depending on the pressure level, the samples may undergo solid–solid phase transitions from ice I into the domains of ice II, ice III or ice V (see Fig. 1). We show that treatments of cells between 250 and 350 MPa at $-25\text{ }^{\circ}\text{C}$ are the most effective in inactivating vegetative *B. subtilis* cells. For these conditions, a double effect of extracellular solid–solid (Ice I–III) phase transition and possible intracellular solid–liquid phase transition is

suggested to be key in mediating the observed drop in viability.

2. Materials and methods

2.1. Strain

The strain used in all of the experiments was *B. subtilis* PS832 (VBBA 1–187, a wild-type trp^+ revertant of strain 168, Unilever Food Research Centre) kindly provided by the group of Prof. Peter Setlow (University of Connecticut Health Center, Farmington, CT USA). This strain is classified as genetically indistinguishable from *B. subtilis* 168 (Kort et al., in press; Oomes & Brul, 2004).

2.2. Microorganism culture preparation

One aliquot of *B. subtilis* PS832 overnight culture was transferred into pre-warmed TSB (Trypticase Soy Broth, Tritium Microbiologie B.V. BBL 4311768) with an initial OD600 of 0.02–0.04. This broth was incubated for about 2.5 h to reach the mid exponential growth phase at an OD600 of 0.4–0.6. This step was repeated once to get to the final culture that was used for the experiments. Cells were then harvested by centrifugation at $4\text{ }^{\circ}\text{C}$ and $5000 \times g$ for 15 min. Cell pellets were re-suspended in 50 mM ACES, pH 7.0 (*N*-[2-Acetamido]-2-aminoethene-sulfonic acid, EEC No. 230-908-4, SIGMA) buffer, at a final concentration of 10^7 – $10^8\text{ cells ml}^{-1}$.

Approximately 3 ml of the suspension was placed in a polyethylene pouch and heat-sealed. This pouch was placed in an aluminum pouch and heat-sealed. It was imperative to eliminate any air bubbles from both bags. After sealing samples were immediately stored at $-80\text{ }^{\circ}\text{C}$.

The cultures were examined for the presence of spores by heating the cell suspension at $80\text{ }^{\circ}\text{C}$ for 10 min, followed by

colony counting on TSA (tryptic soy agar, Tritium Microbiologie B.V. BBL 4311043). The concentration of the spores in all cultures used in the experiments described here was less than 10^2 spores ml^{-1} .

2.3. High pressure treatment at 10 °C

These experiments were carried out in a 1.5 l high-pressure rig (National Forge St Niklaas, Belgium). The initial temperature inside the vessel was set at 10 °C. The cell suspension was exposed to pressure levels of 200, 250 and 350 MPa for 1, 60, 300, and 600 s. In addition, pressure was applied in 2-cycles of 300 s. After treatment, the samples were further processed as described in Section 2.5.

2.4. High pressure treatment at subzero conditions

All experiments were carried out in a high-pressure vessel specially designed for subzero operation (Unipress, Warsaw, Poland), already described in a previous paper (Schlüter et al., 2004). The temperature of the vessel was controlled by immersion either in a cryostat (HAAKE, Germany) or a thermostat (Lauda, Germany). The temperatures (including the outer vessel wall and the bath temperature) and the system pressures during the experiments were digitally recorded each 0.2 s (measure rate of 5.0 Hz). For the control of the sample temperature, a thermocouple measured the temperature of the pressure-transmitting medium surrounding the sample. The double pouch was placed inside the pressure vessel, which was then filled with silicon oil and pre-equilibrated at the desired temperature. After the temperature reached equilibrium, pressure was applied for 1, 20, 60, 300, 600 seconds and 2-cycles of 300 s as described in Section 2.3. As a control, another sample was kept at the desired temperature without pressurization.

Similar to the high-pressure treatment at 10 °C, the *P/T* (pressure/temperature) combinations used were 150, 250, 350 and 450 MPa at initial temperature of -45 and -25 °C. After treatment, samples were stored at -80 °C until further processing as described in Section 2.5.

2.5. Inactivation assessment

Samples treated at subzero temperatures were taken out from the deep frozen storage (-80 °C) and thawed on ice before colony counting. Samples treated at 10 °C were kept on ice for a maximum of 2 h before colony counting. All of these samples were then diluted in ten-fold series and incubated in TSA at 37 °C for 3 days for colony counting. All the experiments were performed in duplicates, except for the *P/T* combinations -25 °C/150 MPa, -25 °C/250 MPa, -45 °C/150 MPa and -45 °C/250 MPa. In these cases, given the particular interest of the first results obtained, the experiments were repeated a total of five times and all repetitions confirmed the first results

obtained. Typical results of these experiments are shown in this paper.

3. Results and discussion

3.1. Determination of process parameters

In Fig. 1, the selected *P/T* combinations are shown in the phase diagram of water.

In all cases, the samples were first frozen at atmospheric pressure and then pressurised until the corresponding set point. After the treatment time, pressure was released to atmospheric level, after which the samples were taken out from the pressure vessel. They were then kept at -25 °C until further analysis of microbial survival. In the cases where pressure was applied at 2 cycles of 300 s, the samples were again pressurized after the first pressure release.

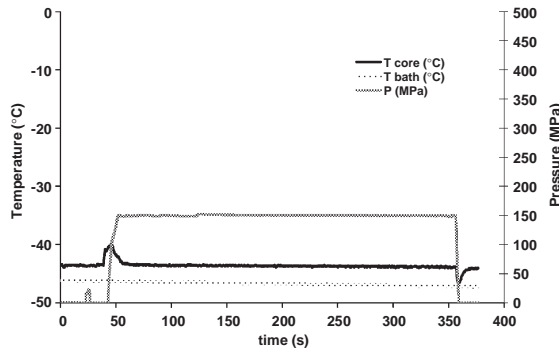
Given the different *P/T* combinations, at which the samples were treated, the *Bacillus* suspensions were subjected to different solid–solid and solid–liquid phase transitions. Depending on the rate of pressurization and pressure release, these solid–solid phase transitions were more or less easily observable in the pressure and temperature profiles. A slower rate of pressure release allowed us to obtain a clearer discontinuity in the *P/T* profiles. It must be pointed out that the temperature measurements correspond to the temperature of the surrounding pressurization medium and not are direct temperature measurement of the microbial suspension. Therefore, the pressure changes (due to phase changes) could not be directly inferred from the temperature changes. Nevertheless, the pressure changes, due to density changes between ice modifications, are still recognizable in most of the pressure profiles.

Different examples of *P/T/t* (pressure/temperature/time) diagrams reflecting the applied processes are shown in Fig. 2. Phase transition events are circled.

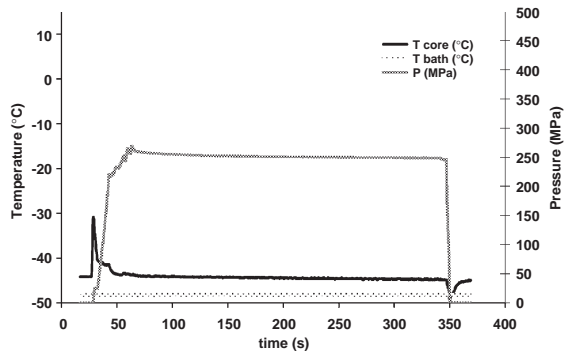
In the experiments described in Fig. 2a, b and e, no solid–solid phase transitions took place, as no discontinuities in either pressure nor temperature profiles were detected. In these cases, the samples kept the initial ice I structure. The analysis of the other experimental conditions (Fig. 2c, f, g, and h) does show solid–solid phase transitions as indicated by the discontinuity in the individual traces (see circles in the figures). The singular points indicating the *P/T* conditions (Fig. 1) for the different experiments lay in some cases in the domain of other ice structures than the ones expected on basis of the ideal behaviour of pure water. For instance the fact that ice V had never obtained all through the experiment as shown in Fig. 3 exemplifies the existence of metastable states of ice III. The specific cases are discussed below.

Fig. 2a shows one of the experiments carried out for the *P/T* combination of -45 °C and 150 MPa. For this *P/T* combination, as it can be seen in Fig. 1, we expected to obtain ice I. The *P/T* profiles experimentally obtained

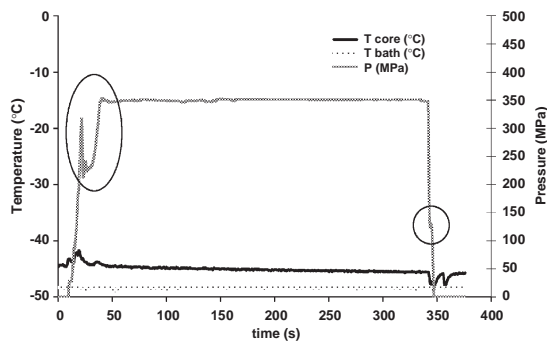
a) $T_i = -45^\circ\text{C}$, $P = 150\text{ MPa}$, $t = 300\text{ s}$



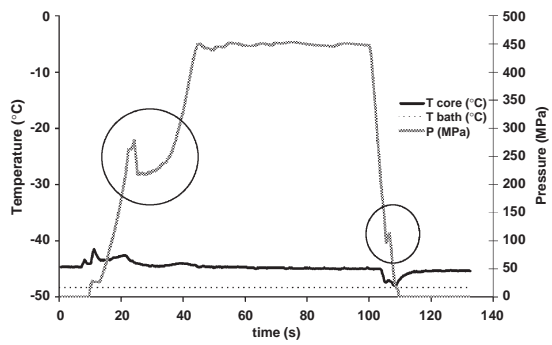
b) $T_i = -45^\circ\text{C}$, $P = 250\text{ MPa}$, $t = 300\text{ s}$



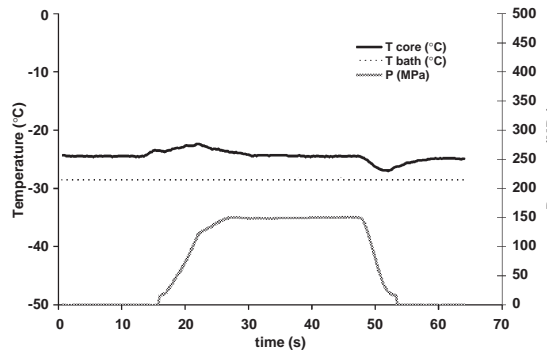
c) $T_i = -45^\circ\text{C}$, $P = 350\text{ MPa}$, $t = 300\text{ s}$



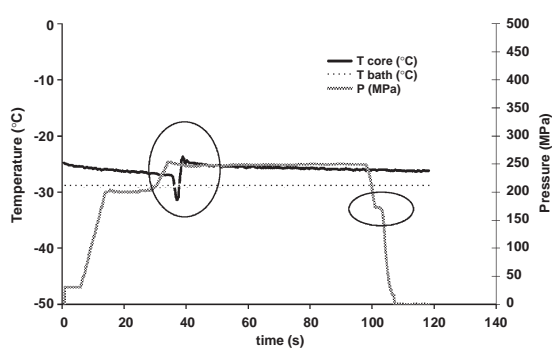
d) $T_i = -45^\circ\text{C}$, $P = 450\text{ MPa}$, $t = 60\text{ s}$



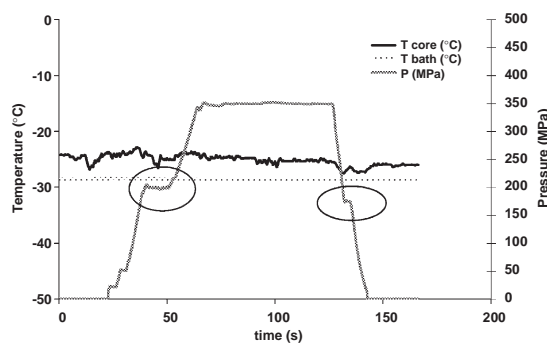
e) $T_i = -25^\circ\text{C}$, $P = 150\text{ MPa}$, $t = 20\text{ s}$



f) $T_i = -25^\circ\text{C}$, $P = 250\text{ MPa}$, $t = 60\text{ s}$



g) $T_i = -25^\circ\text{C}$, $P = 350\text{ MPa}$, $t = 60\text{ s}$



h) $T_i = -25^\circ\text{C}$, $P = 450\text{ MPa}$, $t = 300\text{ s}$

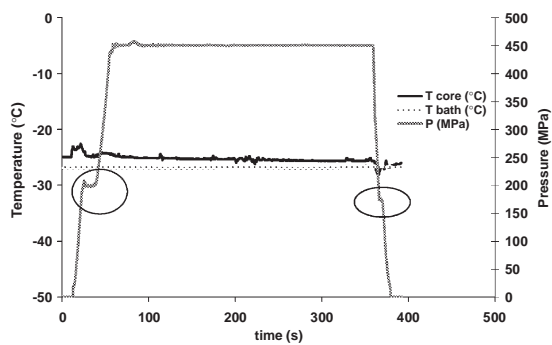


Fig. 2. Examples of one-cycle P/T profiles. *B. subtilis* overnight cell-suspensions in ACES buffer were treated at various time intervals, temperature and pressure levels as outlined in Materials and methods. Phase transition events are indicated with a circle.

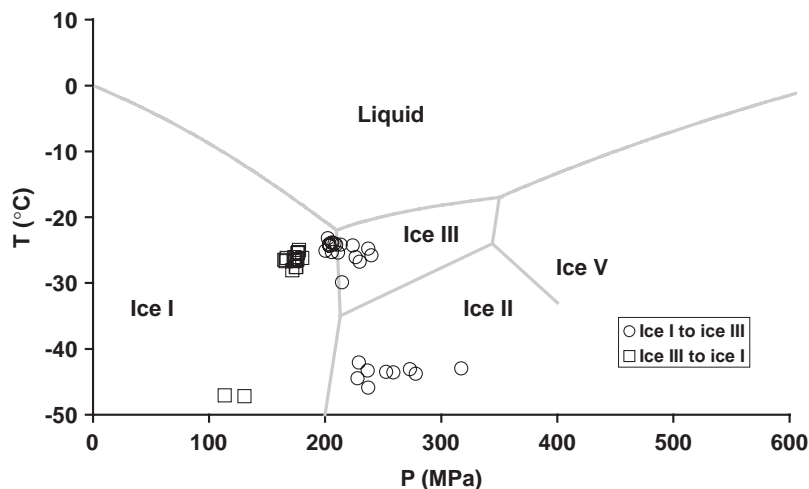


Fig. 3. Solid–solid phase transition registered points for of *B. subtilis* suspensions. Summary for all the accumulated data of solid–solid phase transitions during our experiments. Depending on the direction of the path or pressurisation or pressure release, the positioning of the experimental phase transition points differed. In this case, it should be pointed out that the lines shown for the pure water diagram are average values, as published from Bridgman (1912). Therefore, the range over which solid–solid phase transitions may be obtained can be very wide.

confirmed this. The same is applicable for the example shown in Fig. 2e (−25 °C and 150 MPa). For this P/T combination, the sample is still placed in the domain of ice I. No solid–solid phase transitions were observed.

Fig. 2b shows an unexpected result. For this P/T combination (−45 °C and 250 MPa), the sample is in the domain of ice II, but the experimentally obtained curves indicated that no solid–solid phase transitions occurred. This behaviour gives a strong indication for the existence of a metastable state of ice I in the domain of ice II. It should be taken into account that even the solid–solid phase transition lines for pure water as shown in Fig. 1 are actually the average values from many data points that have an experimental variability of more than 100 MPa in some cases. Fig. 2f and g show two examples of processes in which a solid–solid phase transition from ice I to ice III took place, just as on basis of the positioning of these processes in the phase diagram. In both cases, the phase transition was clearly observed both during pressurisation and upon depressurisation.

Fig. 2h shows a treatment of cells at −25 °C/450 MPa. For this P/T combination, as shown in Fig. 1, it was expected that a second solid–solid phase transition from ice III to ice V would take place. However, as the P/T profiles show, only a phase transition from ice I to ice III was observed. This case again points to the existence of a metastable phase, in this case of ice III in the domain of ice V.

Fig. 2c and d show treatments of cells at −45 °C/350 MPa and −45 °C/450 MPa, respectively. Despite that the ice II stability area is the one present in these experiments, ice III is usually formed when pressurizing ice at about −45 °C above the transition pressure (see e.g. Luscher et al., 2004). To assess the state of ice in our experiments, samples were warmed under constant pressure of 300 MPa above the ice II–ice III phase transition line until reached +10 °C

(unpublished data). No solid–solid phase transition was observed. Instead, the ice directly melted to water at the temperature of the phase transition line of ice III to liquid. This clearly indicated that the ice modification that was present was ice III. Therefore, we assume that ice III was formed in all cases in which solid–solid phase transitions took place.

For the experiments at −45 °C, the fact that in all the experiments carried out at 250 MPa no phase transition was observed highlights the existence of a metastable area of ice I in the domain of ice III. Only when the set point pressure was higher than 250 MPa, the phase transition occurred. Also, the fact that the points registered for the transitions during pressure increase differed significantly with those obtained during pressure decrease demonstrate the existence of kinetically metastable phases, leading to hysteresis processes. In these situations the path of the process under study has an influence on the final registered phase transition points.

Finally, for the experiments at −25 °C, the lack of a phase transition ice III to ice at 450 MPa is evidence for the existence of a metastable phase ice III in the domain of ice V.

Fig. 3 summarises all the accumulated data of solid–solid phase transitions during our experiments. Clearly, depending on the direction of the path or pressurisation or pressure release, the positioning of the experimental phase transition points differed.

3.2. Influence of the HPLT treatment on viability of *B. subtilis* vegetative cells

3.2.1. Effect of HPLT applied on *B. subtilis* cells in the domain of ice I

To assess bactericidal effect of freezing bacteria at atmospheric pressure, samples were frozen and kept for 40

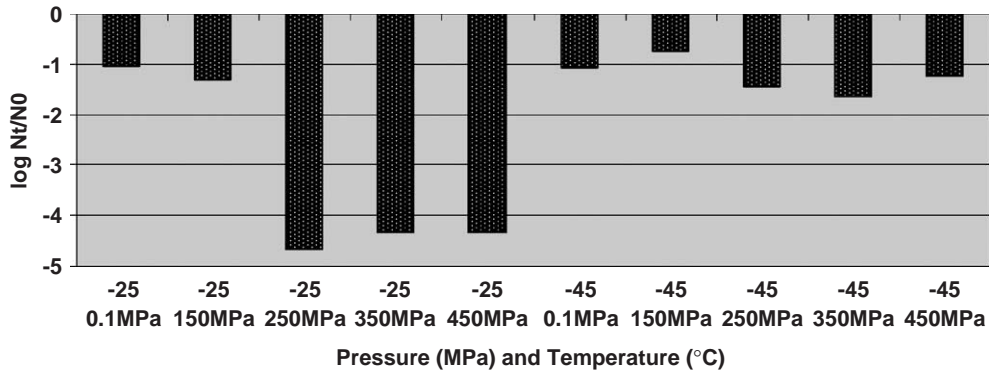


Fig. 4. The effect of lowered temperatures on HPLT inactivation of *B. subtilis* cells. *B. subtilis* strain PS 832 was suspended in ACES buffer at appropriate dilutions. The cultures were then treated in sealed bags for 20 s at the indicated *P/T* combinations as described in Materials and methods. Survival was measured through plate counting.

minutes at $-25\text{ }^{\circ}\text{C}$ or $-45\text{ }^{\circ}\text{C}$, respectively. Additionally, bactericidal effects of long term storage at $-20\text{ }^{\circ}\text{C}$ (over 25–31 days) were also monitored. The number of survivors was assessed by colony counting of aliquots after 3 days of incubation under optimal conditions ($37\text{ }^{\circ}\text{C}$ on TSA plates).

Viability of the cells was not affected by short-term storage at subzero temperatures. After 40 min freezing at $-25\text{ }^{\circ}\text{C}$ and $-45\text{ }^{\circ}\text{C}$, only a small \log_{10} -reduction in colony forming units (CFUs) was found (Fig. 4). Even a long-term storage up to 4 weeks did not lead to further substantial inactivation. A maximum of 1.4 log-reduction in CFU's was found at 31 days of storage at $-20\text{ }^{\circ}\text{C}$.

Next we assessed what the effect was of applying pressure on deep frozen *B. subtilis* cell. We found that 150 MPa pressure at either $-25\text{ }^{\circ}\text{C}$ or $-45\text{ }^{\circ}\text{C}$ led to only a1 log-reduction in CFUs (Fig. 4).

3.2.2. Effect of a combination of high pressure and freezing on microbial survival

3.2.2.1. Effect of temperature and pressure on inactivation. Fig. 4 summarises the results of the inactivation levels observed in *B. subtilis* cells subjected to a 20-s treatment at the various *P/T* combinations. All experiments carried out at 250 MPa and higher and at an initial

temperature of $-25\text{ }^{\circ}\text{C}$, resulted in strong inactivation. In *Bacillus* suspensions subjected to such conditions, a more than 4 log-reduction in cell viability was observed. Considering that the suspensions contained ca. 102 spores/ml, we concluded that a nearly full inactivation was achieved. Other cases ($-45\text{ }^{\circ}\text{C}$ with various pressures, and $-25\text{ }^{\circ}\text{C}/150\text{ MPa}$) resulted in mild to moderate inactivation, characterised by less than 2 log-reduction in viable cells. In addition, the inactivation showed a non-linear relation to treatment pressures at all the subzero temperatures. In contrast to what was observed at -25 and $-45\text{ }^{\circ}\text{C}$, the application of high pressure (200–250 MPa) at $10\text{ }^{\circ}\text{C}$ up to 60 s led only less than one log reduction (Fig. 5). An increasing difference in CFU's was obtained when the pressure treatment was above 250 MPa, or treatment time above 60 s.

3.2.2.2. Effect of the treatment time and application of two cycles of low temperature pressure treatment. In order to study in more detail the kinetics of the HPLT induced microbial inactivation, we next went on to assess the effect of treatment time on the inactivation of the cells. In these experiments, cells were exposed to $-25\text{ }^{\circ}\text{C}$ or $-45\text{ }^{\circ}\text{C}$ at elevated pressures and prolonged pressure holding times or with two cycles of pressure treatment. Unexpectedly, no

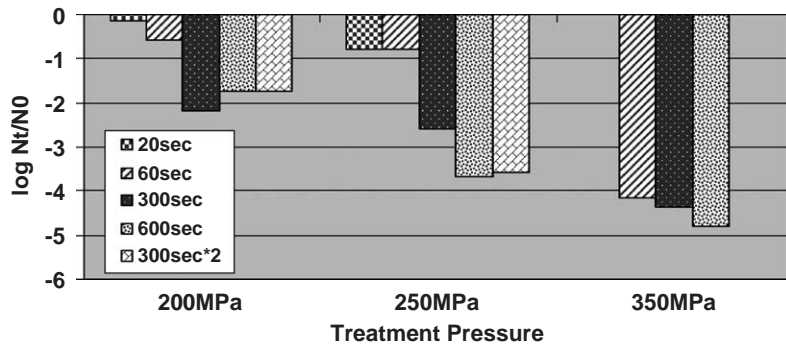


Fig. 5. The effect on high-pressure inactivation of *B. subtilis* cells at $10\text{ }^{\circ}\text{C}$. *B. subtilis* strain PS 832 was suspended in ACES buffer at appropriate dilutions. The cultures were then treated in sealed bags at the indicated *P/t* combinations as described in Materials and methods. Survival was measured through plate counting.

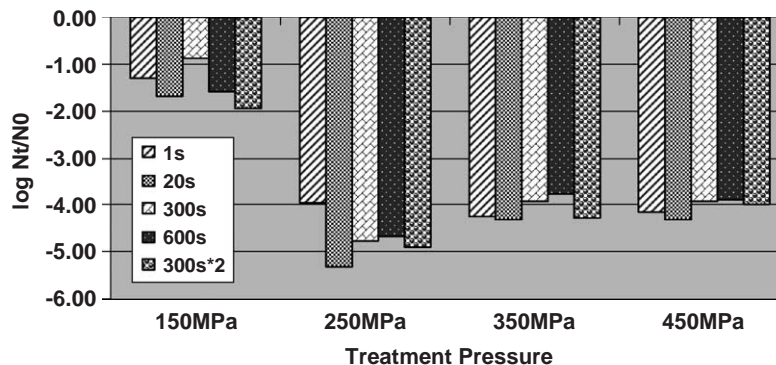


Fig. 6. Influence of treatment time and repeated treatment on the inactivation of *B. subtilis* at $-25\text{ }^{\circ}\text{C}$. *B. subtilis* strain PS 832 was suspended in ACES buffer at appropriate dilutions. The cultures were then treated in sealed bags at the indicated $P/T/t$ combinations as described in Materials and methods. Survival was measured through plate counting.

significant correlation was found between treatment time and loss of viability at all experimental conditions. Fig. 6 summarises the results for the $-25\text{ }^{\circ}\text{C}$ treatments. In a typical experiment, the inactivation of *Bacillus* cells that had undergone 600 s or 2 cycles of 300-s treatment was not enhanced compared to a short-term treatment of only after a few seconds. A similar trend was observed for the $-45\text{ }^{\circ}\text{C}$ treatments (data not shown). Interestingly, the inactivation at $10\text{ }^{\circ}\text{C}$ showed a clear time-dependence, with an increase loss of viability especially for treatment time up to 300 s (Fig. 5). The trend was mostly pronounced at pressures lower than 250 MPa.

4. Discussion

Temperature lowering alone has hardly any effect on the inactivation of *B. subtilis* cells. High pressure alone has a minor effect on cell viability. The combination of high pressure and low temperature, especially when it led to phase transitions, turned out to be most effective in inhibiting outgrowth of the *Bacillus* cells. In discussing our results, both solid–solid state transition and the phase transition from solid to liquid phase should be considered.

Phase transition from ICE I to III at temperature such as $-25\text{ }^{\circ}\text{C}$ was observed. The change of the ice crystal configuration may induce denaturation of the cellular protein, which damages the integrity of the cell membrane, and could lead to enhanced inactivation of crucial intracellular enzymes.

In addition, partial thawing of structures inside the cells may occur as well. Osmotic active substances such as Na^+ and K^+ in *Bacillus* are reported to be present in vegetative exponentially growing cells at concentrations above 350 mM and can reach easily to 500 mM or higher in stressed cells (Holtmann, Bakker, Uozumi, & Bremer, 2002; Whatmore, Chudek, & Reed, 1990; Whatmore & Reed, 1990). According to an ion–solvent interaction model proposed by Frank and Wen (1957), water structure around Na^+ ion resists making ice-like ordered water clusters while in the

frozen environment. On the other hand, ions such as K^+ , Rb^+ , Cs^+ and NH_4^{4+} surrounded by the water are called structure-breaking ions, which make the structure of the ice crystal much “looser” than the ice made up of pure water. It is therefore hypothesized that at the above-mentioned conditions, thawing had already occurred inside the cells while the surrounding water (actually ice) was undergoing Ice I–III solid–solid phase transition. A double effect of extracellular solid–solid (Ice I–III) phase transition and possible intracellular solid–liquid phase transition is suggested to be key in mediating the observed large drop in viability at $-25\text{ }^{\circ}\text{C}$ and $\geq 250\text{ MPa}$ treatment.

On the other hand, *Bacillus* cells treated at $-45\text{ }^{\circ}\text{C}$ had only Ice I–III phase-transition. More cells survived after treatment compared to the same treatment at $-25\text{ }^{\circ}\text{C}$ because the intracellular surrounding was fully frozen. In contrast to the situation at $-25\text{ }^{\circ}\text{C}$, the high intracellular cation levels maybe help to protect cells by preventing protein denaturation (Kinsho, Ueno, Hayashi, Hashizume, & Kimura, 2002).

In contrast to our results, Luscher et al. (2004) found surprisingly that inactivation of *Listeria* by the treatments at $-45\text{ }^{\circ}\text{C}$ were almost as effective as the treatments at $-25\text{ }^{\circ}\text{C}$. This may be caused by the stationary phase *Listeria* not containing very high levels of osmotically active compounds (Fagerbakke, Norland, & Heldal, 1999).

Interestingly we found that the inactivation showed a time-dependence only when the treatment was at $10\text{ }^{\circ}\text{C}$, short-term and in the low-pressure range. All the other cases showed no time-dependence at all.

More than 4-log reduction was achieved after the treatment at $10\text{ }^{\circ}\text{C}$ and 350 MPa. In this case, we considered it likely that most of the survivors are spores (ca. 10^2 ml^{-1}) since the initial inoculum was 10^{7-8} cfu/ml and contained up to 10^2 cfu/ml spores (see Materials and methods). These results are in good agreement with the pressure resistance experiment on *Listeria monocytogenes* performed at $20\text{ }^{\circ}\text{C}$ (Tholozan, Ritz, Jugiau, Federighi, & Tissier, 2000).

To explain the cases at -25 and $-45\text{ }^{\circ}\text{C}$, as discussed above, phase transition (and possible partial thawing)

caused the major inactivation when cells were treated with pressure while in the frozen state. The phase transition effect on cellular viability is much stronger than factors such as treatment time and pressure. Therefore the inactivation we observed was independent on the treatment time, and only little dependent on increased pressure, either. Moreover most likely susceptible cells had undergone (partial) intracellular thawing next to extracellular ice I–III phase transition, which may enhance the (all-or-nothing) effect. Therefore it would also explain why freeze–thaw cycles did not lead to any additional inactivation.

Certainly results from the current study call for a mechanistic evaluation of the effects of applying high pressure at low temperatures as an anti-microbial treatment in the food industry. Such data are currently being gathered and will be very valuable in defining optimal cold pasteurisation process conditions.

Acknowledgements

This work was financially supported by the European Project SAFE ICE (QLK1-2002-02230). Gabriel Urrutia Benet is supported for a PhD program by the Generalitat Valenciana-Beca FPI CTBPRB/2002/303.

We would also like to thank Dr. N. Johnson of Unilever Research and Development, Vlaardingen, The Netherlands, for critically reading the manuscript.

References

- Bridgman, P. W. (1912). Water in the liquid and five solid forms under pressure. *Proceedings of the American Academy of Arts and Sciences*, XLVII(13), 439–558.
- Brul, S., Rommens, A. J. M., & Verrips, C. T. (2000). Mechanistic studies on the inactivation of *Saccharomyces cerevisiae* by high pressure. *Innovative Food Science and Emerging Technologies*, 1, 99–108.
- Cheftel, J. (1995). High pressure, microbial inactivation and food preservation. *Food Science and Technology International*, 1, 75–90.
- den Aantrekker, E. D., Vernooij, W. W., Reij, M. W., Zwietering, M. H., Beumer, R. R., van Schothorst, M., et al. (2003). A biofilm model for flowing systems in the food industry. *Journal of Food Protection*, 66(8), 1432–1438.
- Fagerbakke, K. M., Norland, S., & Heldal, M. (1999). The inorganic ion content of native aquatic bacteria. *Canadian Journal of Microbiology*, 45, 304–311.
- Frank, H. S., & Wen, W. Y. (1957). Ion-solvent interaction. III. Structural aspects of ion-solvent interaction in aqueous solutions: a suggested picture of water structure. *Disc. Faraday Soc.*, 24, 133.
- George, S. (2000). *Impact of high hydrostatic pressure on foods at sub temperatures: Phase Transitions, Modelling Temperature Distribution During Thawing and Inactivation of Microorganisms*. PhD thesis, Berlin: University of Technology.
- Heinz, V., & Knorr, D. (1996). High pressure inactivation kinetics of *Bacillus subtilis* cells by a three-state-model considering distribution resistance mechanisms. *Food Biotechnology*, 10, 149–161.
- Heinz, V., Knorr, D., & Schlüter, O. (1998). Hochdruckbehandlung von Lebensmitteln. *Spektrum der Wissenschaftlicher*, 9/98, 132–136.
- Holtmann, G., Bakker, E. P., Uozumi, N., & Bremer, E. (2002). *KtrAB and KtrCD: Two K⁺ uptake system in Bacillus subtilis and their role in adaption to hypertonicity*, *Journal of Bacteriology*, 185.4, 1289–1298.2003
- Hoover, P. G., Metrick, C., Papineau, A. M., Farkas, D. F., & Knorr, D. (1989). Biological effects of high hydrostatic pressure on food microorganisms. *Food Technology*, 43, 99–107.
- Kinsho, T., Ueno, H., Hayashi, R., Hashizume, C., & Kimura, K. (2002). Sub-zero temperature inactivation of carboxypeptidase Y under high hydrostatic pressure. *Journal of Biochemistry*, 269, 4666–4674.
- Knorr, D. (1995). Hydrostatic pressure treatment of food: Microbiology. In G. W. Gould (Ed.), *New emerging technologies for food preservation*. Blackie Academic & Professional.
- Kort, R., Brien, A. C. O., van Stokkum, I. H. M., Oomes, S. J. C. M., Crielaard, W., Hellingwerf, H. J. et al. (in press). Assessment of heat resistance of bacterial spores from food product isolates by fluorescent monitoring of dipicolinic acid release. *Journal of Food Microbiology*.
- Luscher, C., Balasa, A., Fröhling, A., Ananta, E., & Knorr, D. (2004). Effect of high-pressure-induced ice I-to-Ice III phase transitions on inactivation of *Listeria innocua* in frozen suspension. *Applied and Environmental Microbiology*, 70, 4021–4029.
- Mackey, B. M., Forestière, K., & Isaacs, N. (1995). Factors affecting the resistance of *Listeria monocytogenes* to high hydrostatic pressure. *Food Biotechnology*, 9, 1–11.
- Oomes, S. J. C. M., & Brul, S. (2004). The effect of metal ions commonly present in food on gene expression of sporulating *Bacillus subtilis* cells in relation to spore wet heat resistance. *Innovative Food Science and Emerging Technologies*, 5, 307–316.
- Schlüter, O., Urrutia Benet, G., Heinz, V., & Knorr, D. (2004). Metastable states of water and ice during pressure-supported freezing of potato tissue. *Biotechnology Progress*, 20, 799–810.
- Smelt, J. P. P. M., Hellemons, J., & Brul, S. (2001). Physiological aspects of modelling of high pressure inactivation of microorganisms. *Proceedings of high pressure meeting Japan, November 2000*.
- Tholozan, J. L., Ritz, M., Jugiau, F., Federighi, M., & Tissier, J. P. (2000). Physiological effects of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella typhimurium*. *Journal of Applied Microbiology*, 88(2), 202–212.
- Urrutia Benet, G., Schlüter, O., & Knorr, D. (2004). High pressure–low temperature processing. Suggested definitions and terminology. *Innovative Food Science and Emerging Technologies*, 5, 413–427.
- Whatmore, A. M., & Reed, R. H. (1990). Determination of turgor pressure in *Bacillus subtilis*: a possible role for K⁺ in turgor regulation. *Journal of General Microbiology*, 136, 2521–2526.
- Whatmore, A. M., Chudek, J. A., & Reed, R. H. (1990). The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *Journal of General Microbiology*, 136, 2527–2535.