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**Germination and inactivation of *Bacillus subtilis* spores induced by moderate  
hydrostatic pressure**

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## **Abstract**

In this study, we investigated the mechanisms of spore inactivation by high pressure at moderate temperatures to optimize the sterilization efficiency of high-pressure treatments. *Bacillus subtilis* spores were first subjected to different pressure treatments ranging from 90 to 350 MPa at 40 °C, with holding times from 10 min to 4 h. These treatments alone caused slight inactivation, which was related to the pressure-induced germination of the spores. After this pressure treatment, the sensitivity of these processed spores to heat (80 °C/10 min) or to high pressure (350 MPa/40 °C/10 min) was tested to determine the pressure-induced germination rate and the advancement of the spores in the germination process. The subsequent heat or pressure treatments were applied immediately after decompression from the first pressure treatment or after a holding time at atmospheric pressure. Our results show that high pressure was necessary and very efficient in inducing spore germination. However, it seemed to slow the enzymatic digestion of the cortex, which is required for germinated spores to be inactivated by pressure. Although these results indicate that high-pressure treatments are more efficient when the two treatments are combined, a small spore population still remained dormant and was not inactivated with any holding time or pressure level.

**Keywords: bacterial spores; *Bacillus subtilis*; high pressure; germination; inactivation**

## **Introduction**

Under starvation conditions, *Bacillus* species can form endospores, which are metabolically dormant and physically very resistant. Bacterial spores exhibit a multilayer structure in which each layer plays an important role in protecting the spore against environmental perturbations. Bacterial spores are considered to be the most resistant life form among biological models and can survive for millions of years (Vreeland et al., 2000). The extreme resistance of bacterial spores to many stresses has been reported, including to external desiccation (Dose and Gill,

1995), very high pressure (Margosch et al., 2004), UV radiation, heat, and chemical agents (Setlow, 2006), and this resistance is therefore an important obstacle to food preservation. The only reliable industrial method of spore inactivation is heat treatment for about 20 min at 120 °C, which is widely used for food sterilization in the industry. However, this thermal process has some drawbacks insofar as it modifies not only the sensory qualities (color, flavor, texture) but also the nutrient content of foodstuffs. High-pressure treatments have stimulated considerable interest in the food industry because they not only preserve the nutritional and organoleptic food qualities, but also reduce the energy and time required for processing (Tewari et al., 1999). However, this alternative technology fails to achieve the efficient destruction of bacterial spores. In fact, spore inactivation by high pressure at ambient temperature is not sufficiently efficient and some spores are even reported to survive after a treatment at 1034 MPa for 90 min at 35 °C (Timson and Short, 1965). Combining high pressures with moderate temperatures (Clery-Barraud et al., 2004) or pressure cycling (Furukawa et al., 2003) could improve spore inactivation but the complete destruction of bacterial spores at moderate temperatures has not so far been achieved.

The phenomena involved in the spore inactivation induced by high pressure differ from those identified in vegetative cells. It has been reported that pressure induces cell inactivation by cell permeabilization, the subsequent leakage of internal solutes, and cell shrinkage (Perrier-Cornet et al., 1995, 1999) or by protein denaturation (Alpas et al., 2003). These phenomena are normally reinforced by the increased pressure. On the contrary, Sale et al. (1970) found an optimal pressure for *Bacillus subtilis* spore destruction at about 250 MPa, in the pressure zone ranging from 100 to 800 MPa at 25 °C, with a 1 h holding time (Sale et al., 1970). This atypical inactivation curve seems to be the result of spore germination, which is intimately involved in spore inactivation by pressure. In fact, hydrostatic pressure triggers the spore germination phenomenon and during this process, spores progressively lose their typical

resistance and become more readily inactivated (Moir, 2006; Setlow, 2003), like vegetative cells.

The germination phenomenon can also be induced by other factors (nutrients or abrasion) (Jones et al., 2005; Paidhungat et al., 2002; Rode and Foster, 1960; Setlow et al., 2003). Spore germination is generally divided into different stages but the distinction and the quantification of those germination stages are not clear. The release of dipicolinic acid (DPA) may only be useful in studying the onset of the germination process (Moir, 2006), whereas visualization of protein mobility (Cowan et al., 2004; Moir, 2003), the drop in optical density, or increased spore permeation to fluorescent substances only occur in the late stages. The use of flow cytometry coupled to fluorescent probes has recently been reported to differentiate different spore populations after pressure treatments, but the identification of these populations is complex (Mathys et al., 2007). In this study, we tried to differentiate the different stages in the germination process by identifying the spore sensitivity to heat and high pressure, as shown in Figure 1.

In the first stage, spores partially lose their impermeability to water, leading to water influx (with a slight increase in volume) and solute (DPA,  $\text{Ca}^{2+}$ ) leakage. Consequently, they become sensitive to wet heat (Setlow, 2003). During the second stage, the cortex is enzymatically digested, leading to full core rehydration, greater hydration of the core macromolecules and a greater loss of spore-specific resistance (to high pressure, for example; Wuytack et al., 1998). It has already been reported that pressure (600 MPa)-germinated spores, which were sensitive to heat but still resistant to high pressure (Wuytack et al., 1998), had characteristics similar to those of spores genetically blocked at stage I, and were induced by nutrients (Setlow et al., 2001).

At the end of stage II, the small acid-soluble spore proteins (sasP) are hydrolyzed to amino acids, which are subsequently used in protein synthesis by the growing cell (Moir, 2006;

Setlow, 2003). Protein synthesis and spore metabolism only occur in the outgrowth phase, in which the germinated spore is converted into a growing cell. However, the small endogenous energy reserves (3-phosphoglyceric acid constitutes 0.3% of the spore dry weight and sasP constitute 3.5% of the spore dry weight) do not allow spore outgrowth without external nutrients. In fact, spores lack some enzymes, which require external substrates for their synthesis (Paidhungat and Setlow, 2002). After the germination and outgrowth phases, and under favorable conditions, bacterial cells can undergo a multiplication process and colonize the medium. They may even produce toxins in foodstuffs. Without control, the spore germination induced by high pressure can be a risk to foods.

The aim of this work was to study the germination and inactivation of bacterial spores induced by moderately high pressures and the effects of different factors on these two processes to gain insight into the mechanisms of spore inactivation by high pressure. Our results might help to optimize the barosterilization process.

## **Materials and methods**

### ***Bacterial strains and growth conditions***

The strain used was *Bacillus subtilis* ATCC 31324 (DSMZ 704) obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The sporulation of *B. subtilis* was induced in a reactor in a complex medium at 37 °C, pH 8.0, with an air flow of 4 L min<sup>-1</sup> and with agitation (450 rpm min<sup>-1</sup>) (Nguyen Thi Minh et al., 2008). Sporulation was estimated by counting the spores before and after a heat treatment at 80 °C for 10 min. *Bacillus subtilis* spores were harvested when sporulation reached more than 95% of the cell population (about 48 h). The spores were washed three times with sterile distilled water and spray-dried.

## ***High-pressure treatment***

### *Sample preparation*

Spore powder was suspended in sterile distilled water at a final concentration of approximately  $10^9$  spores  $\text{mL}^{-1}$ . Samples of about 1 mL of the spore suspension were transferred to sterile polyethylene bags, which were aseptically heat-sealed and stored at room temperature until treatment.

### *High-pressure treatments*

High-pressure treatments were applied using a 1.5 L vessel (GEC Alsthom ACB, France), which was maintained at a constant temperature ( $40 \pm 3$  °C) and was capable of operating at up to 600 MPa. Hydrostatic pressure was generated with a hydraulic high-pressure pump (Hydro Process, France) connected to the vessel. The pressure and temperature inside the vessel were controlled by a pressure gauge (Sedeme, France) and a K-type thermocouple (Top Industrie, France), respectively. High-pressure treatments were applied at 90–450 MPa, with holding times ranging from 0 min to 4 h. All high-pressure treatments were applied at 40 °C in this study. Pressure loading and unloading were performed at approximately  $12 \text{ MPa s}^{-1}$ . For treatments with a holding time of 0 min, decompression was initiated immediately after compression.

### ***Heat treatment***

Spore samples, prepared as previously described, were maintained at 80 °C in a water bath for 10 min. The heat-treated samples were cooled by immersion in a water bath maintained at room temperature.

### ***Viability measurements***

Viability measurements were made according to the colony-forming-unit method. After dilution in physiological water (9‰ NaCl w/w), the microbial suspensions were manually

spread over Columbia agar medium (Biokar Diagnostics, France) and incubated at 37 °C for 24 h.

### ***Quantification of different spore populations during germination***

In this study, the germination process was expected to stop in the early phase of stage II because in the absence of nutrients, spores cannot establish the active processes necessary for the recovery of the vegetative state. This fact was verified by microscopic observations. After the pressure treatment, the initial population of native spores (NS) was partially replaced by germinated spores at different stages (stage I [GI] and stage II [GII]) in the suspension, as shown in Figure 1. Each population exhibits corresponding morphological changes and is associated with the loss of specific resistance (Setlow, 2003). Including a heat treatment (80 °C/10 min) after the initial pressure treatment allowed us to distinguish each population of germinating spores, as summarized in Table I. Because spontaneous germination was rare, all heat-sensitive spores must have been induced by pressure and achieved at least stage I of the germination process. We observed that the number of spores sensitive to heat increased progressively during maintenance at atmospheric pressure for 4 h and remained constant thereafter (up to 24 h). Therefore, all the heat-resistant spores observed after this delay were assumed to have remained native spores and their germination was not induced by pressure. In the absence of vegetative and outgrowing cells, the spores inactivated by pressure were in stage II (GII) of the germination process. The number of native spores (NS) was calculated by subtracting the total number of germinated spores from the initial concentration of spores ( $10^9$  spores mL<sup>-1</sup>).

## **Results**



***Germination and inactivation of *B. subtilis* spores immediately after pressure treatment at 350 MPa and 40 °C***

The spore germination and inactivation induced by pressure treatment at 350 MPa and 40 °C, with various holding times, were first studied (Figure 2). Before treatment, we observed that the spores were not inactivated by heat, which means that only native spores were present in the samples. A pressure treatment consisting of a 10 min holding time at 350 MPa (see arrow, Figure 2) directly induced about 1 log of spore destruction. With reference to Table I, this result means that after only 10 min, at least 90% of the initial population had germinated, had reached stage II of germination (GII), and had become sensitive to pressure. At the same time, the destruction rate achieved with a subsequent heat treatment after a 4 h holding period at atmospheric pressure was about 4 log (see arrow, Figure 2). Thus, 4 h after treatment, almost the whole population had already germinated and had reached at least stage I of germination (GI + GII). Because the initial concentration was about  $10^9$  spores  $\text{mL}^{-1}$  and the heat destruction rate was 4 log, only  $10^5$  spores  $\text{mL}^{-1}$  still remained native after the 10 min holding period at 350 MPa.

Increasing the holding time at 350 MPa to 60 min significantly increased the number of pressure-induced germinated spores (GI + GII). In fact, the heat-inactivation rate was about 6.5 log for 60 min at 350 MPa. However, only 2 log were directly inactivated (GII) after such a high-pressure treatment. Germination was induced in the main part of the resistant spore population after treatment with 350 MPa for 60 min, however stage II of germination was not achieved. Because the initial concentration was about  $10^9$  spores  $\text{mL}^{-1}$  and 6.5 log of spores had already been heat inactivated, only a small population (about  $10^3$  spores  $\text{mL}^{-1}$ ) still remained native under these conditions.

A further increase in the holding time had little effect on pressure-induced germination (GI + GII) or on pressure-induced inactivation (GII). As shown by the second arrow in Figure 2, the

direct inactivation of the spores by a pressure treatment of 350 MPa for 4 h was about 2.5 log, whereas the pressure-induced germination of heat-sensitive spores was 6.5 log. A small population of native spores was always observed, even when the holding time was prolonged to 24 h (data not shown). Therefore, pressure appears to rapidly induce spore germination (in the first 30 min). It has been reported that it took about 20–30 min to achieve stage II of germination when spores were induced to germinate by nutrients (Setlow and Johnson, 2007). However, pressure-induced germination was incomplete and the resistant native spores could germinate in the presence of nutrients.

We observed a great difference between pressure-induced inactivation (GII) and pressure-induced germination (GI + GII). Moreover, a long holding time (4 h) at 350 MPa was less efficient in terms of spore destruction (about 2.5 log) than a holding time of equivalent length at atmospheric pressure after a minimum induction of 10 min at 350 MPa and a subsequent thermal treatment (more than 4 log). In the next section, we compare the germination and inactivation that occurred during treatment at 350 MPa with those of previously pressure-induced spores that occurred at atmospheric pressure.

#### ***Comparison of spore germination during high-pressure treatment and after pressure treatment at atmospheric pressure***

Spore germination was first induced by a pressure treatment of 350 MPa at 40 °C for 60 min, and after decompression, the spore suspension was held at atmospheric pressure. After different holding times at atmospheric pressure, the spore suspension was subjected to a heat treatment at 80 °C for 10 min to identify the development of the pressure-induced germination population (GI + GII). The pressure-inactivated population (GII) was estimated by subjecting the previously pressure-induced germinated spores to a pressure treatment of 350 MPa/40 °C/10 min. The results are presented in Figure 3, and compared with those for spore germination and inactivation at 350 MPa for different holding times.

The induction of spore germination after 60 min under pressure, as shown by the GI and GII inactivation curves, did not seem to change, regardless of whether or not the spores were further maintained under pressure. However, germination advancement, as described by the GII inactivation curves, seemed to be more favored when the spores were held at atmospheric pressure than when they were maintained at the same high pressure. After the first pressure treatment for 60 min at 350 MPa, followed by a 60 min holding period at atmospheric pressure, about 5 log of spores were inactivated by a second pressure treatment, compared with the 2 log of spores that were destroyed after a pressure treatment at 350 MPa with a 120 min holding period. The germination process of the GII spores occurred during the first 60 min after decompression and did not increase significantly thereafter (Figure 3).

Therefore, pressure seems to have two opposite effects on spore germination: the rapid induction but slow completion of the spore germination process. High pressure, which was very efficient in inducing spore germination, seemed to slow the latter stage of the germination process, more particularly the passage from stage I to stage II. In the next step, we focused on the effects of the pressure level on these two events.

#### ***Effects of high pressure levels on pressure-induced germination and inactivation***

The effects of high pressure levels on spore germination and inactivation were studied as a function of the pressure level (90–550 MPa). All the experiments were performed with a holding time of 60 min because this time had been observed to be optimal for the induction of spore germination at 350 MPa (cf. Figure 2). In Figure 4, our results for the inactivation of *B. subtilis* spores after 60 min of pressurization graphed against the pressure levels are compared with the results of Wuytack et al. (1998) for the inactivation of *B. subtilis* PS832 spores at 40 °C for 30 min in the same pressure range (Wuytack et al., 1998).

As shown in this figure, Wuytack et al. (1998) found the optimal pressure for spore inactivation near 200 MPa (2 log) (Wuytack et al., 1998). Interestingly, in the present study,

when *B. subtilis* spores were treated for 60 min under pressure, pressure inactivation (GII) was optimal (about 1.82 log) at roughly the same pressure. Therefore, our results agree with those of Wuytack et al. (1998) in that the inactivation of *B. subtilis* spores by pressures below 600 MPa at 40 °C is relatively weak and is not directly proportional to the pressure. Optimal inactivation was observed at moderate pressures (about 250 MPa), which have little effect on the inactivation of vegetative cells (Gao and Jiang, 2005).

On the contrary, pressure-induced germination depended on high pressures of up to 400 MPa. For example, the destruction rate of the total germinated spores (GI + GII) was less than 3 log at 90 MPa compared with 6 log at 350 MPa. Further increases in pressure did not result in greater spore induction. Moreover, a pressure of 550 MPa even slightly reduced the spore population induced by pressure. Therefore, the optimal pressure for spore inactivation (about 200 MPa) is less than the optimal pressure necessary for the induction of spore germination (about 400 MPa) with a holding time of 60 min. Although pressure inactivation was related to the pressure induction of spore germination, these two phenomena do not have the same optima. This observation supports the hypothesis that high pressure inhibits the achievement of stage II of germination.

## **Discussion**

The germination phenomena that occur during the high-pressure process are best characterized using the spores' sensitivity to heat and high-pressure treatments and by comparing these phenomena with and without pressure. The effects of high pressure seem to differ greatly depending on the germination stage. This observation could be related to the cellular phenomena involved during germination, which are detailed in the following sections.

### ***How does pressure induce the germination process?***

The mechanisms underlying the high-pressure induction of spore germination seem to depend on the pressure level used. Moderately high pressures (between 50 to 400 MPa) have been

reported to induce germination through nutrient receptors, and the subsequent events are processed via the nutrient-triggered germination pathway (Black et al., 2005; Pelczar et al., 2007). Very high pressures (above 400 MPa) do not activate nutrient receptors but possibly trigger the release of Ca–DPA and the subsequent events of germination (Black et al., 2007; Paidhungat et al., 2002). Our experiments did not show two separate phenomena as a function of the pressure range, and the rate of total spore induction by pressure increased continuously up to 400 MPa (cf. Figure 4). However, the two mechanisms of spore induction could operate together in a certain pressure range. At pressures above 400 MPa, the induction phenomenon seemed to be optimal and even decreased slightly with further increases in pressure. A small part of the population (1 per  $10^7$  spores) did not appear to be induced by high pressure.

After the decompression of the first germination-inducing treatment, the number of stage II germinated spores continued to increase, even at atmospheric pressure (cf. Figure 3). Because the spores were suspended in distilled water without nutrients, these results seem to demonstrate that spore germination induced by high pressure is an irreversible process in the absence of nutrients. Thus, high hydrostatic pressure, which is a physical factor like heat, has a very different effect on the induction of spore germination from the effect of heat. It has been reported that, in the absence of nutrients, spore germination can also be induced by sublethal heating (about 80 °C for 10 min), but this induction is reversible because the induced spores return to the dormant state without the addition of nutrients after some time in storage (Keynan et al., 1964).

Moreover, pressure-induced germination is rather rapid and efficient. In fact, about  $10^5$  spores  $\text{mL}^{-1}$  were already induced to germinate after a pressure treatment of 350 MPa at 40 °C for 30 min, compared with the initial concentration of  $10^9$  spores  $\text{mL}^{-1}$ . This short period may be attributable to the fact that the first events of the germination process are passive and do not require enzymatic reactions. Because moderately high pressure induces spore germination by

activating the spore's nutrient receptors, increasing the pressure to 400 MPa probably supplies more energy to change the conformation of these receptors and consequently enhances spore induction, as shown by the results presented in Figure 4. Wuytack et al. (1998) found no significant differences in the induction of germination by 100 and 400 MPa at 40 °C (Wuytack et al., 1998). However, their experiments were performed with a shorter holding time (30 min), so the germination rates were low and may not have allowed the observation of differences under these conditions.

We also observed a spore fraction (see the gray area in Figure 5) that was not sensitive to heat immediately after the pressure treatment but germinated later. This spore fraction has some similarities to the activated spores induced by heat and could be called “pressure-activated spores”. However, this pressure-activated process was irreversible and the cells proceeded through the next germination stages, even after the pressure was released. These spores are probably at the very first stage of activation and are not sensitive to heat. Otherwise, they are perhaps activated after the high-pressure treatment by a substance excreted from the germinating spores (DPA,  $\text{Ca}^{2+}$  ...).

Finally, the induction of spore germination by pressure was incomplete because a very small spore population (about 1 per  $10^6$  spores) still remained in their native state in the suspension. However, these spores could germinate in the presence of nutrients after the pressure treatment. Therefore, their nutrient receptors still remained active but were perhaps not sensitive to pressure. This saturation effect could be attributable to the “superdormancy” phenomenon under these conditions but our results did not allow us to confirm this hypothesis. Specific mutants could be useful in evaluating this possibility. Nevertheless, the spore germination induced by moderately high pressure seems slightly different from nutrient-induced germination.

***How can pressure affect the progression from stage I to stage II of germination?***

Stage II of germination is related to the hydrolysis of the cortex, the full hydration of the spore core, the restoration of protein mobility and enzyme activity, and the loss of most spore-specific resistance (Cowan et al., 2003; Paidhungat et al., 2002). This germination stage requires the activity of enzymes and normally takes longer than the first stage. Because the progression from stage I (GI) to stage II (GII) is effectively faster at atmospheric pressure than under high pressure (Figure 3), a pressure of 350 MPa ought to slow the transition from GI to GII, probably by lowering the activities of the cortical lytic enzymes and some core enzymes involved in this stage. It has also been reported that germinating spores induced by very high pressures can complete the germination process, but very slowly (Wuytack et al., 1998). Pressure can affect enzyme structures and functionality as well as the kinetics and specificity of the catalyzed reaction. Indeed, the activities of proteolytic and glycolytic enzymes are significantly reduced at pressures higher than 300 MPa (Malone et al., 2003). Similarly, changes in the treatment temperature or pH can accelerate this progression from stage I to stage II of germination (Stewart et al., 2000). The passage from stage I to stage II of the germinated spores seems to be the limiting step in the germination process under pressure.

#### ***Hypothetical mechanisms of spore inactivation under high pressure***

In summary, the effect of the pressure level on the different stages of spore germination and inactivation are shown in Figure 6. Here, we have reported the variations of spore numbers that correspond to each phenomenon as a function of pressure levels.

As shown in Figure 4, pressure-induced germination increased with increasing pressure up to 400 MPa. On the contrary, the numbers of spores that proceeded from stage I to stage II of germination decreased with increasing pressure levels. Moreover, as shown previously, stage II germinated spores (GII) lost their specific resistance and the number of GII spores destroyed by pressure increased with increasing pressure, like vegetative cells. The destruction of spores, as influenced by the three processes, is shown in Figure 6. Below 300

MPa, pressure only weakly inhibited the progression from stage I (GI) to stage II (GII) of germination, but because pressure had little effect on spore induction or the destruction of GII spores, the spore inactivation rate was not high. Pressures above 300 MPa induced the germination of considerable spores, but because the progression from GI to GII was very limited under pressure, the spore inactivation rate was also low. Spore inactivation by high pressure, which is a combination of these three processes, may be optimal at about 250 MPa (Wuytack et al., 1998).

To optimize spore destruction by hydrostatic pressure, different solutions were used, predominantly with a combination of pressure and temperature or with pressure cycling. On the one hand, the higher spore inactivation rate at high temperatures can be explained by the acceleration of enzymatic reactions during the progression from stage I to stage II of germination but also by the fact that stage I germinated spores are directly inactivated by temperatures above 70 °C. On the other hand, the application of pressure cycling is more efficient than a single pressure treatment, when the total exposure is equivalent (Hayakawa et al., 1994). One explanation of this phenomenon is that the repeated rapid decompression causes injury to and disruption and inactivation of the germinated spores (Furukawa et al., 2003). Our results suggest that after the induction of spore germination by the first pressure treatment, the decompression between the pressure cycles favors the progression from stage I to stage II of the germinated spores. The stage II germinated spores are then inactivated by the subsequent pressure cycles, which would explain the greater spore destruction by pressure cycling. Some authors have observed only small differences in spore inactivation between pressure cycling and continuous pressure, probably because the germinated spores were exposed for a very short period to atmospheric pressure and could not proceed further to stage II of germination. The use of pressure cycling with a precise exposure time could significantly enhance spore inactivation by high pressure. For example, a first pressure treatment for 30



min at 350 MPa and 40 °C, maintenance at atmospheric pressure for 30 min, and a second pressure treatment at 350 MPa and 40 °C for 10 min can induce spore destruction of about 5 log.

### **Conclusion**

The initiation of the germination process is the only way to inactivate spores at pressures below 600 MPa and a moderate temperature (40 °C). Germinated spores were inactivated when they reached stage II of germination. Pressure has two opposite effects on the induction of spore germination and spore inactivation. Pressure is necessary for the induction of spore germination but it slows the progression from stage I germinated spores to stage II germinated spores, and consequently spore inactivation. The completion of spore germination and inactivation by high pressure remains challenging because a small proportion of the spore population (about 1 per 10<sup>6</sup> spores) is not activated by these pressures. These results allow a better comprehension of the effects of pressure on spore germination and inactivation. However, further work is required to better control spore inactivation when applying high-pressure processing for food preservation.

### **References**

- Alpas H, Lee J, Bozoglu F, Kaletunc G. 2003. Evaluation of high hydrostatic pressure sensitivity of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by differential scanning calorimetry. *Int J Food Microbiol* 87(3):229-237.
- Black EP, Koziol-Dube K, Guan D, Wei J, Setlow B, Cortezzo DE, Hoover DG, Setlow P. 2005. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. *Appl Environ Microbiol* 71(10):5879-5887.
- Black EP, Wei J, Atluri S, Cortezzo DE, Koziol-Dube K, Hoover DG, Setlow P. 2007. Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure. *J Appl Microbiol* 102(1):65-76.

- Clery-Barraud C, Gaubert A, Masson P, Vidal D. 2004. Combined effects of high hydrostatic pressure and temperature for inactivation of *Bacillus anthracis* spores. *Appl Environ Microbiol* 70(1):635-637.
- Cowan AE, Koppel DE, Setlow B, Setlow P. 2003. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: Implications for spore dormancy. *Proc Natl Acad Sci U S A* 100(7):4209-4214.
- Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. *Proc Natl Acad Sci U S A* 101(20):7733-7738.
- Dose K, Gill M. 1995. DNA stability and survival of *Bacillus subtilis* spores in extreme dryness. *Orig Life Evol Biosph* 25(1-3):277-293.
- Furukawa S, Shimoda M, Hayakawa I. 2003. Mechanism of the inactivation of bacterial spores by reciprocal pressurization treatment. *J Appl Microbiol* 94(5):836-841.
- Gao YL, Jiang HH. 2005. Optimization of process conditions to inactivate *Bacillus subtilis* by high hydrostatic pressure and mild heat using response surface methodology. *Biochem Eng J* 24(1):43-48.
- Hayakawa I, Kanno T, Yoshiyama K, Fujio Y. 1994. Oscillatory compared with continuous high pressure sterilization on *Bacillus stearothermophilus* spores. *J Food Sci* 59(1):164-167.
- Jones CA, Padula NL, Setlow P. 2005. Effect of mechanical abrasion on the viability, disruption and germination of spores of *Bacillus subtilis*. *J Appl Microbiol* 99(6):1484-1494.
- Keynan A, Evanchik Z, Halvorson HO, Hastings JW. 1964. Activation of bacterial endospores. *J Bacteriol* 88:313-318.

- Malone AS, Wick C, Shellhammer TH, Courtney PD. 2003. High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *J Dairy Sci* 86(4):1139-1146.
- Margosch D, Ganzle MG, Ehrmann MA, Vogel RF. 2004. Pressure inactivation of *Bacillus* endospores. *Appl Environ Microbiol* 70(12):7321-7328.
- Mathys A, Chapman B, Bull M, Heinz V, Knorr D. 2007. Flow cytometric assessment of *Bacillus* spore response to high pressure and heat. *Inn Food Sci Emer Technol* 8(4):519-527.
- Moir A. 2003. Bacterial spore germination and protein mobility. *Trends Microbiol* 11(10):452-454.
- Moir A. 2006. How do spores germinate? *J Appl Microbiol* 101(3):526-530.
- Nguyen Thi Minh H, Perrier-Cornet JM, Gervais P. 2008. Effect of the osmotic conditions during sporulation on the subsequent resistance of bacterial spores. *Appl Microbiol Biotechnol* 80(1):107-114.
- Paidhungat M, Setlow B, Daniels WB, Hoover D, Papafragkou E, Setlow P. 2002. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Appl Environ Microbiol* 68(6):3172-3175.
- Paidhungat M, Setlow P. 2002. Spore germination and outgrowth. In: Hoch JA, Losick R, Sonenshein AL, editors. *Bacillus subtilis* and its relatives: from genes to cells. Washington, DC, USA: ASM. p 537-548.
- Pelczar PL, Igarashi T, Setlow B, Setlow P. 2007. Role of GerD in germination of *Bacillus subtilis* spores. *J Bacteriol* 189(3):1090-1098.
- Perrier-Cornet J-M, Maréchal P-A, Gervais P. 1995. A new design intended to relate high pressure treatment to yeast cell mass transfer. *J Biotechnol* 41(1):49-58.

- Perrier-Cornet JM, Hayert M, Gervais P. 1999. Yeast cell mortality related to a high-pressure shift: occurrence of cell membrane permeabilization. *J Appl Microbiol* 87(1):1-7.
- Rode LJ, Foster JW. 1960. Mechanical germination of bacterial spores. *Proc Natl Acad Sci U S A* 46(1):118-128.
- Sale AJ, Gould GW, Hamilton WA. 1970. Inactivation of bacterial spores by hydrostatic pressure. *J Gen Microbiol* 60(3):323-334.
- Setlow B, Cowan AE, Setlow P. 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. *J Appl Microbiol* 95(3):637-648.
- Setlow B, Melly E, Setlow P. 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *J Bacteriol* 183(16):4894-4899.
- Setlow P. 2003. Spore germination. *Curr Opin Microbiol* 6(6):550-556.
- Setlow P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 101(3):514-525.
- Setlow P, Johnson EA. 2007. Spores and their significance. In: Doyle MP, Beuchat LR, editors. *Food Microbiology, Fundamentals and Frontiers*. Washington, DC, USA: ASM. p 35-67.
- Stewart CM, Dunne CP, Sikes A, Hoover DG. 2000. Sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA 3679 to combinations of high hydrostatic pressure and other processing parameters. *Inn Food Sci Emer Technol* 1(1):49-56.
- Tewari G, Jayas DS, Holley RA. 1999. High pressure processing of foods: an overview. *Sciences des Aliments* 19(6):619-661.
- Timson WJ, Short AJ. 1965. Resistance of microorganisms to hydrostatic pressure. *Biotechnol Bioeng* 7(1):139-159.
- Vreeland RH, Rosenzweig WD, Powers DW. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* 407(6806):897-900.

Wuytack EY, Boven S, Michiels CW. 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl Environ Microbiol 64(9):3220-3224.

## Figure captions

Figure 1. Different stages of spore germination (Setlow, 2003).

Figure 2. Spore germination (◆) and inactivation (▲) immediately after a pressure treatment at 350 MPa at 40 °C for different holding times. (GI) stage I germinated spores; (GII) stage II germinated spores; (P) pressure treatment; and (H) heat treatment.

Figure 3. Comparison of the germination and inactivation of *B. subtilis* spores immediately after pressure treatment at 350 MPa/40 °C (—) and those induced by high-pressure treatment at 350 MPa/40 °C/60 min, followed by atmospheric pressure (---). (▲) inactivation by pressure; (◆) germination induced by pressure; (P) pressure treatment; and (H) heat treatment.

Figure 4. Effects of the pressure level on the inactivation (\*) and germination (◆) of *B. subtilis* spores induced by high-pressure treatments at 40 °C for 60 min observed in this study and the inactivation results for *B. subtilis* spores after high-pressure treatments at 40 °C for 30 min (▲) observed by Wuytack et al. (1998). (P) pressure treatment; and (H) heat treatment.

Figure 5. Pressure inactivation (▲) and pressure-induced germination (◆) as a function of holding time at atmospheric pressure after the pressure treatment (350 MPa/40 °C/60 min) for the induction of germination.

Figure 6. Schematic diagram of the effects of the pressure level on the different stages of spore germination and inactivation.

**Table I. Tests used to quantify each spore population after the pressure-induced germination process. Native spores (NS), stage I germinated spores (GI), stage II germinated spores (GII), pressure treatment (P), and heat treatment (H).**


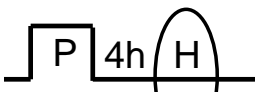
Successive treatment	Test schema	Resistant fraction	Inactivated fraction	Phenomenon
Pressure treatment (350 MPa/40 °C)		NS + GI	GII	Pressure inactivation
Pressure treatment + 4 h (Atmospheric pressure) + Heat treatment (80 °C/10 min)		NS	GI + GII	Pressure-induced germination

Figure 1. Different stages of spore germination (Setlow, 2003)

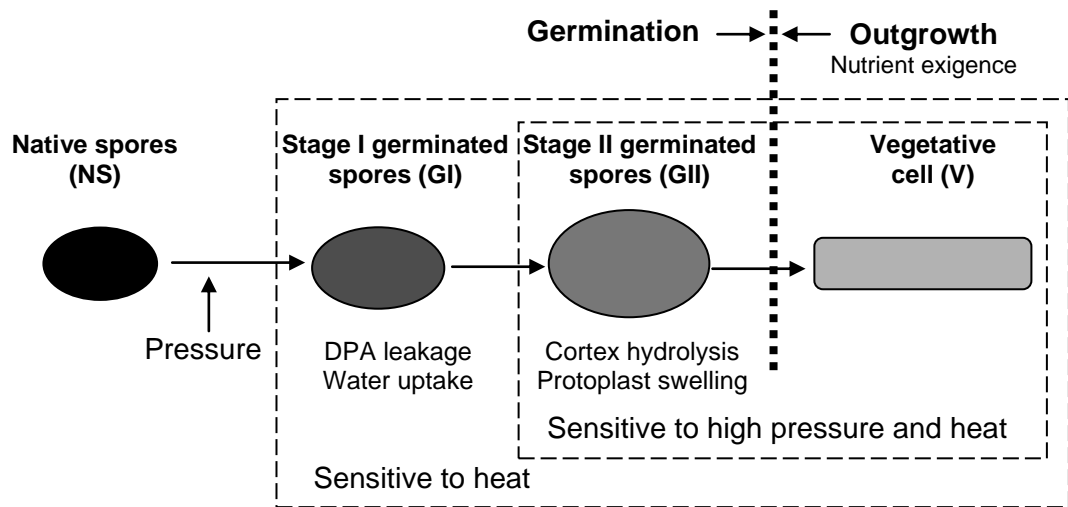




Figure 2. Spore germination (◆) and inactivation (▲) immediately after a pressure treatment at 350 MPa at 40 °C for different holding times. (GI) stage I germinated spores; (GII) stage II germinated spores; (P) pressure treatment; and (H) heat treatment.

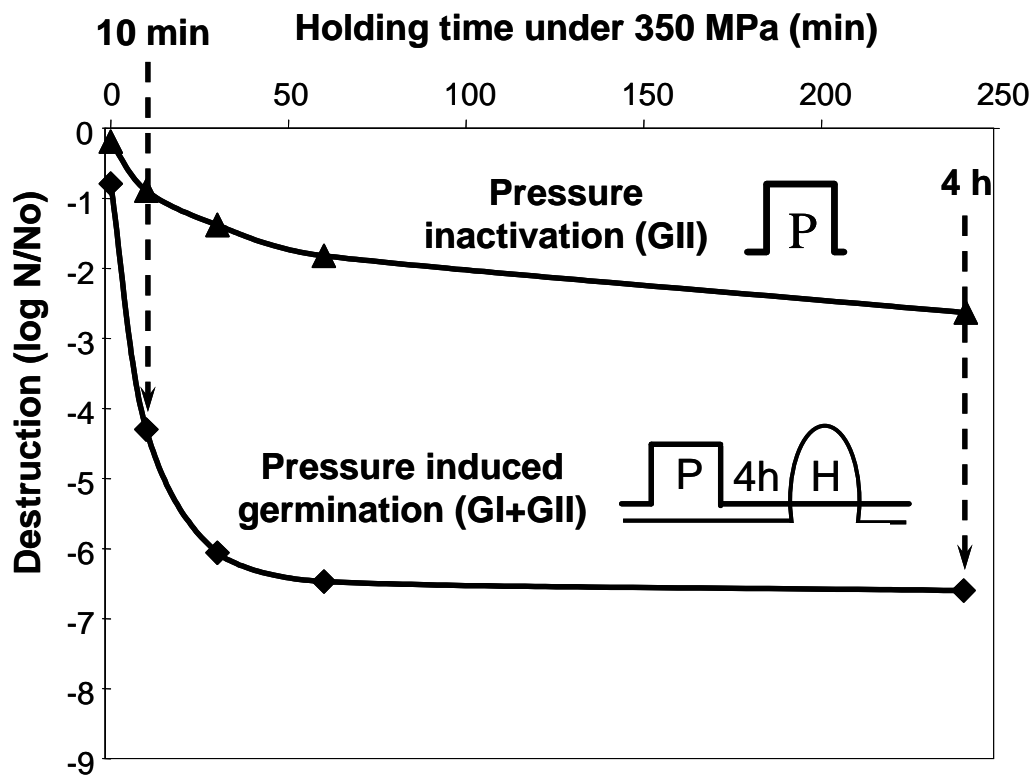


Figure 3. Comparison of the germination and inactivation of *B. subtilis* spores immediately after pressure treatment at 350 MPa/40 °C (—) and those induced by high-pressure treatment at 350 MPa/40 °C/60 min, followed by atmospheric pressure (---). (▲) inactivation by pressure; (◆) germination induced by pressure; (P) pressure treatment; and (H) heat treatment.

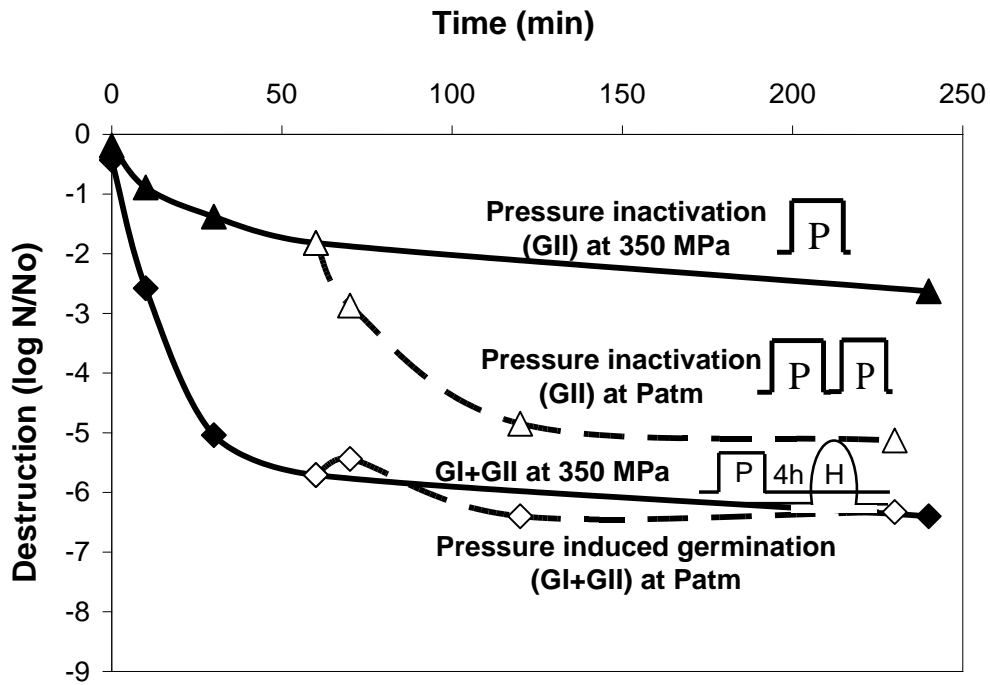


Figure 4. Effects of the pressure level on the inactivation (\*) and germination (♦) of *B. subtilis* spores induced by high-pressure treatments at 40 °C for 60 min observed in this study and the inactivation results for *B. subtilis* spores after high-pressure treatments at 40 °C for 30 min (▲) observed by Wuytack et al. (1998). (P) pressure treatment; and (H) heat treatment.

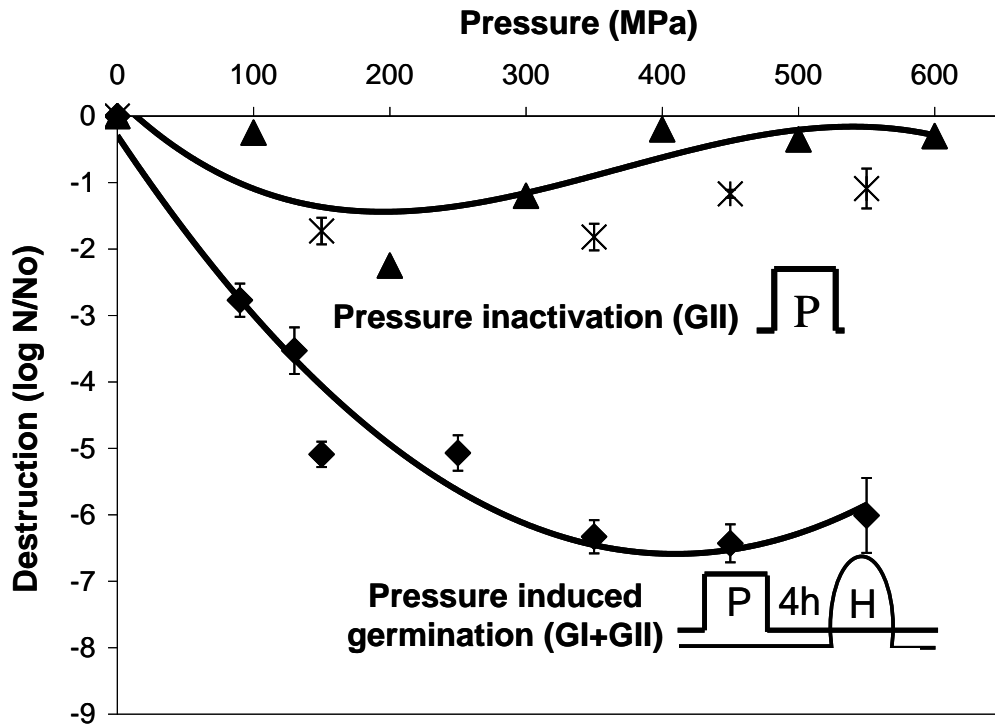


Figure 5. Pressure inactivation ( $\blacktriangle$ ) and pressure-induced germination ( $\blacklozenge$ ) as a function of holding time at atmospheric pressure after the pressure treatment (350 MPa/40 °C/60 min) for the induction of germination.

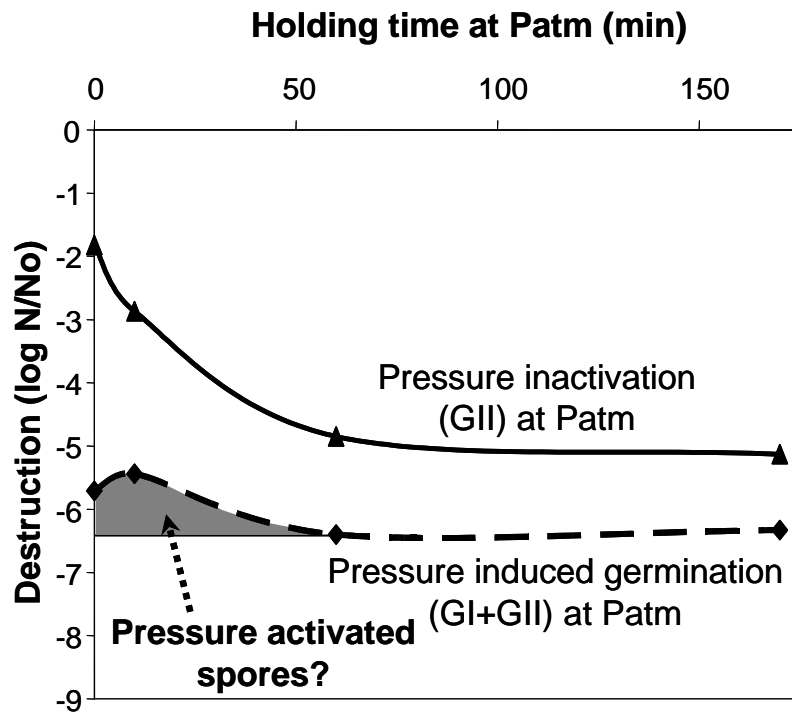


Figure 6. Schematic diagram of the effects of the pressure level on the different stages of spore germination and inactivation.

