Mechanisms of Bacillus spore germination and inactivation during high pressure processing

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Zusammenfassung

Hochdruck in Kombination mit hohen Prozesstemperaturen ermöglicht es, hochwertige sterile Lebensmittel herzustellen. Da unter anderem die Inaktivierungsmechanismen bakterieller Sporen nicht vollständig geklärt sind, wird diese Technologie bisher noch nicht industriell verwendet. Ziel der Arbeit war es, das Keimungs- und Inaktivierungsverhalten von *Bacillus subtilis* Sporen sowie von isogenen mutierten Stämmen, denen ein Teil des Keimungsmechanismus fehlt, in einem großen Druck-Temperatur-Zeitbereich zu untersuchen.

Für reproduzierbare Prozessbedingungen wurden alle Kinetiken unter isothermen und isobaren Bedingungen während der Druckhaltezeit erstellt. Zur Vermeidung systematischer Fehler durch Veränderungen im Medium wurde ein pH-Messsystem für bis zu 130 °C und 1 MPa zur Messung in flüssigen und pastösen Lebensmitteln entwickelt. Es zeigte sich nahezu keine Verschiebung im Dissoziationsgleichgewicht bis 130 °C für Phosphat-Puffer, weshalb dieser für thermische Inaktivierungen verwendet wurde. Ein relativ stabiler pK_a-Wert unter Hochdruck konnte durch die Verwendung von ACES-Puffer gewährleistet werden. Nach den Behandlungen wurde der physiologische Zustand der Sporen durch Keimzahlbestimmungen, durchflusszytomtetrische Analysen und eine Quantifizierung der freigesetzten Menge an Dipicolinsäure (DPA) mittels HPLC bestimmt. Für elektronenmikroskopische Untersuchungen erfolgte die Entwicklung einer Ionenstrahl-Schnittmethode, die es ermöglichte Strukturveränderungen im Inneren der Spore zu untersuchen.

Durch diese Methoden konnte ein Inaktivierungsmechanismus für 150 MPa und 37 °C abgeleitet werden, welcher durch eine physiologische DPA-Ausschleusung ausgelöst wird, über eine Cortex-Hydrolyse und den Abbau der kleinen säurelöslichen Proteine (SASP) zu einer Inaktivierung führt. Eine Druckerhöhung auf 550 MPa verlangsamt die Keimung und ein SASP-Abbau ist nicht mehr möglich. Wird dieser Druck mit Temperaturen über 60 °C kombiniert, induziert dies eine schnelle DPA-Ausschleusung sowie eine Inaktivierung. Daraus lässt sich schlussfolgern, dass unter diesen Bedingungen die empfindlichste Struktur die innere Sporenmembran ist. Unter Verwendung eines Differentialgleichungsmodells konnten Isokinetik-Druck-Temperatur-Diagramme für die DPA-Ausschleusung, die Bildung temperaturempfindlicher sowie inaktivierter Sporen berechnet werden. Es wurde bestätigt, dass der erste Schritt der Inaktivierung unter Hochdruck die Freisetzung von DPA ist.

Ferner bestimmt die Prozesstemperatur oberhalb eines Grenzdruckes von 600 MPa die Keimungs- und Inaktivierungsgeschwindigkeit. Darüber hinaus ermöglichte das verwendete Berechnungsmodell, Kinetikdaten aus zwei verschiedenen Hochdruckanlagen miteinander zu vergleichen. Die Daten dieser Arbeit und die abgeleiteten Inaktivierungsmechanismen können dazu beitragen, diese vielversprechende Technologie zu kommerzialisieren und somit die Lebensmittelsicherheit zu erhöhen.

Abstract

High pressure combined with elevated temperatures can produce low acid, commercially sterile and shelf-stable food. However, this promising sterilization technology has not yet been applied in industrial settings, which is largely due to the unknown inactivation mechanism of highly resistant bacterial spores. This study aimed to clarify the germination and inactivation behavior of *Bacillus subtilis* spores and isogenic mutant strains lacking part of the germination apparatus, within a wide pressure–temperature–time domain.

To ensure reliable process conditions, all kinetic data were derived under isothermal and isobaric treatment conditions during pressure dwell time. To avoid any biasing errors in the experimental results caused by varying matrix conditions, a pH-measurement system for liquid and semi-solid foods up to 130 °C and 1 MPa was constructed. The use of this system showed that PBS buffer had nearly no shift of dissociation equilibrium up to 130 °C, and was therefore used for all thermal inactivation experiments. However, under high pressure ACES buffer was used, due to its relatively stable pK_a value. After the treatment, the physiological state of each spore sample was analyzed using the plate count method and flow cytometry, and the amount of dipicolinic acid (DPA) released was quantified by HPLC. In order to optimize scanning electron microscopic evaluations, a focused ion beam section method was developed to investigate the internal structural changes in spores.

Based on this set of methods, a mechanism was proposed in which 150 MPa at 37 °C induces a physiological-like DPA release, followed by cortex and small acid soluble protein (SASP) degradation, resulting in a subsequent inactivation. A pressure increase to 550 MPa retarded the completion of germination and disabled SASP degradation. High pressures in combination with high temperatures (≥ 60 °C) caused a rapid DPA release and spore inactivation. This suggests that the most sensitive structure under these conditions is the inner spore membrane. By using a multiresponse kinetic model, isorate pressure–temperature diagrams for the release of DPA, the formation of heat-sensitive and inactivated spores were calculated. It was confirmed that the first step of inactivation under high pressure is the release of DPA. Above a threshold pressure of 600 MPa the treatment temperature dominates germination and inactivation rate. The model used enabled the comparison of kinetic data derived in two different high pressure units with different compression rates. The kinetic data from this study and the suggested mechanisms can aid in optimizing this promising sterilization technology and will increase food safety.

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List of abbreviations

Abbreviation	Description
ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid
AMP	Adenosine 5`-(tetrahydrogen monophosphate)
ATP	Adenosine 5`-(tetrahydrogen triphosphate)
CFD	Computational fluid dynamics
CFLE	Cortex fragment lytic enzyme
CLE	Cortex lytic enzymes
Ca-DPA	Ca^{2+}- pyridine-2,6-dicarboxylic acid
DNA	Deoxyribonucleic acid
DPA	Pyridine-2,6-dicarboxylic acid
FB113	*Bacillus subtilis* mutant strain (Δ*cwlJ*; Δ*sleB*)
FB114	*Bacillus subtilis* mutant strain (Δ*ger*; Δ*sleB*)
FB115	*Bacillus subtilis* mutant strain (Δ*ger,* Δ*cwlJ*)
FCM	Flow cytometry
FDA	Food and Drug Administration
HP	High pressure
HPTS	High pressure thermal sterilization
NIST	National Institute of Standard and Technology
PATS	Pressure assisted thermal sterilization
PBS	Phosphate buffer system
PE	Polyethene
PET	Poly(ethylene terephthalate)
PTFE	Polytetrafluoroethylene
pTTi	Pressure-temperature-time indicator
SASP	small acid soluble protein

Symbol	Description	Unit
A^-	Base	
b	Weibull scale parameter	
c_p	Specific heat capacity	J kg^{-1} K^{-1}
D	Decimal reduction time at specific temperature	min or s

Symbol	Description	Unit
E_a	Activation energy	kJ mol^{-1}
G	Gibbs free energy	kJ kg^{-1}
HA	Acid	
k	Rate constant	
K_a	Acid dissociation constant	
N	number of microorganisms	
n	reaction order	
n	Weibull shape parameter	
p	Pressure	Pa
pH	Decadic logarithm of the activity of hydroxonium ions	
pK_a	Decadic logarithm of acid dissociation constant	
q	Heat energy	kJ kg^{-1}
R_m	Molar gas constant	8.3145 cm^3 MPa K^{-1} mol^{-1}
S	Entropy	kJ kg^{-1} K^{-1}
T	Temperature	K or °C
t	Time	s
V	Volume	m^3
$\Delta V^{\#}$	Reaction volume at atmospheric pressure	cm^3 mol^{-1}
ΔV^{*}	Activation volume of the reaction	cm^3 mol^{-1}
W	Volumetric work	kJ kg^{-1}
W_{compr}	Specific work of compression	kJ
z	z-value at specific pressure, dT for decimal D-value reduction	°C
β	Compressibility	Pa^{-1}
γ_i	Activity coefficient for molecule i	
Δ	Increment or gradient	
μ	Chemical potential	
ρ	Density	kg m^{-3}
σ	Sigma factor, protein subunit	
∂	Partial derivative	

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1. Introduction

The consumer's demand for minimally processed high quality food has increased drastically in the past decade. To meet this goal and to guarantee food safety, various different preservation methods and technologies have been developed. Many of the traditional preservation methods rely on the inactivation of microorganisms, in which microbial safety is usually only made possible by increasing the process intensity, which is often accompanied by a decrease in the nutritional value and sensory properties. To overcome these unwanted side effects, high pressure processing is often regarded as one of the major recent technological innovations in food preservation.

During the last years high pressure has emerged as a viable commercial alternative for pasteurization for high quality food. Pressures above 300 MPa enable the inactivation of viruses, enzymes, vegetative bacteria yeast and molds at ambient temperatures. This process is already used at an industrial scale in more than 190 high pressure machines, which produce more than 300,000 tons of pressure-pasteurized food per year (Tonello Samson, C. 2012, NC Hiperbaric, Spain, personal communication). The shelf-life of most of these products, however, is limited to between several days and some weeks under chilled conditions. There is a huge demand for high quality low acid food with a more extended shelf-life at ambient temperatures.

One of the most promising technologies in this regard is the combination of high pressure with elevated initial product temperatures. This combination can homogenously heat processed food up to sterilization temperatures (Heinz & Knorr, 2002). This process, which is called high pressure assisted thermal sterilization (PATS), was established and certified by the Food and Drug Administration (FDA) of the United States in February 2009. It is not yet used at an industrial scale, however, and nor its alternative process, the high pressure thermal sterilization (HPTS), which tries to use the beneficial effects of pressure and temperature on the inactivation of bacterial endospores. The reasons for this lack of up-scaling could be many, but one of the major challenges is to understand the underlying inactivation mechanisms of endospores during the process. This knowledge is not only necessary for up-scaling, but also for evaluating the impact of the temperature inhomogeneities occurring during the pressure treatment, and for optimizing this alternative sterilization process. The determination of an ideal range of process conditions for endospore inactivation would raise the reliability of cost–benefit calculations for PATS or HPTS processes, and moreover would reduce the hazards of product recalls and the subsequent destruction of public confidence in the company´s products.

The main objective of this basic study was the development and application of new analytic methods to investigate the mechanistic pathways that are in active in spore germination and

inactivation during high pressure processing. The study aimed to ensure reliable kinetic data for endospore germination and possible subsequent inactivation under various pressure temperature conditions. It was therefore essential to use reproducible process parameters, such as isothermal and isobaric treatment conditions, a simple liquid treatment medium with a nearly stable pH value under all tested conditions, as well as defined resistant properties of the used spore suspension. A range of analytic methods were used, including particle image analysis systems, the construction of pH measurement equipment for retorting conditions, flow cytometry analysis, as well as various microscopic and analytic techniques. This opened up the possibility of obtaining fundamental insights into the spore inactivation mechanisms under pressure, and of calculating non-linear $\log_{10}$ reductions. An empirical multiresponse kinetic model, based on a mechanistic approach, was used to identify the rate-limiting process conditions of inactivation, and furthermore the universal applicability of the model's kinetic data from different high pressure systems was demonstrated, which may help to upscale the process.

2. Theory and literature review

2.1 Bacterial endospores and their consequences for food preservation

In general endospore-forming bacteria belong to two classes of the phylum Firmicutes that include the aerobic Firmicatus (namely Bacilli) and the anaerobic Firmicutes (namely Clostridia) clusters. Both classes can be divided into a number of orders, some with sporulating genera and some with non-sporulating genera. It is generally assumed that the common ancestor of all Bacilli and Clostridia was an endospore-forming organism, even though several related genera important to food processing, such as *Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Listeria*, have lost their ability to form spores (Maughan et al., 2009).

The formation of highly resistant endospores by stressed vegetative cells results in perfect vehicles for the spoilage of food and/or infection of humans. Indeed, even in ancient literature diseases caused by spore-forming bacteria are described. In Chapter 9 of the biblical book of Exodus, the fifth and sixth plague visited upon Egypt was the death of livestock and boils in humans. Some biblical scholars believe that the fifth plague was animal anthrax and the sixth plague was cutaneous anthrax (Blane, 1890; Hort, 1958). Hence, this is probably the first written report of a true epizootic outbreak (Torred et al., 2012). Another example for the remarkable resistance of spores that is more relevant for food processing and storage is the often-fatal disease of the nervous system, Botulism. This disease is caused by *Clostridium botulinum* and has probably accompanied mankind since we began to preserve and store food, first in the home and later with industrial canning. The first reported cases of Botulism were observed in Europe in 1735 (Erbguth, 2004). These cases were suspected of being associated with a German sausage and Botulism was thus named after the Latin word for sausage, "*botulus*". At the end of the eighteenth century, the number of cases of fatal food poisoning throughout southwest Germany increased as a result of increased consumption of canned blood sausage (Torred et al., 2012). In 1895, an extraordinary outbreak of Botulism occurred in Belgium. After the consumption of smoked ham, diners started to feel muscle paralysis and three people died. Based on the observation of this outbreak, Emile von Ermengem published that Botulism was intoxication and that the toxin itself was heat-labile and produced in preserved food products. He further attributed the toxin production to a specific anaerobic bacterium *Bacillus botulinus* (*Clostridium botulinum*) (Erbguth, 2004). By 1925, outbreaks of Botulism from industrial canned foods essentially ceased (Tanner, 1935), due to the widespread adaption of standards for the necessary heating times and temperatures for industrial-scale food canning.

Although the infections from spore-forming bacteria can be traced back for thousands of years, the discovery of spores was not reported until Perty's work in 1852 (Perty 1852) and that of Robert Koch in 1876, who discovered the first pathogen, *Bacillus anthracis.* In 1872 Ferdinand J. Cohn established the name *Bacillus* and theorized that there might be a special "germ" that survives boiling. The bacteria that appeared after boiling were bacillus rods, which he called *Bacillus subtilis.* Cohn reported that during cultivation, many of the bacilli would swell at one end and became filled with oval, strongly refractive little bodies. Believing that these bodies represented a stage in the life-cycle of the bacilli, he suggested that they "were real spores, from which new Bacilli may develop", which also survive the boiling process and germinate later on to form new bacteria (Torred et al., 2012). In this study, *Bacillus subtilis* and isogenic mutant strains were investigated. The genus *Bacillus* includes in total 139 species (GBIF, 2012) and is classified as:

Kingdom: Bacteria

Division: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

Out of all of the spore-forming *Bacillus* genuses, *Bacillus subtilis* is the best understood in terms of its molecular and cell biology.

2.1.1 Sporulation of endospores

The formation of endospores is a complex, highly coordinated process, which allows the developing spore to survive when nutrients and moisture are scarce. However, because sporulation is an extremely energy-consuming process, cells avoid sporulation under mild stress conditions.

Sporulation has been reviewed by several authors (Phillips & Strauch, 2002; Setlow, 2003; 2007; Robleto et al., 2012) and due to extensive research it is a well-characterized process. It is initiated in a vegetative cell by environmental conditions that limit growth and leads to the formation of two cell compartments: the fore spore and the mother cell. The final stage of sporulation results in the lysis of the mother cell and the release of the mature spore. This developmental program is completed in 8-10 hours for *Bacillus subtilis* (Robleto et al., 2012).

In more detail, the sporulation is subdivided into seven states, which are temporally controlled by five distinct sigma factors (de Hoon et al., 2010). The sporulation differentiation

program in a vegetative cell is initiated by the activation of the Spo0A response regulator. In *Bacillus subtilis*, different sensor kinases (*kin* genes) phosphorylate Spo0A to SpoA-P, which then binds to the DNA and directly affects the transcription of 121 genes. In the undifferentiated vegetative cell, with two DNA molecule coils along the central axis (stage 0-I, in Figure 2. 1), the sporulation is induced by the activated transcription factor Spo0A and the sigma factor σ^H. These two factors promote the development of an asymmetric cell septum, which separates the cell into the mother cell and the fore spore (stage II) (Robleto et al., 2012). Further to this the asymmetric cell septum entraps incompletely a DNA plasmid into the forespore compartment. In this regard, the expression of low levels of the activated Spo0A-P retards the sporulation and/or promotes the formation of biofilms.

The asymmetric compartment splits the cell into a small and large compartment, each of which engages in differential activation of the genes because of the compartment-specific sigma factors (stage II, Figure 2. 1). In the developing forespore, σ^F activates the transcription of 48 genes. Once σ^F is active, transcription of genes that facilitate different functions takes place in the forespore (Wang et al., 2006). The genes regulated by σ^F are involved in DNA binding, synthesis of germinant receptors, development of spore cortex components, DNA repair, and communication with the mother cell compartment. Furthermore, the σ^F factor also directs the transcription of genes that mediate the engulfment of the forespore into the mother cell (stage III).

After engulfment, σ^E from the compartment of the mother cell controls the gene expression required for cortex formation. This factor regulates more than 260 genes and influences further the formation of the spore coat and the mother cell's metabolism (Robleto et al., 2012). In stage IV, the mother cell membrane grows around the forespore, which is already surrounded by an intact membrane. The synthesis of a thick peptidoglycan cortex between the outer and inner forespore membranes instigates the formation of the cortex and the germ cell wall (stage IV). At the same time as cortex formation, the forespore loses water and ions like potassium (K^+), prompting a large decrease in volume and a simultaneous decrease in pH by approximately one unit. The loss of water is a response to the uptake of cations like Ca^{2+}, Mg^{2+} or Mn^{2+}, and pyridine-2,6-dicarboxylic acid (dipicolinic acid, Ca-DPA or DPA), all of which are synthesized in the mother cell.

Due to progressive core dehydration, DPA concentration exceeds its saturation level and it is assumed that Ca^{2+}-DPA complexes are generated. The sigma factor σ^E further triggers the activation of σ^G in the maturing spore (stage IV-V). The genes activated by σ^E mediate the continued morphogenesis of the spore cortex and the spores´ resistance properties and germination functions (*ger* genes), as well as the activation of the last sigma factor of the sporulation program in the mother cell, σ^K (Robleto et al., 2012).

Within stage IV-V small acid-soluble proteins (SASPs) are synthesized, which remodel the forespore nucleotide into a ring. Hence, σ^E controls the gene expression for DNA protection and repair (Setlow, 2007). Furthermore, at stage V, the protoplast becomes increasingly resistant to high pressure, heat, radiation and the attack of chemical agents or enzymes. This resistance is due mainly to the spore´s high degree of compartmentalization.

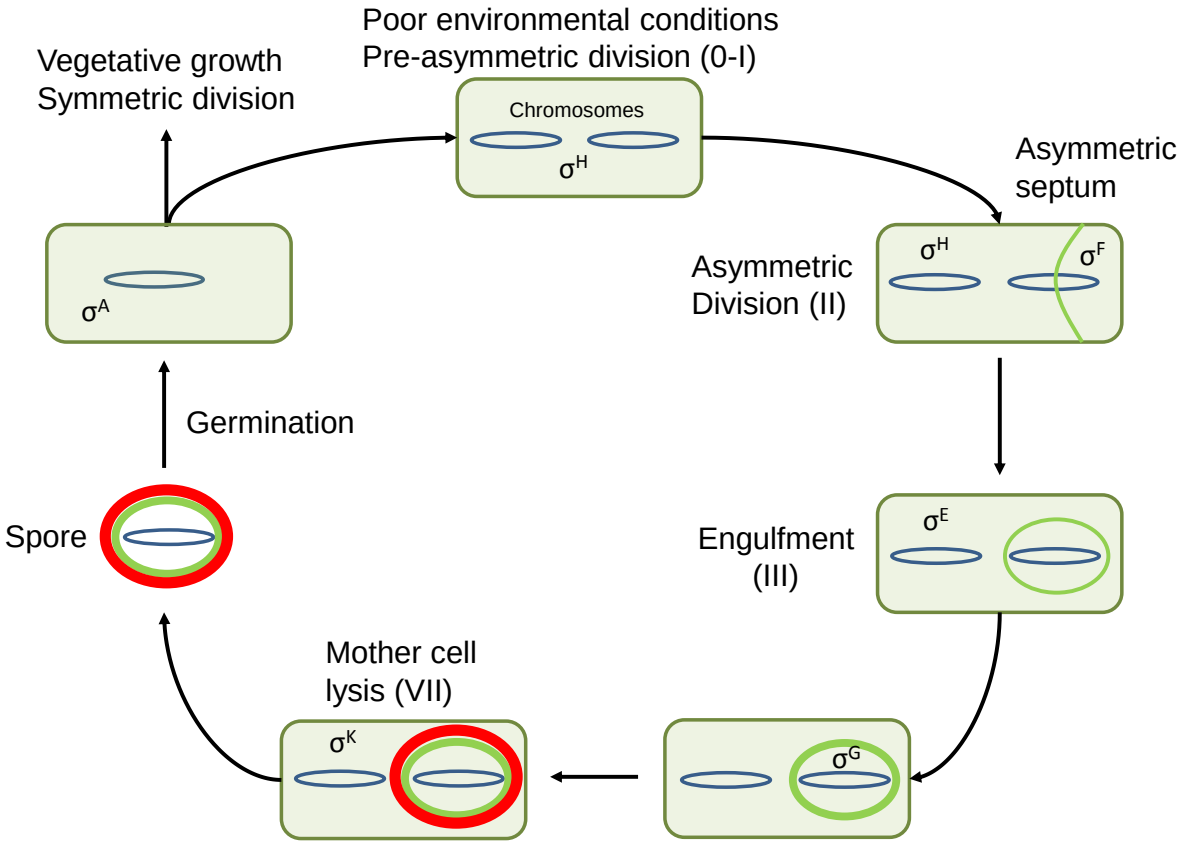

Figure 2. 1. Schematic drawing of the sporulation process of *Bacillus subtilis* (adapted from (de Hoon et al., 2010).

The transcriptional activity mediated by σ^K directs the formation of spore coat proteins, mother cell lysis and spore release.

During sporulation a complex proteinaceous coat, with coat proteins from the mother cell, is layered on the outer surface of the spore (stage VI), affecting the specific hydrophobicity of bacterial spores (Wiencek et al., 1990). This proteinaceous coat could cause a possible spore agglomeration (Mathys et al., 2007b) and is also responsible for the magnitude of adhesion forces to e.g. packing materials and surfaces. After the mother cell lyses, the spore is released (stage VII). The spores formed have no metabolism and enable the microorganism to survive over long periods of time and under extreme environmental conditions (sometimes thousands of years).

2.1.2 Structure of spores and mechanisms of endospore resistance

After the sporulation process, the matured spore has a well-structured multilayer morphology with several mechanisms to withstand multiple environmental stress conditions. The resistant properties of spores are remarkable. *Bacillus* spores trapped in amber or salt crystals are easily revived after perhaps millions of years. Furthermore, genomic examinations of prehistoric spores show amazing similarities with modern organisms (Torred et al., 2012). Endospores are thus perfectly adapted vehicles for spoiling food or subsequently causing foodborne diseases. A deeper understanding of the mechanisms involved in long-term survival is therefore extremely important for developing strategies for killing spores.

Working from the outside in, endospores generally consists of the exosporium (not present in e.g. *Bacillus subtilis*), coats, outer membrane, cortex, germ cell wall, inner spore membrane, and spore core (Figure 2. 2). The most external layer of an endospore is the exosporium, a large loose-fitting structure found in some organisms of the *Bacillus cereus* group (Waller et al., 2004). This multilayered shell surrounds the spore but is not attached to it. However, spores of many species, including *Bacillus subtilis*, either do not contain an exosporium or it is greatly reduced in size and tightly associated with the outer coat (Pedraza-Reyes et al., 2012).

Proceeding inward, the next structure is the multilayered proteinaceous coat, consisting of the crust, the outer and inner coat, and the basement (Setlow, 2012). The coat is mostly composed of cysteine- and tyrosine-rich proteins, and small amount of lipids and carbohydrates. This coat assembly represents the first line of defense of a spore, as its multiple layers of protein act as a chemical filter and play a minor role in spore resistance to UV radiation and wet heat (Pedraza-Reyes et al., 2012; Setlow, 2012). Furthermore, they protect the cortex against peptidoglycan lytic enzymes (like lysozyme) and predation by protozoa (Dirks, 1999). The coat is also believed to facilitate permeation of nutrient germination molecules to the inner spore membrane (Setlow, 2003), but it is not considered as a permeability barrier for small molecules (Setlow, 2006).

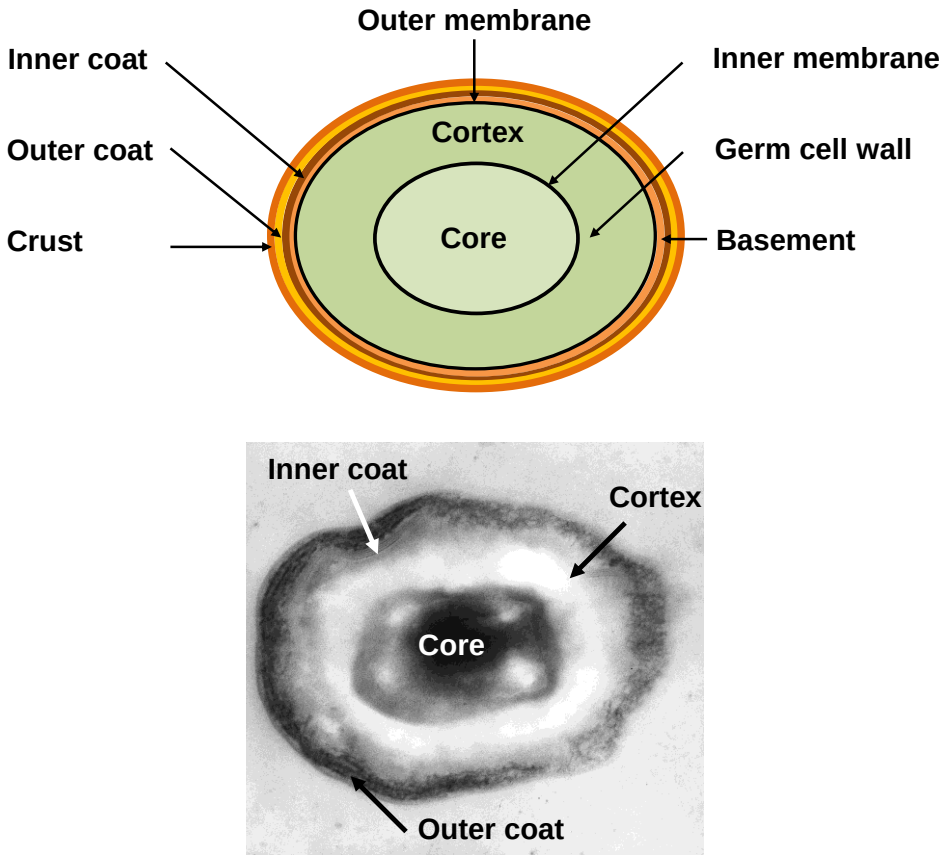

Figure 2. 2. Schematic structure of a dormant Bacillus spore (adapted from (Setlow, 2003; 2012) and a transmission electron micrograph (63,000 fold magnification) from a dormant *Bacillus subtilis* spore.

The next structure, the outer membrane, is an essential structure during the spore formation, but does not retain its integrity in the matured spore (Popham, 2002). For *Bacillus subtilis*, the cortex-lytic enzyme CwlJ is present in the spore coat layer, which is adjacent to the spore cortex. Even though the precise location of CwlJ in the coats is not known, it has been shown that CwlJ is adjacent to the coat protein GerQ, which is needed for the presence of CwlJ in spore coats (Setlow, 2003).

Beneath the outer membrane is a thick peptidoglycan layer, the cortex. Its structure is similar to the cell wall of vegetative cells, but contains some spore-specific modifications (Popham, 2002). In the cortex around 50 % of the peptide side-chain muramic acid residues are replaced by muramic acid lactam, which results in a more flexible and loosely cross-linked peptidoglycan (Warth & Stroming.Jl, 1972). The cortex thus presumably serves as a retaining structure to withstand the turgor pressure generated by the high concentration of solutes in the core (Popham, 2002).

Present in the cortex and at the cortex–coat boundary is the second cortex lytic enzyme of *Bacillus subtilis*, SleB, and the YpeB protein needed for the appearance of SleB in spores. These are also present in the structure below the cortex, the inner spore membrane (Setlow, 2003). Located between the cortex and the inner spore membrane is the germ cell wall,

which has a similar structure to the cell wall of vegetative cells. The germ cell wall serves as the initial cell wall during spore outgrowth (Pedraza-Reyes et al., 2012). The inner spore membrane is the analogue of the growing cell cytoplasmic membrane and represents a strong permeability barrier. It hence plays a key role in the resistance of spores to many DNA-damaging chemicals (Nicholson et al., 2000; Cortezzo & Setlow, 2005). Moreover, the inner spore membrane is the determining factor in the maintenance of the low water content in the spore core, although the exact mechanism through which the spore achieves dehydration remains unclear (Popham, 2002).

In the dormant spore, the inner membrane is in a gel phase and is a very compressed structure (Cowan et al., 2004), although its composition is similar to that of the plasma membrane of growing cells (Pedraza-Reyes et al., 2012). During the first minutes of germination (and in the absence of ATP) its volume increases two-fold, whereas in the dormant spore the lipid molecules remain largely immobile (Cowan et al., 2004). Consistent with the immobilization state of the membrane lipids, the water permeability of the inner membrane is 3–4 orders of magnitude lower than prototypical lipid bilayer membranes (Sunde et al., 2009). A rapid diffusion of water into the spore coat and cortex layers (50 s) was measured, whereas the time for diffusion through the inner membrane into the spore core was found to be approximately 8 minutes. Furthermore, the inner spore membrane is the location of the spore´s different types of nutrient receptors (Setlow, 2003).

The analogue to the protoplast of a vegetative cell is the spore´s core, which has several characteristics that contribute to the spore's high resistance level. These unique properties of the core are the low water content, the high levels of minerals and DPA in the core, the decreased spore core permeability, and the saturation of the DNA with SASPs (Pedraza-Reyes et al., 2012). One of the key factors contributing to the spore's enzymatic dormancy and its heat resistance is the low water content in the core. In comparison to growing cells, which have 75–80 % water content, water in the spore core constitutes only 27–55 % of the spore's wet weight. The low permeability of the inner spore membrane and the accumulation of DPA are both essential to maintaining this low water content. Contrary to the glassy state of the spore core proposed several years ago (Sapru & Labuza, 1993; Ablett et al., 1999), recent spin relaxation rates in H_2O / D_2O exchange in *Bacillus subtilis* spores indicate that water in the core has a high mobility through the spore. Hence, the spore's dormancy is probably linked to the dehydration-induced conformational changes of enzymes (Sunde et al., 2009). Further to this Sunde et al. (2009) established that the core water molecules are bound to the hydration layers of the proteins, but still remain mobile. They supposed that the spore core is an insoluble heat-stable gel, which exchanges water with the external environment.

A second factor that contributes to the wet heat resistance of *Bacillus* and *Clostridium* spores is the amount of minerals in the core. The mineralization is mainly determined by the high amount of DPA (5-15 % of spore dry weight), which is at a 1:1 ratio associated as a chelate with predominantly Ca^{2+} (Setlow et al., 2006). Whereas the total concentration of Ca-DPA in individual spores differs, its concentration (> 800 mM to 1 mM) is well above the Ca-DPA solubility level, indicating that most of the Ca-DPA in the spore core is in an insoluble form (Huang et al., 2007).

The third type of core molecules that play a role in spores' resistant properties are the SASPs, especially the α / β – type (Setlow, 2007). These small proteins are highly concentrated in the spore core and constitute 3–6 % of the total spore protein. The α / β SASP bind to and saturate the spore DNA (Setlow, 2007), causing structural changes in the DNA and altering the DNA's photochemistry (Lee et al., 2008). Spore mutant strains that lack α- and β- type SASPs show a reduced UV-light resistance as well as a higher sensitivity to heat, peroxides, ionizing radiation, and other sporicidal treatments (Setlow et al., 2003; Pedraza-Reyes et al., 2012).

Table 2. 1 shows an overview of the different defense mechanisms against common inactivation methods used in the food industry during food production, for preservation and cleaning. One of the most common methods of inactivating spores to increase the shelf-life of low acid food is the application of wet heat. However, the mechanisms for wet heat-killing of spores, as it occurs during retorting, are still not fully understood. It is well known that wet heat does not inactivate spores by DNA damage or oxidative damage (Setlow, 2006). Instead, wet heat inactivation of spores is mostly associated with enzyme inactivation and protein denaturation (Warth, 1980; Coleman et al., 2007; Tabit & Buys, 2010). Wet heat inactivation is also often accompanied by the release of Ca-DPA (Janssen et al., 1958), although the exact mechanism responsible for the Ca-DPA release is still not known. It is presumably caused by a rupture of the inner spore's membrane (Coleman et al., 2007). In addition, the relationship between protein denaturation and Ca-DPA release is still unclear, although Coleman et al. (2007) reported that the inactivation of core enzymes of *Bacillus cereus* and *Bacillus megaterium* is faster than the release of Ca-DPA.

In contrast to inactivation by wet heat, inactivation by dry heat is mostly attributed to DNA damage and strand breaks. Hence, the DNA protecting α / β –type SASPs and DNA repair systems, which are active during outgrowth, are important for the dry heat resistance of spores.

Endospores are additionally extremely resistant to a variety of chemicals, including bases, acids, oxidizing and alkylating agents, as well as aldehydes and organic acids. An overview of the spore's defense mechanisms against some chemicals and the impact of irradiation is given in Table 2. 1, and a detailed discussion of target structures and repair mechanisms is

given by Setlow (2007) and Pedraza-Reyes et al. (2012). The only chemical which is discussed in more detail is hydrogen peroxide, due to its relevance for surface decontamination and as an analytical tool for an estimation of SASP degradation during spore germination. In contrast to other oxidizing agents, which mainly damage the inner spore membrane, hydrogen peroxide could permeate the inner spore membrane and act directly in the core (Melly et al., 2002). Hence, the only defense mechanism of the spore is the saturation of the DNA with α / β –type SASPs, which could protect the DNA *in vitro.*

Table 2. 1. Mechanisms of endospore survival against physical and chemical treatments.

Sporicidal treatment	**Defense mechanism / factor influencing the resistance**	**Reference**
Wet heat	• Sporulation temperature	(Raso et al., 1995; Melly et al., 2002)
	• Core´s level of DPA and Ca^{2+}	(Setlow et al., 2006)
	• α- and β- type SASP	(Setlow, 2007)
	• Low water content in the core	(Gerhardt & Marquis, 1989)
Dry heat	• DNA protection by α- and β- type SASP	(del Huesca Espitia et al., 2002) (Setlow, 2006)
	• DNA repair enzymes ExoA and Nfo (active during germination)	(Salas-Pacheco et al., 2005) (Barraza-Salas et al., 2010)
Desiccation	• DNA protection by α- and β- type SASP	(Setlow, 1995)
Chemical resistance		
Sodium chloride/ Hypochloride	• Spore coat – coat proteins react and detoxify	(Setlow et al., 2006)
Hydrogen peroxide	• DNA protection by α- and β- type SASP	(Melly et al., 2002)
Ionizing radiation	• DNA repair enzymes ExoA and Nfo (active during germination)	(Salas-Pacheco et al., 2005) (Barraza-Salas et al., 2010)
	• Decreased level of core water	(Nicholson et al., 2000)
	• Sulphur-rich spore coat proteins and DPA	(Nicastro et al., 2002)
	• Increased levels of Mn^{2+} and other divalent cations	(Granger et al., 2011)
UV - radiation	• UV-Photochemistry of DPA - DNA – formation of “spore photoproduct”	(Nicholson et al., 2000) (Douki et al., 2005a)
	• Error free repair “spore photoproducts”	(Douki et al., 2005b)
	• DNA protection by α- and β- type SASP	(Setlow et al., 2006)
	• DNA repair enzymes ExoA (active during germination)	(Nicholson et al., 2000)
	• Specific DNA repair system for “spore photoproduct”	(Nicholson et al., 2000)
High pressure	• Sporulation temperature	(Raso et al., 1998b)
	• Demineralization of the core	(Igura et al., 2003)
	• Ability to retain DPA	(Margosch et al., 2004) (Reineke et al., 2013b)

A multiple target inactivation is caused by the use of cold atmospheric plasma. This non-thermal technology can be described as quasineutral particle system in the form of gaseous

or fluid-like mixture of free electrons and ions. Frequently a plasma contains also neutral particles (atoms and molecules), with a large kinetic energy of the electrons as well as free radicals and reactive oxygen species (Knorr et al., 2011).

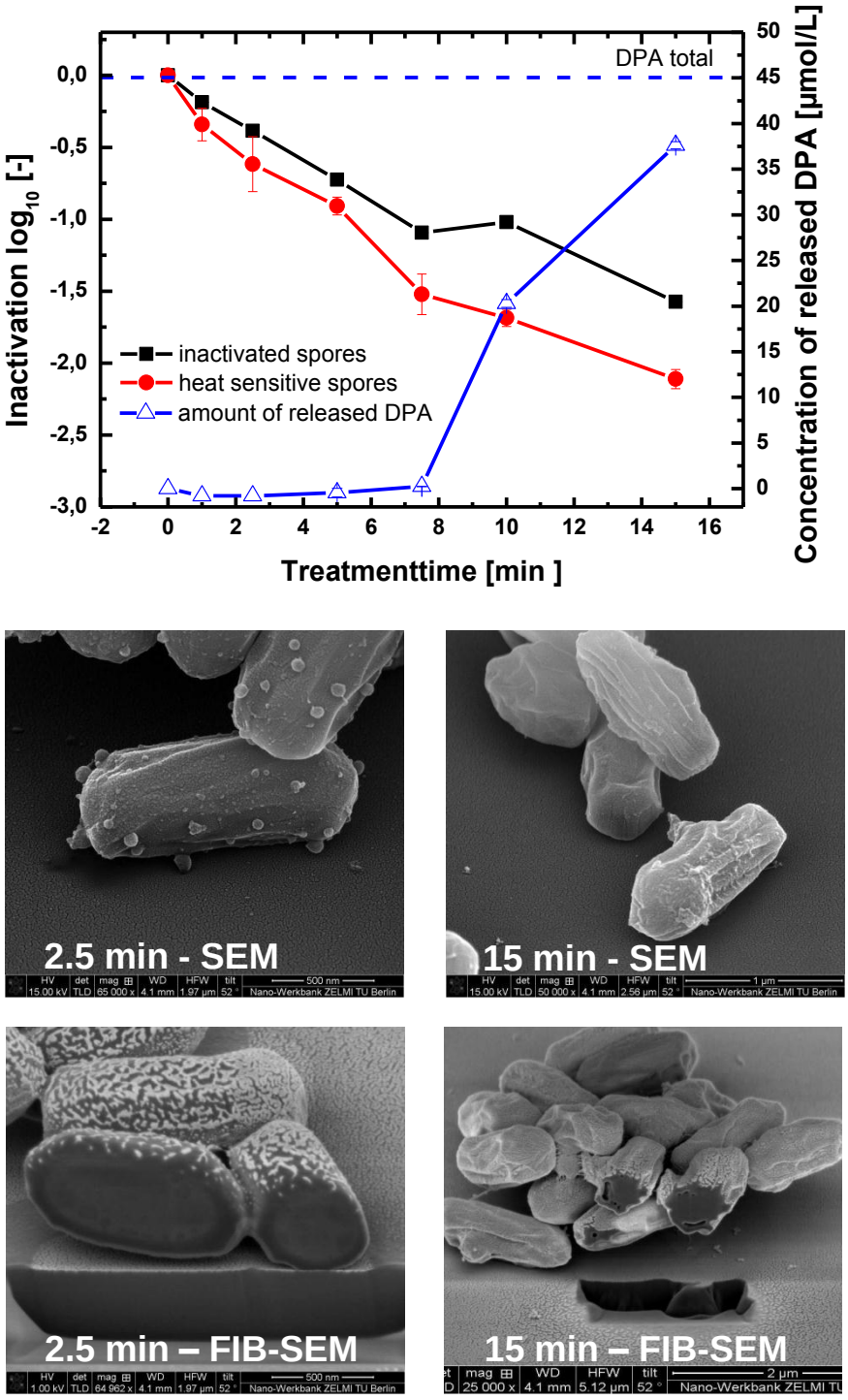

Figure 2. 3. Kinetic data for heat (red, •) and inactivated *Bacillus subtilis* (PS832) spores treated with cold atmospheric plasma (plasma jet, 6 L h^{-1} gas flow rate consisting of 99.99 % argon and 0.01 % oxygen) in distilled water and corresponding FIB section SEM pictures. The blue line (Δ) denotes the amount of released DPA after the plasma treatment (Reineke unpublished).

The entire mechanisms regarding spore inactivation by cold atmospheric plasma are not fully understood, but UV irradiation, photodesorption and an effect called “etching” are supposed to be responsible for the inactivation (Moisan et al., 2001). Hence, the mode of inactivation is various and affects the outer spore structure, like it is shown in Figure 2. 3. By using a high resolution SEM combined with a focused ion beam cross section method, developed by Reineke et al. (2013a), it was possible to visualize the impact of plasma on *Bacillus subtilis* spores suspended in distilled water. As the destruction, presumably caused by reactive oxygen species, degrades the spore from the outside, a Ca^{2+}-DPA release after a sufficient treatment time was measurable, due to structural changes in the inner spore membrane. However, further research is needed to fully understood, e.g. the impact and interaction of all compounds present in the plasma on the inactivation mechanisms of endospores.

2.1.3 Parameters effecting spore resistance against high pressure

Within the last decades some research groups have investigated factors influencing the resistant properties of endospores to high isotactic pressure. In general, no correlation between heat and pressure resistance has been found for several *Bacillus* (Nakayama et al., 1996) and *Clostridium* strains (Margosch et al., 2004b).

However, as with wet heat resistance, especially the sporulation temperature and the composition of the sporulation media were identified as influencing the pressure resistance of spores. Raso et al. (1998b) reported that *Bacillus cereus* spores prepared at 20 or 30 °C are more pressure resistant than those sporulated at > 30 °C after a treatment at 690 MPa and 60 °C for 30 s. Melly et al. (2002) performed a detailed investigation on the effect of the sporulation temperature on *Bacillus subtilis* spore resistance and spore composition. The temperature of sporulation was found to affect the pressure resistance and also to result in significant alterations in the spore coat and cortex composition. Further, modifications of the sporulation conditions might suppress some Ger proteins needed for pressure induced germination (Minh et al., 2011).

In another study, Igura et al. (2003) sporulated *Bacillus subtilis* at 30, 37, and 44 °C, and investigated the effect of minerals on the resistance. Spores sporulated at 30 °C had the highest resistance to treatments with high hydrostatic pressure (100 to 300 MPa, 55 °C, 30 min). Pressure resistance increased after demineralization of spores and decreased after remineralisation of spores with Ca^{2+} or Mg^{2+}, but not with Mn^{2+} or K^{+}. This may suggest that Ca^{2+} or Mg^{2+} are involved in the activation of cortex-lytic enzymes during germination. Furthermore, *Bacillus cereus* spores sporulated at pH 6.0 were more resistant to pressures up to 600 MPa than those sporulated at pH 8.0 (Oh & Moon, 2003). By contrast, Minh et al. (2011) reported that *Bacillus subtilis* spores produced at pH 10 showed increased pressure

resistance after a pressure treatment at 350 MPa at 40 °C after 60 min. Furthermore, Margosch et al. (2004) tested in total 19 *Bacillus* spore strains and stated that endospore resistance to a combined high pressure (> 600 MPa) and temperature (> 60 °C) treatment depends on the spore´s ability to retain DPA, and on the heat resistance of the DPA-free spores. If this is the case, variations in the fluidity of the inner spore membrane (Abee et al., 2011) may be involved in the pressure resistance of spores. Because high pressure reduces membrane fluidity (Kato & Hayashi, 1999; Winter & Jeworrek, 2009), a higher fluidity of the inner spore membrane might maintain its barrier properties at higher pressure levels and hence increase the spore's resistance to pressure.

2.1.4 Bacterial spore germination

In its state of metabolic dormancy the spore is monitoring its environment, and when conditions are again favorable for growth it germinates and goes through outgrowth, thus being converted back into a growing cell. The step of germination is the crucial step with regards to loss of resistance, and is therefore of great interest for a variety of sterilization techniques and research. The germination process has been reviewed by several authors (Moir et al., 2002; Setlow, 2003; Moir, 2006; Abel-Santos, 2012) and will be summarized in the next chapter due to its important role for the pressure-induced physiological germination and non-physiological germination of spores (Reineke et al., 2013a).

After the detection and binding of appropriate nutrients an irreversible cascade of biophysical and hydrolytic reactions is initiated, which constitutes the germination process. Spores of *Clostridium* and *Bacillus* rely on similar germinants, which include amino acids, ribosides, sugars, ions and peptidoglycan fragments. To induce the germination process, exposure times of only a few seconds are sufficient for some cases of appropriate germinant molecules, which results in a rapid loss of resistance properties (Setlow, 2003; Christie, 2012). Under physiological conditions, two germination pathways have been identified in spores of *Bacillus* species. The best understood is the binding of nutrients to the nutrient receptors that are bound to the inner spore membrane (Ross & Abel-Santos, 2010). The second and more recently discovered pathway is mediated by serine/threonine kinase (PrkC), which initiates germination after the binding of defined peptidoglycan fragments.

After the initiation of the germination process it can be subdivided into two distinct stages, which are termed stages I and II (Figure 2. 4) (Setlow, 2003).

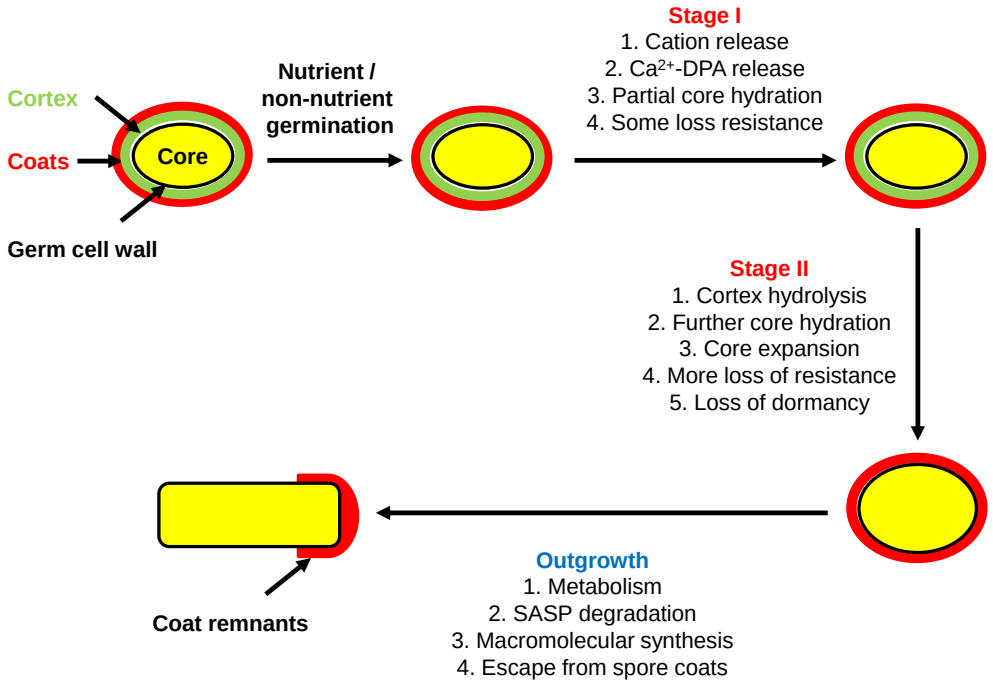

Figure 2. 4. Stages of spore germination according to and adapted from Setlow (2003).

After the germination receptors have been triggered, the release of monovalent cations (H^+, K^+ and Na^+) from the spore core denotes the beginning of stage I. This is accompanied by an increasing internal pH of the core to levels that are suitable for the re-initiation of metabolism, when hydration levels are sufficient (Swerdlow et al., 1981). Cation release is followed by the release of DPA, which contributes ≈ 25 % of the core dry weight and is predominantly associated with Ca^{2+} and other divalent metal ions (Johnstone et al., 1982). Parallel to the release of Ca-DPA, free amino acids such as arginine and glutamic acid are released (Setlow et al., 2008).

The spore core is partially rehydrated when the DPA is released and hence at this point the heat resistant properties of these partially germinated spores are reduced (Setlow et al., 2001; Cowan et al., 2003). This partial core hydration is achieved roughly 4–6 min after the germination was induced, but it can also be retarded for individual spores (Kong et al., 2010), as will be discussed in more detail in chapter 2.1.6, as heterogeneities in endospore populations.

The core hydration level, however, is still insufficient for any enzyme activity, and although the local mobility of some vital protein(s) is such that they could be inactivated by heat (Sunde et al., 2009), the general protein mobility and diffusion in the core remains extremely restricted at this point (Christie, 2012).

The major event that defines the start of stage II of germination (Figure 2. 1) is the degradation of the spore´s thick cortex by specific petidoglycan hydrolases (Setlow, 2003). In *Bacillus subtilis*, two semi-redundant cortex-lytic enzymes (CLEs), SleB and CwlJ, are

responsible for the initiation of this process. The process is also facilitated by the protein SleL, whose activity appears to vary between different species of *Bacillus* (Ishikawa et al., 1998; Setlow et al., 2009; Christie et al., 2010). CwlJ is located at the cortex coat junction and is DPA-responsive. SleB is a lytic transglycosylase, which is present at the inner spore membrane and the outer layers. SleB is more resistant to wet heat than CwlJ (Moir, 2006). Structural genes encoding homologues of CwlJ and SleB are also found in several *Clostridium* species (Paredes-Sabja et al., 2011).

The hydrolysis of the cortex permits further uptake of water and expansion of the spore core, which involves the expansion of the inner spore membrane and the germ cell wall. The hydration level at this point becomes high enough to resume protein mobility and enzyme action (Christie, 2012). During all of these mechanisms no detectable energy metabolism (Paidhungat & Setlow, 2002) takes place. SASPs, which saturate the spore DNA (Setlow, 2007), are degraded and hydrolyzed by germination protein Gpr in the outgrowth phase (Figure 2. 4). This follows a macromolecular synthesis and enables them to escape from the spore coats. For an individual spore these events may take only few minutes, but because of the significant variation of spores, these mechanisms may take much longer for the whole population (Setlow, 2007), as will be discussed in chapter 2.1.6.

2.1.4.1 Initiation of physiological germination

Whereas the general mechanism for triggering germination may be largely conserved between *Bacillus* and *Clostridium* species, a number of different mechanisms involving GerA-type nutrient receptor proteins also exist, and are employed by at least some pathogenic *Clostridia*. The assumption of a common germination mechanism for both classes has therefore been challenged by recent insights derived from different species (Paredes-Sabja et al., 2011).

GerA-type nutrient receptor proteins are integral membrane proteins, which are only abundant in the order of tens of molecules per spore (Paidhungat et al., 2001). They have been suggested to be an evolutionary strategy to ensure that germination is only triggered when the environmental conditions are conducive to sustaining vegetative growth (Christie, 2012). The germination receptors are located deep in the inside of the spore, on the inner spore membrane (Paidhungat & Setlow, 2000). Hence, germinant molecules have to traverse the outer layers of the spore and the cortex to bind at the cognate receptor side at the inner membrane. This interaction, in a process that is poorly understood at the molecular level, somehow activates the receptors and irreversibly commits the spore to proceed through the germination process (Christie, 2012). This process of commitment is the first measurable event during germination (Yi & Setlow, 2010).

The germinant receptor proteins of *Bacillus* species are encoded by the *gerA* operons, and cluster into four distinct clades. These clusters are: the GerA/L group, which response to L-alanine; the GerB group, which response to a mixture of asparagine, glucose, fructose and K^+ (AGFK), the GerH(I)/ Y clade, which recognizes aromatic and related compounds; and the GerK clade, which is associated with the recognition of glucose (Christie, 2012). In contrast to the subsequent events in germination, the presumed binding of the germinant and the mechanism through which it stimulates the receptor function are mostly unknown. It is even not known how many receptors have to be activated to induce the following cascade of events, or how the signal is transduced to the presumed Ca^{2+}-DPA channels (Li et al., 2012) (and possibly other ion channel proteins), which are thought to be significantly more plentiful (Vepachedu & Setlow, 2005). The amount of activated receptors per spore does however appear to be a major determinant in the rate of spore germination heterogeneity (Yi 2010). Additionally, the amount of activated germinant receptors seems to play a role in the timing of the subsequent DPA release, and may also be involved in DPA release itself (Christie, 2012).

In contrast to *Bacilli*, where the germinant receptors appear only to have a role in germination, these receptors may play a crucial role also in post-germinative outgrowth in species of *Clostridia*. This difference perhaps illustrates a fundamental distinction in the receptor functions between the major endospore-forming genera (Paredes-Sabja et al., 2009a). Furthermore, recent data has revealed that several *Clostridium* species encode only a single protein-A receptor subunit (Paredes-Sabja et al., 2011), which may also be a reason for the different germination behavior of *Clostridium* spores under high pressure.

2.1.4.2 Initiation of germination by high pressure

In this chapter non-nutrient pathways to induce germination are discussed, with a focus on high pressure. Non-nutrient germination can be further categorized into a (recently discovered) second physiological and several non-physiological routes. The physiological routes include germination initiated by eukaryotic-like serine/threonine kinase, which is located in the inner spore membrane like nutrient receptors. This kinase, which is present in *Bacillus* and *Clostridium* species, recognizes peptidoglycan fragments (Shah et al., 2008).

Non-physiological germination pathways initiate spore germination by bypassing individual germination steps. This could be stimulated by physiochemical agents, such as exogenous Ca-DPA (Paidhungat et al., 2001), which directly activates the CLE CwlJ, or cationic surfactants such dodecylamine (Setlow et al., 2003), which interferes with the inner spore membrane and causes a direct release of Ca-DPA. This type of germination shares many parallels with the germination route induced by high pressures, above 500 MPa.

High pressure-stimulated germination is probably the best studied non-nutrient germination pathway, due to its potential application for high pressure thermal sterilization in food processing. Exposure of spores to the moderate pressures of 100–150 MPa (Figure 2. 5) has been shown to trigger the nutrient receptors of *Bacillus subtilis* and *Bacillus cereus* (Gould & Sale, 1970; Wuytack et al., 1998; Paidhungat et al., 2002) and by inference all spore-formers (Christie, 2012). Under these moderate pressures, individual germinant receptors show differing levels of pressure sensitivity. In *Bacillus subtilis* GerA is more sensitive to pressure than GerB, which itself is more sensitive than GerK (Black et al., 2005). Research has shown that a variation of the GerB receptor (GerB*) was > 5 times more responsive to pressure than the native GerB receptors, implying that it is not the amount of receptors but rather their specific structure and/or function that are most responsible for influencing the pressure responsiveness (Black et al., 2005; Christie, 2012).

Higher sporulation temperatures further increased the receptor responsiveness with the moderate pressure, compared to spores sporulated at lower temperatures. Despite the possible lower expression of germinant receptors, the sporulation temperature has an impact on the fluidity (Kato & Hayashi, 1999) or others aspects of the composition of the inner spore membrane (Black et al., 2005). Wei et al. (2009) worked with *Bacillus cereus*, which lacks its GerN germinant receptor. These spores showed a reduced germination rate under moderate pressure compared to the wild-type strain. A depletion of the GerT homologue further enhanced this effect, indicating that spores' associated monovalent cation transporters are also significantly involved in the moderate high pressure germination response. Hence, spore strains that lack the major nutrient receptors, like the *Bacillus subtilis* strains FB114 and FB115 (Figure 2. 5), do not germinate under moderate high pressure (< 200 MPa) (Paidhungat et al., 2002; Reineke et al., 2012).

Pressures above 500 MPa and 25 °C do not initiate a germination mediated by nutrient receptors. Under these conditions, Paidhungat et al. (2002) were able to germinate *Bacillus subtilis* spores that lack all major nutrient receptors. In such conditions Ca-DPA is released, which activates the cortex lytic enzyme CwlJ (Figure 2. 5), and the initiated germination process continues after decompression. The Ca-DPA release under these very high pressure conditions has been attributed to an assumed opening of the spores' Ca-DPA-channel proteins (Paidhungat et al., 2002), which are presumably located in the inner spore membrane (Li et al., 2012). This assumption is confirmed by the release of arginine and glutamic acid from the spore core, but not the slightly larger adenosine monophosphate (AMP), which indicates that channels proteins are involved in the Ca-DPA release.

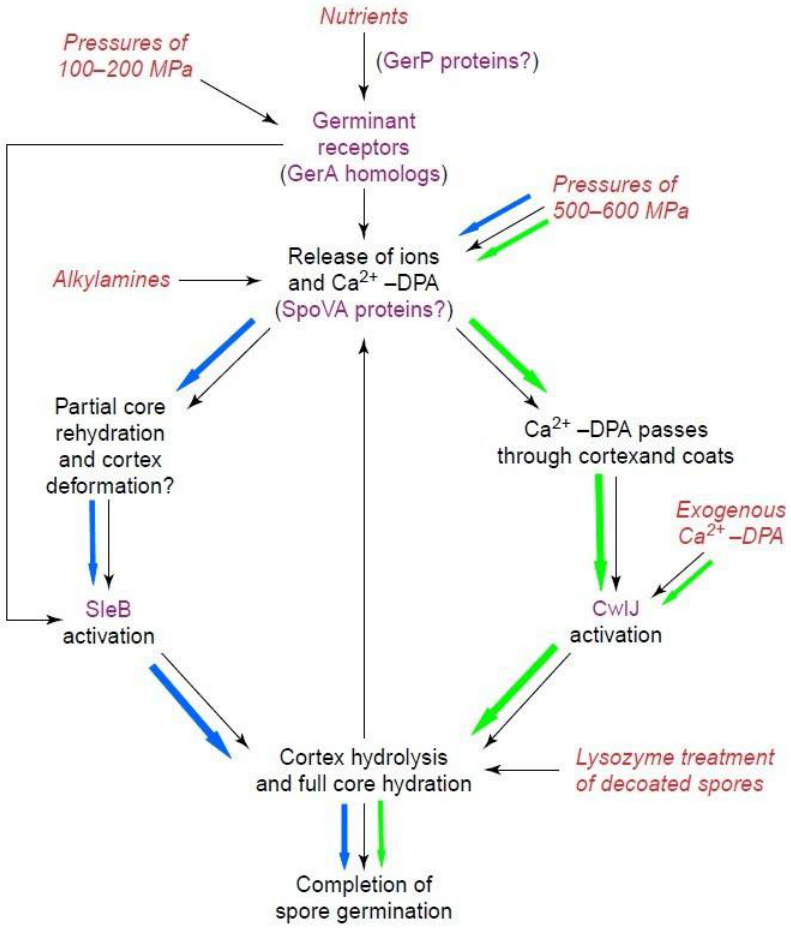

Figure 2. 5. Nutrient and non-nutrient germination pathways according to Setlow (2003). Green arrows indicate the active germination pathway for spores lacking the genes encoding the CLE SleB as well as the major nutrient receptors (like *Bacillus subtilis* FB114). Blue arrows indicate the active germination pathway for spores lacking the genes encoding the CLE CwlJ as well as the major nutrient receptors (like *Bacillus subtilis* FB115).

As exogenous DPA could trigger the germination of spores (Setlow, 2003) by activating the CLE CwlJ, as with pressures above 500 MPa, a superimposition of both mechanisms during the pressure treatment could be possible. This combination could have an impact on germination and inactivation rates of highly concentrated spore suspensions in buffer solution. To prove its possibility, Reineke (unpublished) investigated Ca-DPA release rates and their impact on cell counts of *Bacillus subtilis* PS832 (wild-type) for 10^6 CFU mL^{-1} and 10^8 CFU mL^{-1} (Figure 2. 6). This was for an isothermal (60 °C) and isobaric (600 and 700 MPa) pressure treatment in ACES buffer solution (pH7, 0.05 M). The amount of heat-sensitive as well as inactivated spores (Figure 2. 6 a) differed only slightly between the initial spore concentrations of 10^6 CFU mL^{-1} and 10^8 CFU mL^{-1} after the pressure treatment at 600 and 700 MPa, whereas the released amount of Ca-DPA after 10 min nearly reached the level needed to trigger germination by exogenous Ca-DPA (Figure 2. 6 b). Hence, a systematic overestimation of germination rates derived under these conditions can be excluded if the spore concentration does not exceed 10^8 spores mL^{-1}.

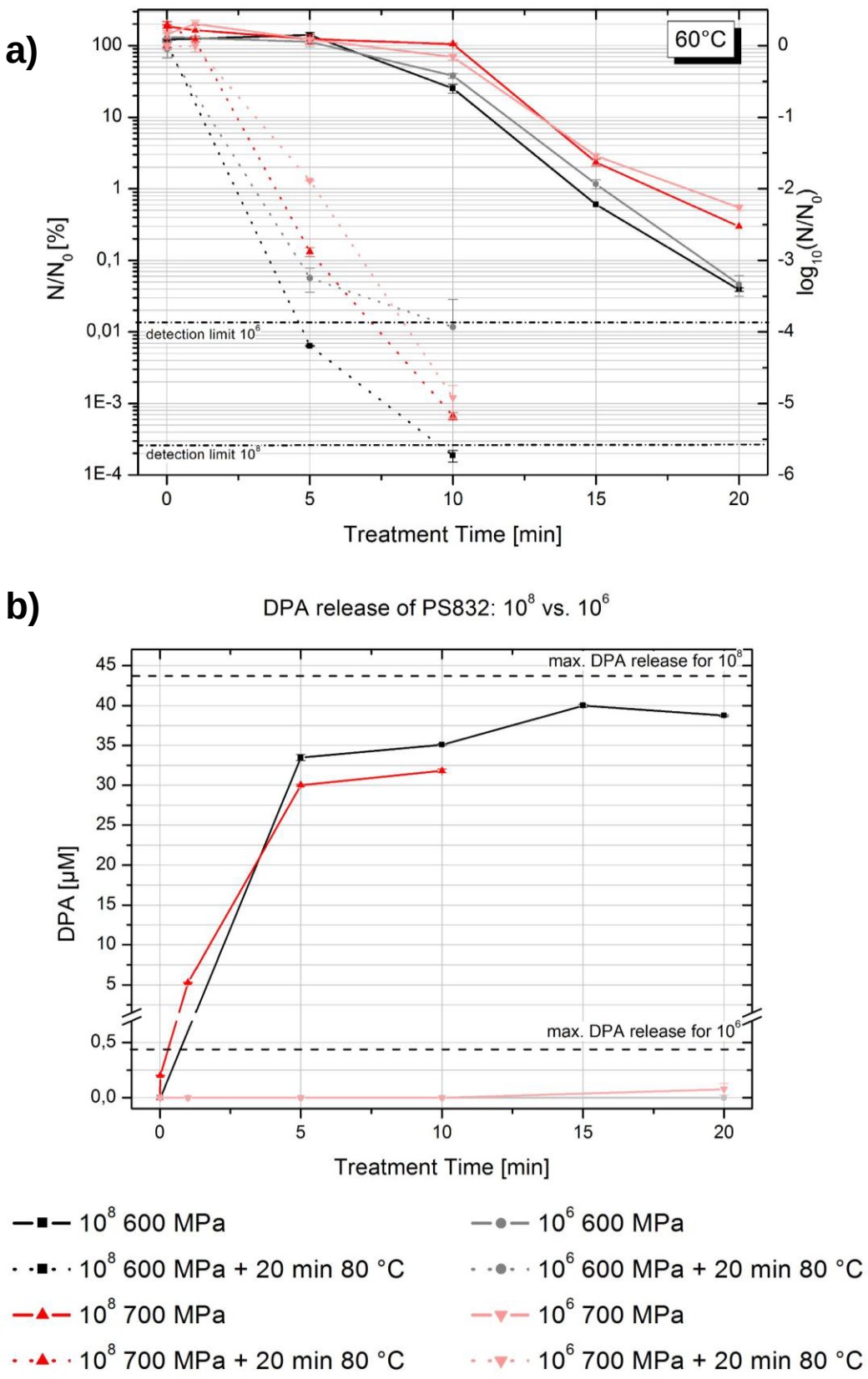

Figure 2. 6. Kinetic data for heat-sensitive and inactivated *Bacillus subtilis* (PS832, wild-type) spores at isothermal (60 °C) and isobaric (600 and 700 MPa) conditions during pressure dwell times for 10^6 and 10^8 spores mL^{-1} as initial cell count. Solid lines denote the amount of DPA released as measured after decompression.

2.1.4.3 Water and cations flux during germination

After nutrients crossed the outer spore layers, which are semipermeable and allow the passage of molecules with a molecular mass < 5 kDa (Henriques & Moran, 2007), nutrients pass through the cortex and activate the nutrient receptors. This causes changes in the inner spore membrane into a more fluid and permeable state (Stewart et al., 1980), which in turn allows ion movement (K^+, Na^+ and H^+) and the release of Ca-DPA, by opening the Ca-DPA-channels. The release of Na^+ and K^+ denote the first step of germination and cause a rise of the spore core´s pH. This leads to a restoration of ion composition, and ionic and osmotic strength in the core, as in the beginning of the outgrowing phase (Bassi et al., 2012).
Immediately after this water goes into the core, followed by activation of the CLEs (Setlow et al., 2003) and a loss of heat resistance. During and after cortex degradation, within minutes a further water flux into the core occurs, and the inner spore membrane expands its surface area almost 4-fold (Setlow et al., 2003). This restores the water content of the growing cells, with the resumption of enzymatic activity and energy metabolism (Setlow et al., 2003).

2.1.5 Degradation of spore cortex during germination

The spore cortex is a thick layer of peptidoglycan with structural modifications that differentiate it from a vegetative cell wall. Since no protein synthesis can take place during germination prior to completion of stage II, cortex lytic enzymes (CLEs) must be produced during sporulation and packaged into the dormant spore in an inactive and highly stable form. In most of the spore forming genera two redundant acting CLEs are present: CwlJ and SleB for *Bacillus subtilis.* A lack of one CLE slows down the rate of germination, but only minimal lytic activity is required to allow a completion of germination (Popham et al., 2012). The CLE SleB is a lytic transglycosylase that cleaves glycan strands. For a stable incorporation of SleB in the dormant spore, YpeB is required, but little is known about the activation of SleB during germination (SleB-Y in Figure 2. 7). Most studies propose that activation by turgor-induced stress on the cortex is important for SleB substrate recognition (Popham et al., 2012). The activation could also be the result of an interaction with the Ger receptors, the ion flux out of the core, or the result of the recovery of the inner membrane's fluidity upon core rehydration (Cowan et al., 2004; Popham et al., 2012).
The second CLE in *Bacillus* spores, CwlJ, is associated with GerQ in the spore coat layers (CwlJ-Q in Figure 2. 7). The activation mechanism is most likely the response to Ca-DPA, which is released during germination (Paidhungat et al., 2001). It has been suggested that CwlJ might have N-acetyl-glucosaminidase (Christie et al., 2010) or muramidase (Heffron et al., 2010) activity. The redundancy of these two CLEs is depicted in the germination rate of

strains that lack both CwlJ and SleB (Paidhungat et al., 2001). Such spores, like the *Bacillus subtilis* FB113 used in this study, initiate germination with a release of their Ca-DPA pool, which results in partial rehydration, but apparently not enough to allow resumption of metabolism (Setlow et al., 2009).

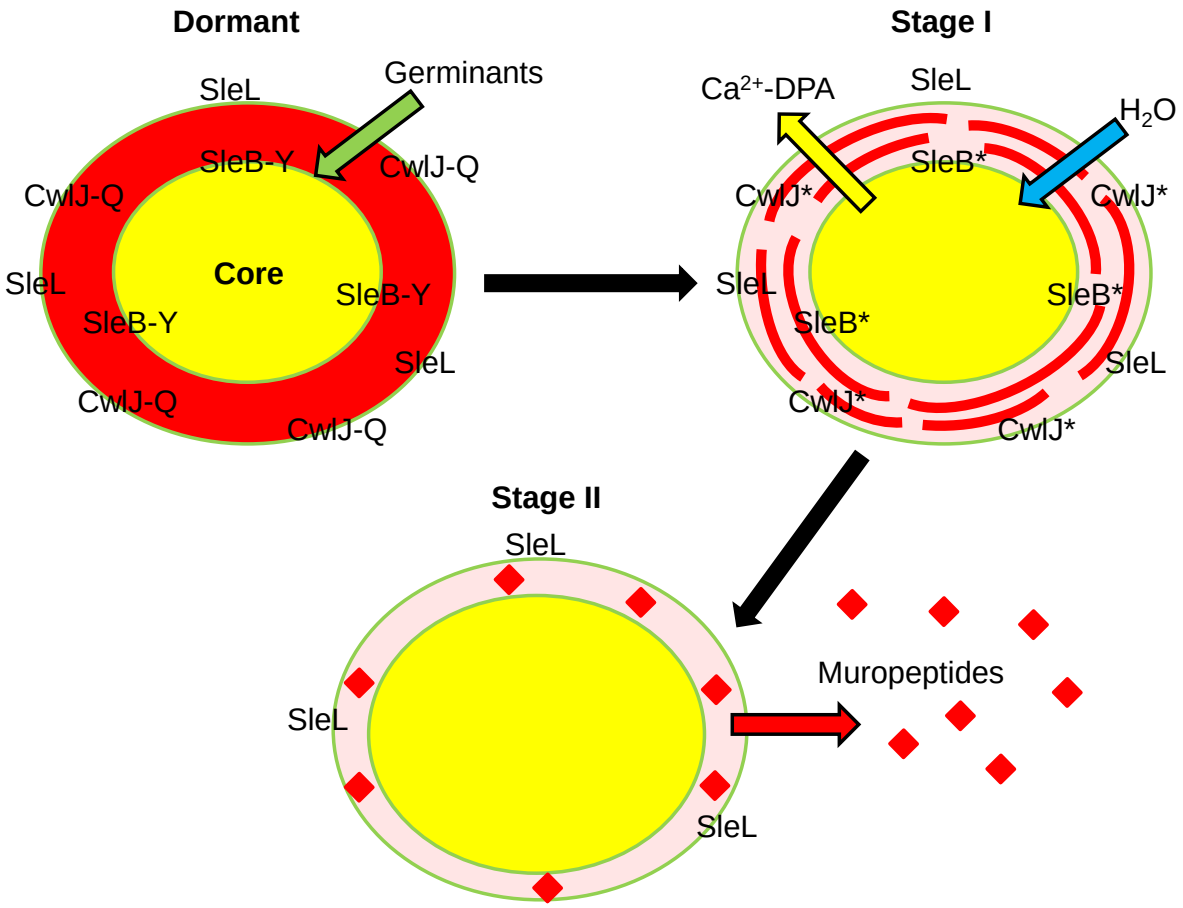

Figure 2. 7. Model for CLE activation and spore cortex hydrolysis; adapted from Popham et al. (2012).

Spores' CLE can act on intact spore peptidoglycan, and must act first during germination, whereas cortical fragment lytic enzymes (CFLEs) can act only on fragments of spore peptidoglycan. The CFLE in *Bacillus cereus*, SleL, was first purified by Chen et al. (2000) and later by Chirakkal et al. (2002) for *Bacillus subtilis*. They found out that it could act on cortex fragments, but not on an intact cortex. Hence they proposed that SleL acts in a secondary step of cortex lysis.

A model for cortex degradation is given in Figure 2. 7, the release of Ca^{+2} and DPA results in activation of CwlJ, and by an unknown mechanism SleB is activated. Through the dissociation of YpeB from SleB (SleB* in Figure 2. 7) and of GerQ from CwlJ (CwlJ* in Figure 2. 7), both enzymes are activated. Prior to this they were physically separated from the cortex, by the inner forespore (SleB) and outer spore membrane (CwlJ), whereas now they can reach and degrade the cortex (Stage I in Figure 2. 7). The resulting cortex fragments are cleaved by the CFLE SleL. This is already present in an active form but it can only act on

peptidoglycan that has been previously cleaved by CLEs. The smaller muropeptides are readily released from the spore and the inner spore membrane can then expand (Popham et al., 2012).

Structural genes encoding homologues of CwlJ and SleB in *Bacillus* and *Geobacillus* species are also found in several *Clostridium* species (Paredes-Sabja et al., 2011). Furthermore, another enzyme, SleC, is present in *Clostridium* spores and plays an essential role in cortex hydrolysis (Miyata et al., 1995; Paredes-Sabja et al., 2009b). Whereas CLEs in the spore are present as inactive but fully matured enzymes, SleC is inactive but has to be proteolytically cleaved into its functional form during germination. Mutant *Clostridium perfringens* spores that are unable to activate SleC do not release Ca^{2+}-DPA, whereas spores without SleC (as well as *Bacillus* spores without SleB and CwlJ) do release Ca^{2+}-DPA. This may represent a fundamental difference in the sequence of physiological events that constitutes the germination process of *Clostridium* and *Bacillus* species (Paredes-Sabja et al., 2009c).

2.1.6 Heterogeneity in endospore populations

Genetically identical populations of *Bacillus* and *Clostridium* species, grown from one single cell, exhibit significant heterogeneities, including wide variations in wet heat resistance as well as in speed of germination. In particular, superdormant spores, which are spores that germinate extremely slowly or are more resistant to environmental stress conditions, greatly complicate spore inactivation in food processing (Setlow et al., 2012). The outcome of these heterogeneities is that killing curves of spores during a treatment, such as high temperature or high pressure treatments, often shows tails that are caused by apparently more resistant spores.

For these tailing effects, several explanations are possible. One is the formation of large aggregates, which could offer a protective effect for spores in the center, or the disaggregation of a spore clump during processing. The disaggregation of spore agglomerates could mathematically describe the shoulder formation that often occurs in spore inactivation kinetics (Mathys et al., 2007b), but as an explanation for tailing this is only partly suitable. Furthermore, even in the absence of spore clumps, it is not uncommon for a small percentage of spores to have a much higher resistance (Setlow et al., 2012).

Spores that are cultivated from a single cell under defined conditions show a large variety in their physical and chemical properties. For instance, the overall spore size could vary ± 25 % for individual spores in a population, and significant variations have also been observe in the coat, cortex and core (Setlow et al., 2012). Furthermore, the Ca-DPA concentration in single spores differs, which may correspond to the variation in spore size. The highest impact on

higher wet heat resistance of individual spores, however, is the variation in core water content (Ghosh & Setlow, 2009).

Superdormant spores

Superdormant spores represent the most extreme examples of spore heterogeneity. These spores germinate many hours or days after the majority of spores (Gould, 2006). While the existence of superdormant spores has long been known, it is only recently that such spores have been isolated by repeated germination of a spore population (Ghosh & Setlow, 2009; 2010). The extracted superdormant spores of *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus cereus* germinated extremely poorly with the germinant used initially to germinate them, although they germinated fairly well with mixtures of several germinants (Setlow et al., 2012). More interestingly, with high pressures of 150 MPa at 37 °C or 500 MPa at 50 °C, Wei et al. (2010) rapidly germinated superdormant *Bacillus subtilis* and *Bacillus cereus*. This indicates that the presence and/or amount and type of expressed nutrient receptors during the sporulation contribute to the superdormancy (Wei et al., 2009; Wei et al., 2010; Setlow et al., 2012).

The yield of superdormant spores in a population could be decreased by:

(i) Heat activation
(ii) Increased levels of nutrient germinants targeting one nutrient germinant receptor
(iii) Mixtures of nutrient germinants that target several nutrient germinant receptors
(iv) Increased average levels of nutrient germinant receptors per spore

(Setlow et al., 2012). Interestingly, these are the same factors that decrease the lag time to induce germination in the entire spore population (Figure 2. 8), suggesting that superdormant spores are generally those with the lowest level of nutrient germination receptors per spore.

Heat activation

It has been known for several years that spore germination can be greatly stimulated by a moderate wet heat treatment (70-80°C) for several minutes (Keynan et al., 1964). The mechanism of this heat activation is still not known, but the process is reversible and may involve changes in the protein structure (Keynan et al., 1964; Zhang et al., 2009) or a phase transition of the cortex. Ghosh et al. (2009) further reported that superdormant spores of *Bacillus* species needed an 8-15 °C higher temperature for optimal heat activation than the initial spore population. This difference was probably not genetic and was attributed to a significantly lower water content in the superdormant spores (Ghosh & Setlow, 2009; Ghosh et al., 2009).

Wet heat resistance

A review of the factors influencing wet heat resistance is given in chapter 2.1.2. Among all of these factors the sporulation temperature is constant for all individuals in a population, at least for spores prepared in the lab. Others factors can vary significantly, such as the level of α / β-type SASPs, Ca-DPA concentration, and water content in the core, although the core water content probably has the highest impact on the heterogeneity in wet heat resistance (Setlow et al., 2012). Zhang et al. (2010b) investigated the release of Ca-DPA for individual spores by laser tweezers and Raman spectroscopy, finding that the release of Ca-DPA and the great majority of protein denaturation took place in only 1-2 minutes in water at elevated temperatures. However, the time when this event began varied greatly between individual spores. No correlations have yet been made between the precise time at which Ca-DPA is released from the core and other variable spore properties, such as core water content (Setlow et al., 2012).

Spore germination

It is well known that individual spores in a population germinate in a quite heterogeneous manner (Indest et al., 2009), which can be affected by heat activation, the level of nutrient germinants, and the number of nutrient germinant receptors (Setlow, 2003; Zhang et al., 2010a). Of highest impact is the average level of nutrient germinant receptors, which can vary three- to four-fold depending on the sporulation medium (Hornstra et al., 2009). The expression level of nutrient receptors is fairly low and was reported to be in the range of 24–40 molecules per spore for the GerB receptor (Paidhungat & Setlow, 2001). Other nutrient receptor levels are expected to be similar (Setlow et al., 2012).

After the addition of germinants to a spore suspension, the highest heterogeneity was reported for the commitment and lag period (Figure 2. 8), whereas the T_{lag} values could vary tremendously for individual spores (Zhang et al., 2010a). The time for the rapid release of > 85 % of Ca-DPA (stage III in Figure 2. 8) is relatively constant at 0.5–3 minutes at 37 °C, and was unaffected by heat activation, nutrient level or amount of nutrient germinant receptors. The mechanism of commitment (nutrient binding / triggering of the germinant receptor) is not known, but it seems to be identical to those influencing the germination rate for *Bacillus subtilis* and *Bacillus cereus* in general.

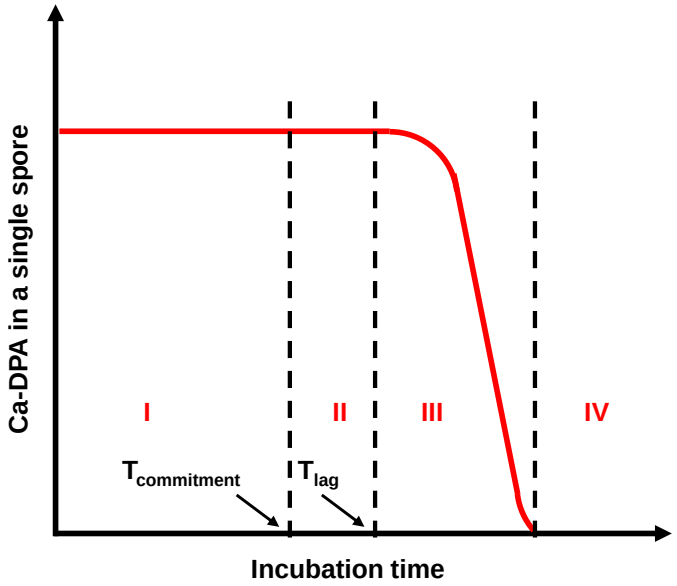

Figure 2. 8. Four stages of spore germination with nutrient receptors: I commitment to germination; II pre-Ca-DPA release; III Ca-DPA release; and IV post Ca-DPA release. Adapted from (Setlow et al., 2012).

The reasons for heterogeneity between individual spores in a population could be caused by: (i) differences in the environment to which populations' sporulating cells are exposed, or extrinsic factors; and (ii) stochastic effects, or intrinsic factors (Setlow et al., 2012). A remarkable impact on spore properties is made by sporulation conditions, including growth medium, temperature, pH, and solid or liquid sporulation media (Melly et al., 2002). Since the sporulation is not a synchronous process, even under defined conditions in a laboratory the early sporulating cells will alter the growth medium, hence causing changes in the environmental conditions for the later formed spores. This effect is by far more distinct for spores formed in nature, which will cause even higher heterogeneity (Setlow et al., 2012). The stochastic variations in the level of key spore proteins are the second reasonable explanation for heterogeneity. A number of studies indicate that stochastic variations in chemical reactions, and/or the amount of transcription and/or translation of key gene products, can play an important role in population heterogeneity of spores (de Jong et al., 2010; Setlow et al., 2012).

In conclusion, the ultimate goal for food processing would be to create a predictive model that relates the heterogeneity of spore properties in a spore population to its germination and/or inactivation behavior. Such a model would provide a mechanistic understanding and would be highly interesting for increasing food safety.

2.2 High isotactic pressure

Pressures currently used in the food industry range from tens of MPa in common homogenizers or supercritical fluid extractors, up to 350 or 800 MPa in ultra-high pressure homogenizers or high pressure (HP) pasteurization units, respectively. Laboratory-sized HP research units can reach up to 1400 MPa and temperatures up to 200 °C (Reineke et al., 2008). These HP units are principally used for the inactivation of vegetative microorganisms to extend the shelf life of the food treated. However, there are numerous other interesting food applications for HP, such as food structure engineering (Knorr, 2002; Diels & Michiels, 2006; Knorr et al., 2006; Sharma & Yadav, 2008), enhanced food quality (Ludikhuyze et al., 2002; Oey et al., 2008), stress response utilization (Kato & Hayashi, 1999; Ananta & Knorr, 2004; Pavlovic et al., 2005), and the control of bioconversion reactions (Knorr et al., 2006; Picard et al., 2006).

The majority of isostatic HP applications in the food industry involve the pasteurization of foods at ambient temperature. This principle was first reported by Hite (1899), who achieved an extended shelf life of bovine milk after HP treatment. Later, Bridgman (1914) reported that HP could coagulate egg albumin, producing gels different from those obtained by heat coagulation. In addition, he presented an extensive data set for the phase diagram of pure water under pressure (Bridgman, 1912). Since the early 1980s, growing consumer demand for minimally processed, fresh-like, safe, and high quality food products has triggered research efforts in the field of alternative food processing technologies (Hendrickx & Knorr, 2002).

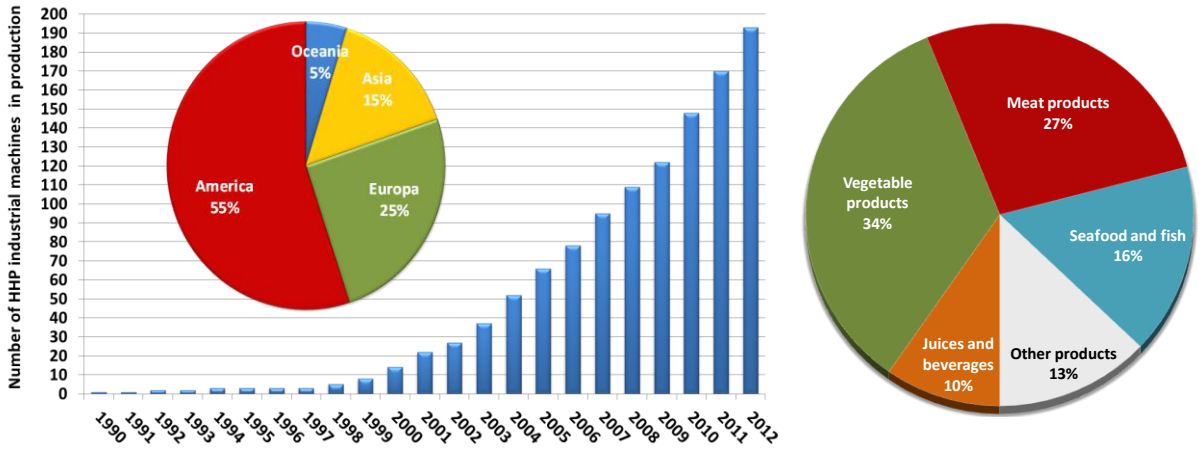

Figure 2. 9. Industrial scale high pressure machines used worldwide and total vessel volume used for different food products; not including 15 dismantled machines installed before 2003 (Tonello Samson, C. 2012, NC Hiperbaric, Spain, personal communication).

The first industrial HP application for the commercial preservation of food was installed in Japan in 1991 (Yaldagard et al., 2008). Due to further extensive research, HP pasteurization

technology was then extended to include a broad range of products, and the number of industrial HP systems for food pasteurization has steadily increased during the past 20 years.

At present, more than 193 industrial scale installations with a maximum volume of 687 L are in use worldwide, with an annual production of more than 300,000 t pasteurized products (Tonello Samson, C., 2012, NC Hyperbaric, Spain, personal communication).

The application of HP in combination with elevated processing temperatures has been evaluated as a promising food processing alternative to classical retorting technologies in several studies (Lau & Turek, 2007; Heinz & Knorr, 1998). High pressure thermal sterilization brings the benefits of an enhanced product quality and its application to packed food, and in addition to this has the huge advantage of the homogenous heating of the entire product during compression and the instantaneous cooling during decompression, due to the adiabatic heating, which will be discussed in chapter 2.2.1.

The combination of moderate pressures and ambient temperatures offers the possibility of a significant spore reduction. However, if a process has sterilization as its goal, very high pressures (> 500 MPa) have to be combined with elevated initial starting temperatures (> 80 °C). This will be discussed in chapter 2.2.7. In addition to the underlying inactivation mechanisms of bacterial endospores under pressure, matrix effects in the real food must also be taken into account. These pressure effects could be beneficial to spore inactivation if bactericide compounds are present in the food (Ratphitagsanti et al., 2010), or a low pH (Wuytack & Michiels, 2001) may offer protection against spore inactivation (Olivier et al., 2011).

2.2.1 Thermodynamics of high pressure: adiabatic heat of compression

A significant role is played by adiabatic heating during the inactivation of microorganisms under high –pressure, due to its thermodynamic effects. The adiabatic heat of compression which occurs during the pressure build-up can also maximize the benefits of high pressure technology (Toepfl et al., 2006).

During the compression, the zeros, first and second law of thermodynamics has to be taken into account in their functional relationship to pressure and temperature. The zeros law of thermodynamics states that if two systems are in an equilibrium with a third one, they are in equilibrium with each other. The first law of thermodynamics is the law of conservation of energy, where dU is the increase in internal energy of the system (Eqn. 1).

$$\boldsymbol{dU = dw + dq + \sum_{i=1}^{j} \mu_i dN_i} \quad \text{Eqn. 1}$$

The term dw here represents the amount of volumetric work done in the system, as occurring during compression during food pasteurization or sterilization. Dw denotes the amount of heat energy added to the system. The sum chemical potential, μ, and the number of particles, N, of type *i* is described by the last term of equation 1. The second law of thermodynamics (Eqn. 2) summarizes the tendency of intensive thermodynamic properties, such as temperature and pressure, to equalize as time goes by, or $dS \geq 0$, where S is the entropy of the system. The heat term is generally related to the entropy by:

$$\boldsymbol{dq = Tds} \quad \text{Eqn. 2}$$

Combining the first and the second law of thermodynamics (Eqns. 1 and 2), where the term –pdV represents the change of volumetric work dw, results in an equation suitable for describing variations of the internal energy, with changes in volume V and entropy:

$$dU = -pdV + TdS \quad \text{Eqn. 3}$$

During the pressure build-up, these fundamental thermodynamic relationships have to be taken into account, in terms of their functional relationship to pressure (p) and temperature (T). Through using the first and second law of thermodynamics, and by a re-arrangement of the Maxwell equations (Perry, 1984) (Eqn. 4):

$$\left(\frac{\partial T}{\partial p}\right)_S = \left(\frac{\partial V}{\partial S}\right)_P \quad \text{Eqn. 4}$$

the heating during the compression and the cooling during the decompression can be described as functions of thermo-physical properties of the compressible product.
Making use of the definition of the thermal expansion coefficient β as a function of temperature and the specific volume V:

$$\beta = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_P \quad \text{Eqn. 5}$$

the relation for enthalpy (H) of the second law of thermodynamics:

$$\left(\frac{\partial H}{\partial T}\right)_P = T \cdot \left(\frac{\partial S}{\partial T}\right)_P \quad \text{Eqn. 6}$$

and the definition of the specific heat c_p:

$$c_p = \left(\frac{\partial H}{\partial T}\right)_P \qquad \text{Eqn. 7}$$

equation 8 can be derived:

$$\left(\frac{\partial T}{\partial p}\right)_S = \frac{\beta}{\rho \cdot c_p} \cdot T \qquad \text{Eqn. 8}$$

Here β, the density ρ and c_p are pressure-temperature dependent. This equation describes the temperature increase upon compression in adiabatic isentropic situations. Hence, equation 8 states that all compressible materials change temperature during physical compression, depending on their compressibility and specific heat (Ting et al., 2002). Aqueous systems show a volume contraction of approximately 20 % when pressurized to 800 MPa (NIST). The specific work of compression can be expressed as:

$$\boldsymbol{W_{compr} = -\int V \cdot dp} \qquad \text{Eqn. 9}$$

If 1 kg of water is compressed up to 1400 MPa, W_{compr} is not higher than 124 kJ. This value has been obtained by integrating equation 9 under isothermal situations using the functional relation V = f(p) for pure water, provided by the NIST formulation (Ardia, 2004).
By using equation 8 the thermal profile during the compression phase can be calculated. The differences in adiabatic heating between several pressure transmitting media and sucrose solution are shown in Figure 2. 10.

Figure 2. 10

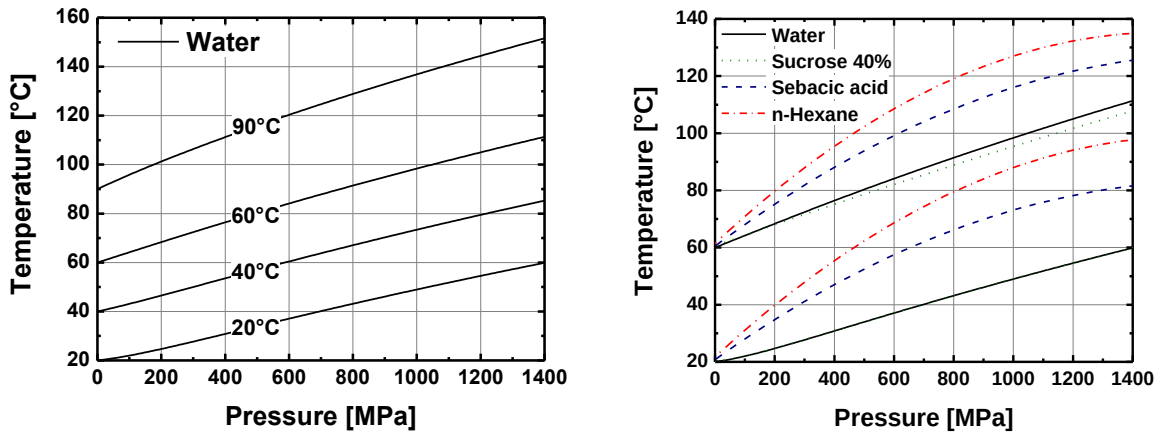

Figure 2. 10. Adiabatic heat of compression in water, sucrose solution with 40 % solid content, sebacate and n-hexane [from (Ardia, 2004)].

The adiabatic heat of the fluid food system sucrose is obtained through the help of formulas used to estimate the physical properties of a mixture of pure substances. The main ingredient in most food is water and thus the thermodynamic properties of water can be utilized to estimate the temperature increase upon compression of high moisture food (Ardia, 2004).

Table 2. 2. Overview of temperature increase during compression for some foods (Ting et al., 2002)

Substances at 25°C	**Temperature increase per 100 MPa [°C]**
Water	~3,0
Mashed potato	~3,0
Orange juice	~3,0
Tomato salsa	~3,0
2%-Fat milk	~3,0
Salmon	~3,2
Chicken fat	~4,5
Beef fat	~6,3
Olive oil	From 8,7 to <6,3[a]
Soy oil	From 9,1 to <6,2[a]

After compression, the product temperature increases to a value that depends on the geometry of the system and on the required time for pressure build-up. Immediately after the decompression the product returns to its initial temperature, or even to a lower value, as a consequence of thermal equilibration established during dwell time. This high cooling capacity is very valuable for the production of high quality foods and the preservation of food ingredients. During compression there are no shear forces inside the liquids and solids, and the transport of force is practically instantaneous. Consequently, each volume element of the product is characterized by the same pressure level, and, under adiabatic conditions, by the same temperature.

A temperature gradient occurs when there is a heat flux across the boundary of the system (Figure 2. 11). Under these conditions, the resulting transient temperature field in the product must be taken into account (Ardia et al., 2004b).

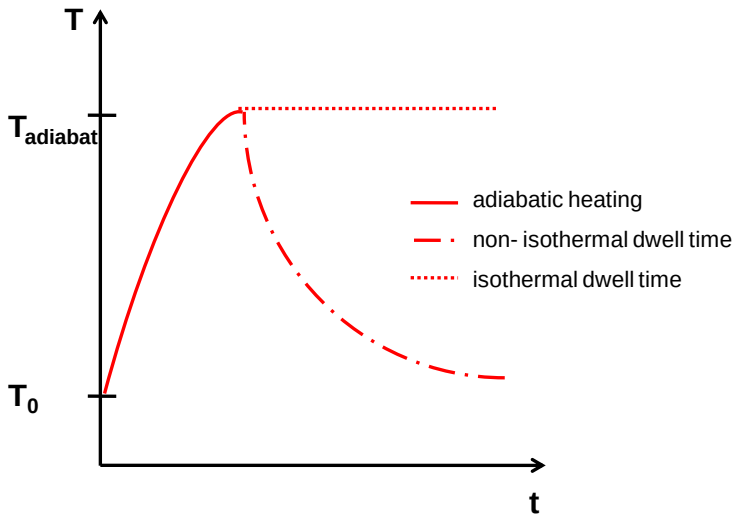

Figure 2. 11. Temperature profile in high pressure vessel under non-isothermal and isothermal conditions during dwell time.

Consequently, under non-isothermal conditions thermal equilibration occurs during dwell time and even during the pressure build-up, ultimately causing a non-uniform microbial inactivation during the pasteurization and sterilization processes. In this context, a higher compression rate results in a higher absolute value of temperature and a more homogenous temperature distribution inside the product, since less time is given to thermal equilibration. In each case, the product is cooled soon after compression, due to the thermal gradient between the product and the pressure vessel if the metal-made pressure vessel is not heated or if there is no insulating layer inside the pressure vessel (Ardia, 2004).

2.2.2 Temperature inhomogeneities

In contrast to conventional heat processing, where convective heating causes significant temperature gradients in the product, all volume elements in an HP vessel are subjected to the same pressure at the same time, due to the isostatic principle of pressure transmission (Rastogi et al., 2007; Heinz et al., 2009). Since pressure and temperature are closely connected physical parameters, the thermodynamic effect of the adiabatic heating, which occurs during the compression and decompression of the treated food and the pressure transmitting media, must be taken into account. This effect can also be utilized to rapidly achieve sterilization temperatures. Hence, unwanted over-processing, as occurs during thermal retorting, could be reduced thereby significantly improving the food quality.

However, temperature peaks do occur during high pressure processing, due to the high initial starting temperatures before pressure build-up and the adiabatic heat of compression during compression. These temperature peaks can vary significantly, based on the composition of the food treated (Table 2. 2), especially at higher temperatures. Hence, identical pressure profiles with the same initial starting temperature could by completely different in different matrices. The impact of such non-isothermal temperature profiles on the inactivation rate of *Bacillus amyloliquefaciens*, in comparison to isothermal pressure dwell times, was investigated by Rajan et al. (2006). They found differences of more than 1.5 $\log_{10}$ inactivation after 7 min holding time at 105 °C. To avoid such temperature peaks, especially in basic research related to the inactivation kinetics of vegetative microorganisms, isothermal treatment conditions during pressure dwell time are essential for bacterial spores and enzymes, in order to be able to compare data derived in different high pressure systems. Such a system, allowing a nearly adiabatic compression and decompression as well as isothermal dwell time, was described by Reineke et al. (2008) for a micro high pressure system. This equipment could reach isothermal isobaric conditions in the range of 50-130 °C and up to 1.4 GPa within a few seconds (Figure 2. 12).

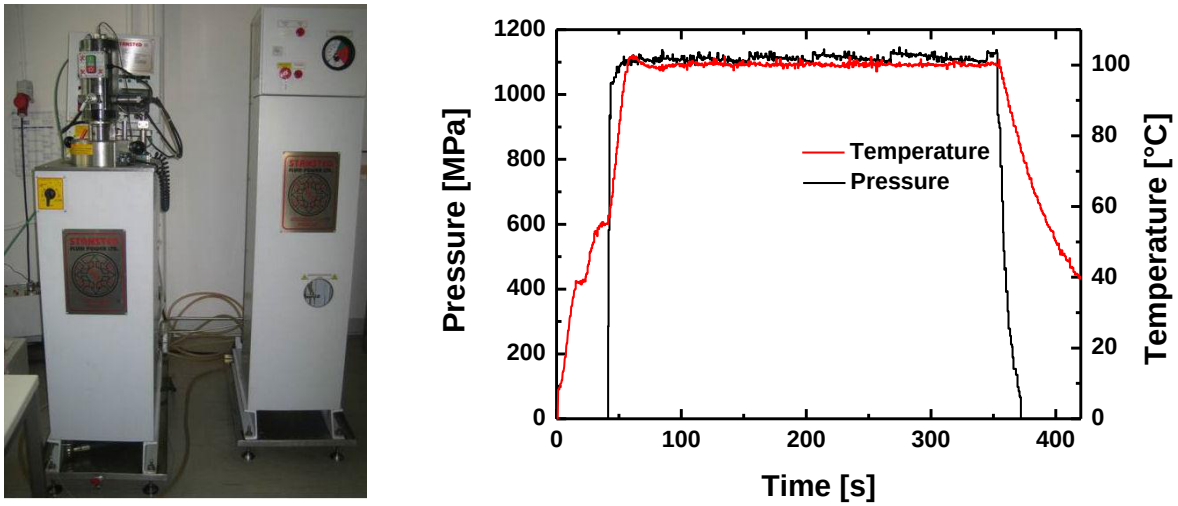

Figure 2. 12. Stansted Mini Foodlab FBG5620 high pressure unit with pressure temperature profile measured inside the pressure vessel. Adapted from Mathys (2008a).

At an industrial scale installation, however achieving such a homogenous temperature during the pressure dwell time is nearly impossible, but could be influenced by the vessel design. Ardia et al. (2004b) modelled the temperature inhomogeneities in a PET bottle with a diameter of 80 mm, a length of 100 mm and a wall thickness of 0.5 mm. They coupled the model with the inactivation kinetics of *Alicyclobacillus acidoterrestris* spores in orange juice, and compared the inactivation data from the vessel wall with the inactivation achieved in the centre of the bottle. Because of the heat flux to the “cold” metallic pressure vessel wall, the

resulting temperature inhomogeneities led to a difference of approximately 6 $\log_{10}$. Later, several groups calculated the temperature field in pressure vessels using “Computational Fluid Dynamics” (CFD) (Hartmann, 2002; Knoerzer et al., 2007; Juliano et al., 2009). For example, Juliano et al. (2009) modelled the temperature field for a vertical 37 L vessel and coupled the data with the inactivation kinetics for *Clostridium botulinum*. The model further included the temperature at the vessel wall, the closures, the metal valve at the bottom, the carrier wall temperature, and the temperature inside model food packs. Within this study, it was shown that the addition of packs significantly reduced the temperature difference between the bottom and top of the high pressure vessel to 3.5 K, and confirmed the insulating effect of PTFE carriers. The temperature inside the packages was relatively stable and varied between 119.2 and 121.6 °C for a pressure cycle of 600 MPa for 220 s and an initial product temperature of 90 °C. There were variations in the tested models, which were used to predict the inactivation of *Clostridium botulinum.* The log-linear model predicted a 17 $\log_{10}$ reduction for the model temperature profile inside a package (which is fare beyond the maximum packaging density of 10^{12} spore mL^{-1} that can occur), whereas the Weibull-model predicted only a 9 $\log_{10}$ inactivation. However, the variation in inactivation for all tested models inside the package was only ~0.5 $\log_{10}$ (Juliano et al., 2009).

In addition to CFD modelling to estimate the temperature fields during high pressure processing, a second approach is the direct measurement inside the vessel. As data transmission from a temperature probe through the pressure vessel is quite difficult, the application of protein-based indicator systems has been tested. Grauwet et al. (2010a), for example, used solvent engineering to modify the thermal resistance of the enzyme ovomucoid to a process window suitable for a high pressure thermal sterilization and α-amylase for a high pressure pasteurization process (Grauwet et al., 2009). Furthermore, they prooved the possibility of using such enzyme-based pressure-temperature-time integrators (pTTis) to map temperature inhomogeneities for high pressure pasteurisation conditions in industrial scale high pressure equipment (55 L vessel volume, horizontal vessel) (Grauwet et al., 2010b). The disadvantage of such systems, however, is that each batch of enzyme has to be calibrated and checked for its possible use as a pTTi. Consequently, there is a need for a reliable, robust and rapid online method to track the temperature during processing. The high pressure equipment manufacture, NC Hiperbaric, have therefore developed a wireless K-type thermocouple, which measures the temperature every 3 s. The results from this measurement have confirmed that along the vertical axis the temperature distribution is relatively homogenous (± 4 °C), whereas great variations were measured in the horizontal axis (up to ± 12 °C), for an initial starting temperature of 90 °C (Tonello Samson, C., 2011, NC Hyperbaric, Spain, personal communication).

Such variations in the processing temperature could have a significant effect in regard to spore inactivation and successful sterilization of the treated food.

2.2.3 High pressure thermal sterilization

Ambient preheating to 80-90 °C, combined with the compression heating during pressure build-up, allows the HP treated food to reach sterilization conditions within a short time. During the dwell time, the denaturation of proteins (e.g. enzymes) and a significant reduction of microorganisms were observed, without affecting molecular bonds and inhibiting some chemical reactions. For example, Maillard reactions, which can produce off-flavors or the destruction of vitamins, can be reduced under HP conditions (Heremans, 2002; Indrawati et al., 2005; Jaeger et al., 2009).

In addition to giving an enhanced product quality and functionality, the HP sterilization process also needs to ensure microbial safety. Currently, the commercial sterility of low-acid (pH > 4.5) canned foods is often achieved by conventional heat processing, in order to give a non-refrigerated, shelf-stable product. Sterilization of food products includes the inactivation of yeasts, fungi, viruses, vegetative microbial cells, and spores. Bacterial endospores require special attention, because of their high resistance to wet and dry heat, freezing and thawing, chemical agents, disinfectants, irradiation, and pressure (Setlow, 2003). Based on extensive research in recent decades, a pressure-assisted thermal sterilization (PATS) process was established and certified by the Food and Drug Administration (FDA) of the United States in February 2009. This process involves thermal sterilization temperatures being reached during pressure dwell time. In addition, a high pressure thermal sterilization (HPTS) process was proposed, which takes into account the impact of pressure on spore inactivation.

A major concern hampering the industrialization of this technology, however, is that the exact inactivation mechanisms of bacterial spores by HP are unknown. In this regard a lot of kinetic data have been published concerning the inactivation of bacterial endospores under pressure. Only a few authors, however, have coupled this data to industrial-scale pressure–temperature profiles (Mathys, 2008a; Juliano et al., 2009), which is essential for upscaling the process. Currently, industrial HPTS equipment tends to have volumes up to 150 L, a maximum working pressure of 700 MPa, and initial temperatures of up to 95 °C (Heinz 2010, German Institute of Food Technology, personal communication). The industrial relevance of the study of Mathys (2008a) was exemplified by implementing the data generated for a *Geobacillus stearothermophilus* spore strain with certified thermal resistance into the process charts of an industrial scale HPTS unit (Figure 2. 13). One of the few existing pilot systems is the Flow Pressure Systems QUINTUS (Food Press Type; 35 L, 600 MPa) sterilization machine (Avure Technologies, Kent, WA, USA), which is described elsewhere in the

literature (Knoerzer et al., 2007). In Figure 2. 13 a and Figure 2. 13 b (location two is near top closure; (Knoerzer et al., 2007), two different pressure and temperature profiles are shown from an HPTS process with (10 L) and without (35 L) a polytetrafluoroethylene (PTFE) carrier. Temperature decrease and inhomogeneities during the dwell time can be reduced when an insulating carrier is used (Juliano et al., 2009). Alternative possibilities for ensuring a homogenous temperature during dwell time would be the application of an internal heater, or heating the vessel wall to an appropriate temperature level that minimizes the temperature gradient between pressure vessel and treated product (Ardia, 2004).

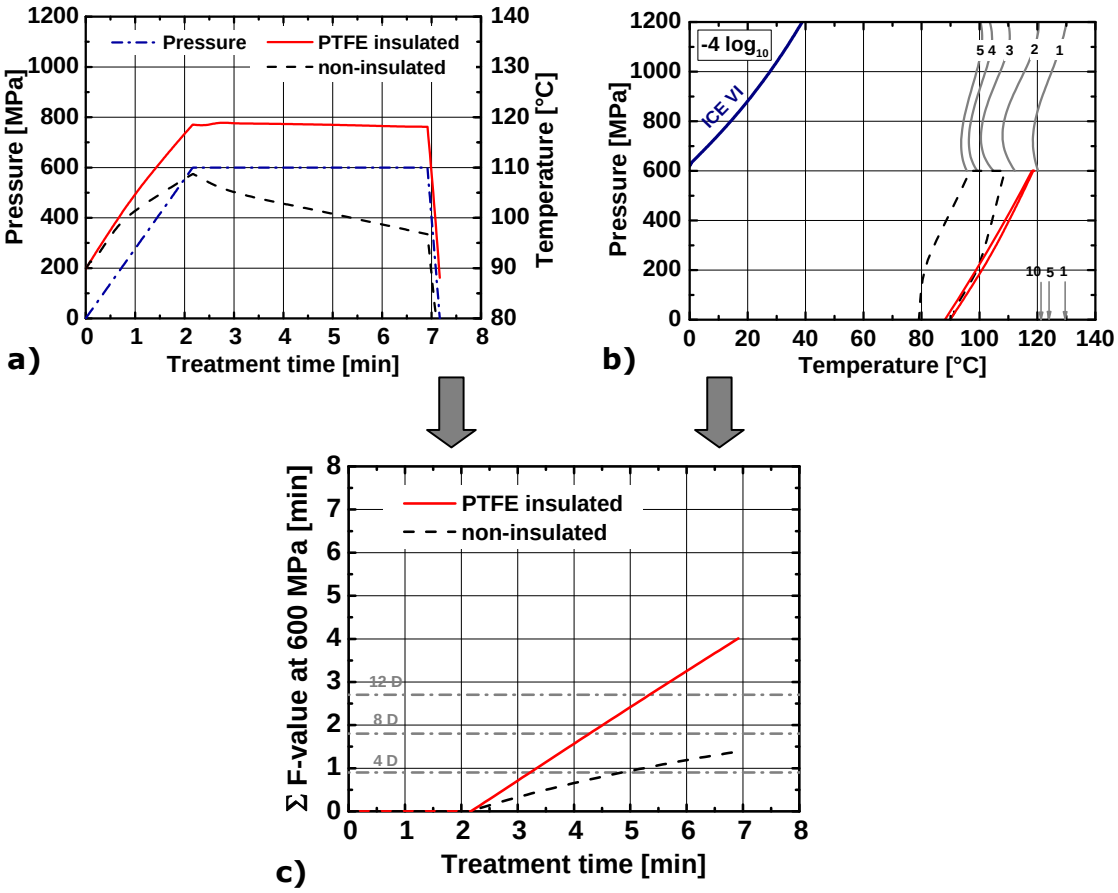

Figure 2. 13. Industrial process analysis of a vertical 35 L vessel (- -, black) and with a 10 L insulated carrier (–,red), using an F-value (c), T_{ref} = 121.1 °C, z_{600MPa} = 35.36 °C, $D_{121.1\,°C}$ = 13.52 s at 600 MPa in 0.05 M ACES buffer (pH 7), and pressure holding times $T(t)_p$. Pressure and temperature profiles (a) were taken from the literature (Knoerzer et al., 2007). The desired inactivation level of (b) can be varied by multiplying the dwell times of the isorate lines with the same value (Mathys et al., 2009).

In Figure 2. 13 b the target inactivation level of – 4 $\log_{10}$ can be varied because of a first-order kinetic approach. Process conditions were as follows: 90 °C initial temperature, 600 MPa final process pressure, and 285 s dwell time, with water as the pressure-transmitting medium (Knoerzer et al., 2007). Using a modified $F_{121.1\,°C}$-value concept for the thermal inactivation of *Geobacillus stearothermophilus* spores (Eqn. 10) in Figure 2. 13 c,

$$F = \int_0^t 10^{\frac{T(t)_p - T_{ref}}{z_{600MPa}}} dt = D_{ref} log_{10}\left(\frac{N}{N_0}\right) \quad \text{Eqn. 10}$$

where reference temperature T_{ref} is 121.1 °C, $T(t)_p$ is the dwell time, $z_{600\ MPa}$ = 35.36 °C, $D_{121.1\ °C}$ = 13.52 s at 600 MPa, N_0 is the initial count, and N is the survival count. Both processes, with or without an insulating carrier, can be adequately analyzed and compared. In thermal processes the F-value is the accumulated lethality, expressed as an equivalent time at a specific reference temperature (T_{ref}) with a specific z-value. The F_0-concept is accepted for the thermal sterilization in the food industry for the case where T_{ref} is 121.1 °C, the $D_{121.1\ °C}$-value is 13.8 s (for *Clostridium botulinum*), and the z-value is approximately 10 °C (Stone et al., 2009). In contrast to conventional thermal processes at ambient pressures with an expected $z_{0.1MPa}$ = 10 °C for spore inactivation, the z-value at 600 MPa (z_{600MPa} = 35.36 °C for *Geobacillus stearothermophilus*) was higher (Mathys et al., 2009). This results in a lower temperature dependence of the inactivation reaction rate. Because of the pressure dependence of the z-value, the preheating as well as compression and decompression phases could not be included in the calculation of the spore inactivation level of *Geobacillus stearothermophilus*. However, the additional processing time can be considered a safety factor. Using a PTFE carrier, commercial sterility (> 12 D) could be achieved within 3 min dwell time at 600 MPa and 90 °C initial temperature (Mathys, 2008a). An alternative process for a HPTS unit with a 55 L horizontal vessel, 700 MPa working pressure, and 95 °C initial temperature was also calculated by Mathys et al. (2008a). He concluded that more than 12 log_{10} inactivation of *Geobacillus stearothermophilus* spores can be achieved in this machine without any insulation within 3 min at 700 MPa.

2.2.4 Pressure equipment for high pressure thermal sterilization

In order to inactivate bacterial and fungal spores, a combination of HP and elevated initial temperature (> 80 °C) seems to be a promising improvement to traditional heat sterilization. For food processing, two different concepts of high pressure units have been developed: batch units with an external pump and intensifier (Figure 2.13 a), and high pressure units with an internal intensifier (Figure 2.13 b) (Rovere, 2002).

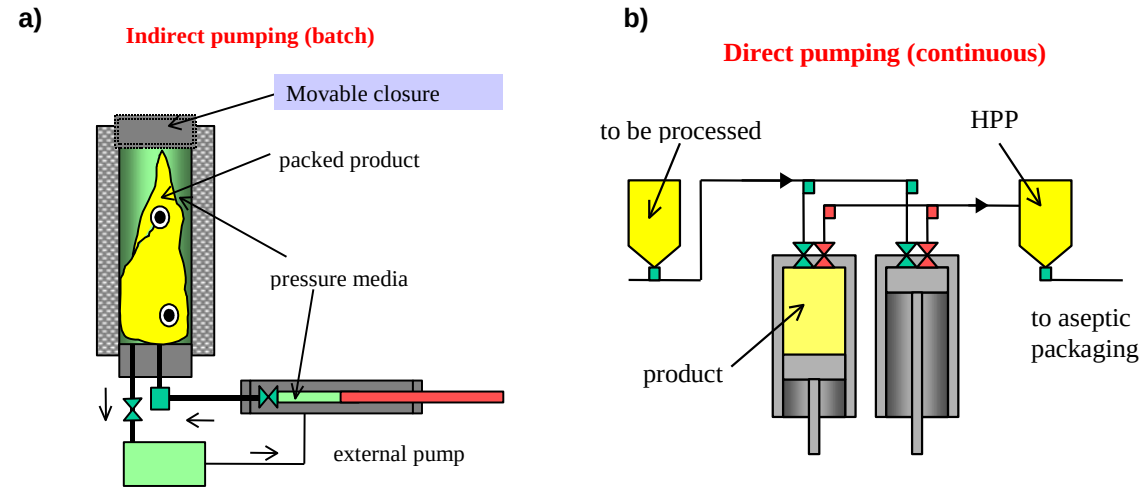

Figure 2. 14. The two different concepts of high pressure units: a) indirect (batch) and b) direct (continuous) pumping (Rovere, 2002).

Both concepts include an intensifier, which basically consists of a low-pressure and a high-pressure cylinder (Figure 2. 15). The simplest practical system is a single-acting hydraulically driven pump. To reach a quasi-continuous product flow, a double-acting arrangement can be used. While one high-pressure vessel is charged, the other can be processed.

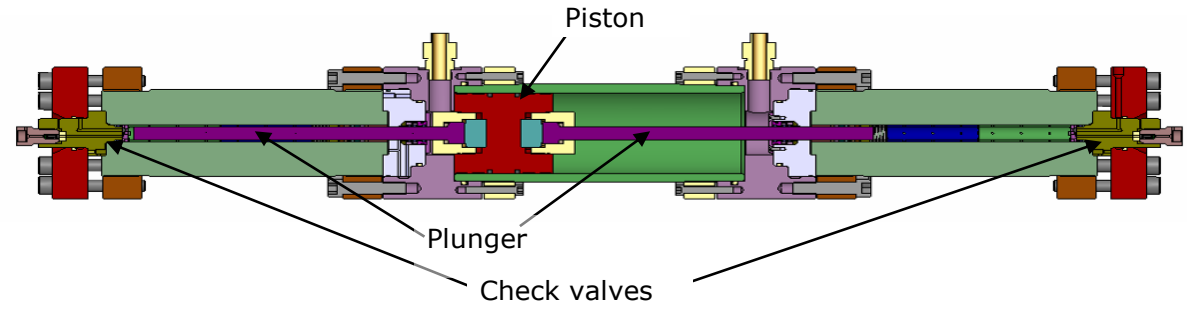

Figure 2. 15. Double-acting intensifier (Hernando, A., 2007, NC Hyperbaric, Spain, personal communication).

Both high-pressure concepts (batch and continuous) are suitable for possible high-pressure thermal sterilization. Batch units have an advantage because they can process all kinds of packed foods. Packing is necessary to avoid direct contact between the pressure-transmitting media (often water or mixture of water and oil) and the processed food. The disadvantage of a batch unit is that the inflow of cold pressure media causes a temperature increase in homogeneities during pressurization (Figure 2. 14 a).

In a continuous high pressure unit packing is not necessary because the high pressure vessel is sealed, such that no contact between the processed food and the pressure-transmitting media is possible (Figure 2. 13 b). A continuous unit is limited, however, by the

size of particulates, and it is mostly used to process fluids. For industrial use (pasteurization processes), batch units with a maximum pressure of 800 MPa (35 L) and vessel volumes up to 420 L and 600 MPa are available (Figure 2. 16 a). To reach higher pressure values or shorter pressurization times, continuous units with a direct pump are preferred (Hendrickx & Knorr, 2002).

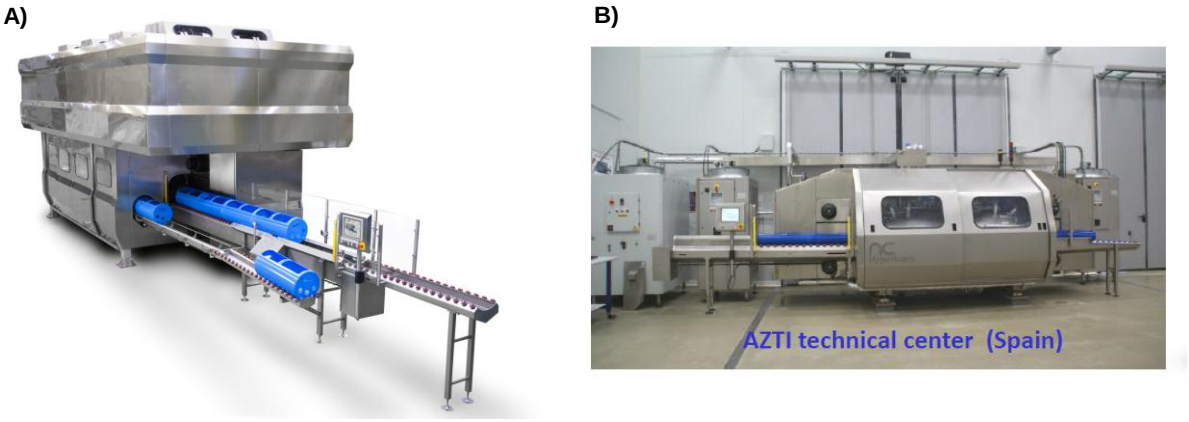

Figure 2. 16. NC Hiperbaric Wave 420 high pressure pasteurization equipment (420 L vessel volume) (A); and NC Hiperparich 55HT high pressure equipment (55 L vessel volume, maximum pressure 630 MPa and maximal achievable temperature under pressure 121 °C) (B) (Tonello Samson, C., 2012, NC Hyperbaric, Spain, personal communication).

The Hiperbaric/55HT high pressure sterilization unit (Figure 2. 16 B), developed in 2009, is the most advanced piece of equipment able to combine pressure (630 MPa working pressure) and temperature (5-90 ºC).

However, especially under the conditions needed to achieve “commercial” sterility, unwanted chemical and physical changes of the high pressure-treated food could occur and must therefore be taken into account.

2.2.5 Effects on water and the dissociation equilibrium

Water is essential to all known forms of life and it represents the major component of most food systems. In industrial scale high pressure equipment, water is used as the pressure transmitting media. The first who presented data on the behavior of water under pressure was Bridgeman (1912), who determined the phase diagram of water as a function of temperature and pressure. The phase transition lines of water and its different ice modifications, according to Bridgeman, are shown in Figure 2. 17. At present, 16 different ice crystal and 3 amorphous structures are known (Zheligovskaya & Malenkov, 2006). At the transition from the liquid to the solid state, ice I represents a specialty since it is the only ice modification to show a positive volume change, ΔV.

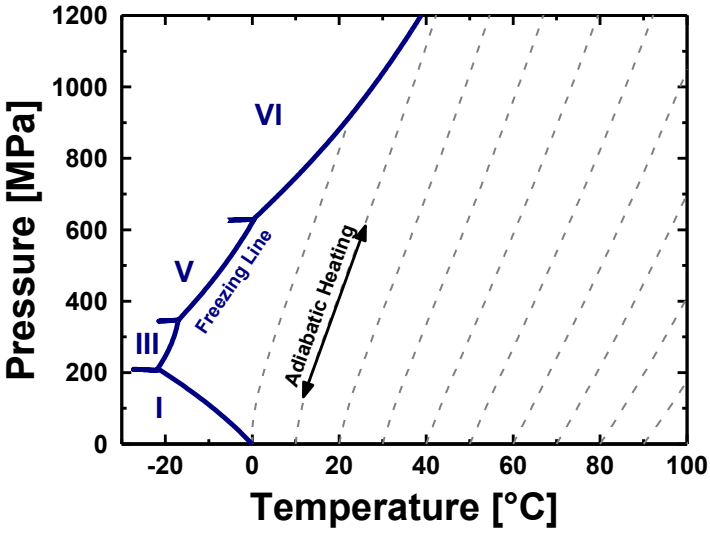

Figure 2. 17. Phase diagram of water with different ice modifications and lines (--) for the adiabatic heat of compression for water in the pressure-temperature landscape (Bridgman, 1912).

Based on data from the “International Association for the Properties of Water and Steam” and the “National Institute of Standards and Technology”, Mathys et al. (2009) calculated some thermodynamic properties of water and illustrated them in a pressure–temperature landscape.

One of the most relevant changes of aqueous systems under pressure is the shift of the dissociation equilibrium, both of water and of simple chemical systems like buffer solutions. The thermodynamic background of a typical dissociation reaction in diluted solutions is described in Eqn. 11,

$$HA + H_2O \leftrightarrow A^- + H_3O^+ \qquad \text{Eqn. 11}$$

where HA is an acid, H_2O is water, A^- is the conjugated base, and H_3O^+ is oxonium ion. The concentration of water remains essentially unchanged throughout dissociation of a base or acid, because it is very large (55.5 M) in relation to the other concentrations, and can therefore be omitted in the law of mass action. Thus the dissociation equilibrium constant K_a for the dissociation of an acid can be calculated with,

$$K_a = \frac{\gamma_{H_3O^+} \cdot \gamma_{A^-}}{\gamma_{HA}} \cdot \frac{[A^-] \cdot [H_3O^+]}{[HA]} \qquad \text{Eqn. 12}$$

with the activity coefficients γ_i. The dissociation equilibrium constant, K_a, indicates the extent of dissociation of oxonium ions from an acid. Because this constant differs for each acid and varies over many degrees of magnitude, the acidity constant is often represented by the additive inverse of its common logarithm (Degner, 2009), represented by the symbol pK_a (Eqn. 13).

$$pH = -log_{10}(pK_a)$$ Eqn. 13

The activity of hydroxonium is a key factor in describing the thermodynamic and kinetic properties of processes occurring in aqueous solutions, and can also vary over a wide range. Consequently it is common to express the activity of hydroxonium in a logarithmic form as pH (Eqn. 14),

$$pH = -log_{10}\left(\gamma_{H_3O^+} \cdot \frac{[H_3O^+]}{1\ mol\ L^{-1}}\right)$$ Eqn. 14

where 1 mol L^{-1} makes the parameter dimensionless (Degner, 2009). During the shift of dissociation equilibria the pH value would be changed with all reaction partners (Eqn. 11), but no change of the concentration difference on one site occurs during the reaction. For example, in water at 0.1 MPa and 20 °C with nearly equal hydroxonium ($[H^+]$) and hydroxide ($[OH^-]$) concentrations, there would be neutral conditions ($[H^+] = [OH^-]$). Under pressure there are increased hydroxonium and hydroxide concentrations, but still "neutral" conditions. Consequently, the pH shift alone cannot exactly describe the dissociation equilibrium shift, and thus the pK_a shift should be used (Mathys, 2008a).

The dissociation equilibrium varies with pressure and temperature, which can strongly influence thermal process, such as pasteurization, sterilization or freezing. Moreover, for basic inactivation studies of microorganisms, buffer solutions are commonly used to achieve constant pH-values and medium properties. In view of the dissociation equilibrium shift during heating, a biased error in the results of inactivation experiments occurs, leading to incomparable results if they are not designed correctly. Several authors have shown that the thermal inactivation of bacterial spores (Sognefest et al., 1948; Loewick & Anema, 1972; Mathys, 2008b), vegetative microorganisms (Deng et al., 1998) or protein and enzyme denaturation (de Wit, 2009) strongly depends on the pH of the suspending media. According to Goldberg et al. (2002) the known temperature dependence of the acid dissociation constant, $K_a(T)$, for different buffer systems could be interpolated up to 60 °C. However, for more complex systems like real food, especially at the high processing temperatures found in the retorting process, the pH value cannot be calculated and instead has to be measured. The pH value plays an important role in determining the amount of heat required to ensure a safe product during industrial food processing.

The effect of a varying dissociation equilibrium is also important for high pressure-treated food, because the pH value, pressure and temperature can act synergistically on the inactivation of microorganisms. However, for basic kinetic studies like the inactivation of bacterial endospores, constant conditions and medium properties are essential, and pressure-dependent changes like of the pK_a value could influence them (Mathys, 2008b).

To measure the variations of the pK_a value under pressure Distèche (1959) developed one of the first pressure-resistant pH-glass electrodes, to measure the pH-value of sea water in the Marianna Trench at 110 MPa. At present, the dissociation equilibrium shift under pressure cannot be measured in solid food, but a few optical measurement methods have been developed for in situ pH measurement of liquids. They can operate in pressure treatments up to 250 MPa (Hayert et al., 1999; Quinlan & Reinhart, 2005) and 450 MPa (Stippl, Delgado & Becker, 2002; 2004). However, these experimental methods are still limited and not suitable for the investigation of combined thermal and pressure effects, but the dissociation equilibrium shift under pressure should not be neglected. This was confirmed by Clousten and Wills (1969), who found different logarithmic reduction of *Bacillus licheniformis* spores in water and phosphate buffer after low pressure inactivation up to 170 MPa.
Hence, if the shift of dissociation equilibrium during heating and/or compression is not taken into account, the results of inactivation experiments are prone to error if they are not designed correctly.

2.2.6 Impact of high pressure on food constitutes

The primary structure of low molecular weight molecules like peptides, lipids, vitamins and saccharides is rarely affected by isostatic high pressure because of the very low compressibility of covalent bonds at pressures below 2 GPa (Gross & Jaenicke, 1994; Cheftel & Culioli, 1997; Van den Broeck et al., 1998; Oey et al., 2008). Conversely, the native structure of macromolecules such as starch can be changed by high pressure, and thermally-induced structural changes can also occur. Hence, a potential use of high pressure in food structure engineering implies diverse advantages because high pressure induces changes to the state of the food ingredients, when applied at moderate temperatures.
As for inorganic systems, such as water or air, the state and stability of biomaterials is regulated by an interaction between internal and external parameters. Merely by changing a single parameter (e.g. pressure or temperature) in one direction, the whole system disequilibrates and could change its phase or state. Pressure, as a basic thermodynamic parameter, affects biomolecules and can change the state of molecular organization (e.g. crystallization of fats or phase changes of phospholipids) (Macdonald, 1992). In food systems such changes can either be positive (e.g. inactivation of microorganisms or gel formation) or negative (e.g. texture defects). These HP effects are governed by three general principles, which are outlined below.

(I) The Principle of Le Chatelier and Braun states that if a chemical system at equilibrium experiences a change, the equilibrium will shift in order to minimize that change. If this

change is an increase of pressure, any phenomenon that is accompanied by a decrease in reaction volume (e.g. chemical reactions, phase transitions or changes in the molecular configuration) is enhanced, and *vice versa* (Cheftel, 1995). Hence, high pressure will shift the equilibrium towards the system with the lowest volume. Moreover, when the activation volume (ΔV*) of an activated complex is smaller than that of the initial reactants, a pressure increase and a temperature decrease will increase the reaction rate constant $k_{kinetic}$ and therefore also the reaction rate, according to Eqn. 15:

$$lnK_{kinetic} = lnk_0 - \Delta V^* \frac{p}{RT} \qquad \text{Eqn. 15}$$

where *p* is pressure, *T* is temperature, and *R* is the molar gas constant. However, an increase in temperature also increases the rate of reaction according to the Arrhenius´law (Cheftel, 1995).

(II) The isostatic principle implies that pressure is instantaneous and uniform, and is transmitted independently of the geometry and size throughout the whole biological sample (Balny & Masson, 1993).

(III) The microscopic ordering principle means that at a constant temperature, an increase of pressure increases the degree of the molecular ordering of a substance (Urrutia Benet, 2005).

The main reason for the dynamic behavior of biomolecules under pressure is the free volume of the system, which is expected to decrease with increasing pressure (Heremans, 2002). The equation for the Gibbs free energy (Eqn. 16) expresses the transition for a general equilibrium process from state A to state B:

$$\Delta G = -RT \cdot lnK = \Delta H - T \cdot \Delta S \qquad \text{Eqn. 16}$$

with *H* as enthalpy and *S* as entropy.

This model can be seen as a general approach, where state A and state B could denote molecular or physicochemical states. In the case of protein denaturation, state A may represent the native and B the denatured state, respectively. The transition line would then run along the equilibrium, ΔG=0, in an elliptical shape at various pressure and temperature conditions (Figure 2. 18) (Heremans, 2002).

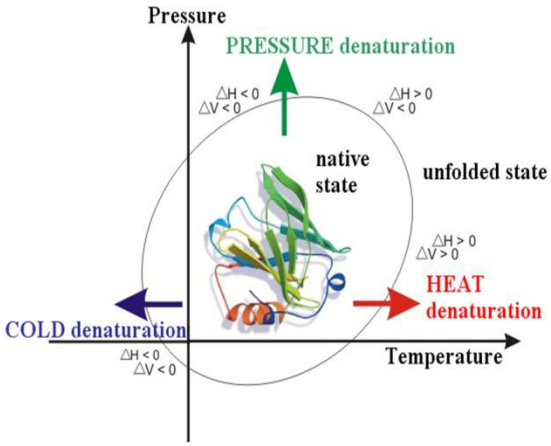

Figure 2. 18. Relation between cold, pressure and heat denaturation of proteins (Smeller, 2002) and changes in enthalpy and volume, redrawn from Heremans (2002).

High pressure can therefore induce reversible or irreversible changes in the native structure of proteins, which are similar to changes occurring at heat and in the presence of chemicals, but the residual molecular structure after a pressure treatment can vary significantly. In this regard, Knorr et al. (2006) published a detailed review of pressure-induced changes in the structure of proteins, and Jaeger et al. (2012) reviewed the impact of these on food structure. An overview of the pressure effects on proteins is given in Table 2. 3.

Besides the effect on proteins, high pressure could also significantly influence the gelatinization of carbohydrates such as starch. The gelatinization induced by pressure differs from that which is thermally induced (Stute et al., 1996). High pressure-induced gelatinization is characterized by a limited swelling of the starch granules, which is accompanied by a retaining of its structure and a loss of birefringence under polarized light (Buckow et al., 2007). The extent of starch gelatinization is dependent on the applied pressure, the pressure dwell time, and the treatment temperature (Rumpold, 2005), although the type of starch has the highest impact on its gelatinization behavior.

Table 2. 3. Impact of high pressure on the structure and properties of proteins (Masson, 1992; Heremans, 2002; Kessler, 2002b; Balny, 2004).

Pressure impact on the protein structure	*Quaternary structure:* • Is stabilized by noncovalent hydrophobic interactions, which are sensitive to high pressure • p < 50–200 MPa favor the dissociation of oligomeric proteins • Dissociation can be followed by subunit aggregation or by precipitation
	Tertiary structure: • Significant changes p > 200 MPa • Complex denaturation affects • Sometimes intermediate states (e.g. molten globule state), leading to multiple denatured forms
	Secondary structure: • Significant changes p > 300-700 MPa • Irreversible denaturation • Effect depends on the applied pressure and on the extent of the secondary structure rearrangements
	Primary structure: • ΔV for exchanges in covalent bonds are nearly zero • Covalent bonds are pressure insensitive up to p > 1000–1500 MPa
Pressure impact on the properties of proteins	• Denaturation, depending on pressure and nature of protein • Smaller than 150 MPa, often reversible • Pressure may produce crystallization • Gel formation • Gelling temperature of gelatin raised • Improved digestibility

The pressure treatment of lipids is often linked to crystallization effects. Differing from thermal treatments, high pressure treatment allows crystallization of the inner phase of o/w emulsions independently from the fat globule size. In addition, shorter treatment times are required and crystallization can be induced at temperatures that would not allow a phase change at atmospheric pressure (Buchheim & Abou El-Nour, 1992).

A much greater impact of the phase transition of lipids, however, is that on biological membranes (Kato & Hayashi, 1999). Besides the inactivation of enzymes, membrane damages are considered as one of the key events related to microbial death.

2.2.7 Germination and inactivation mechanisms of endospores under pressure

In 1970 Gould was the first to report that endospores could be triggered to germinate by a high pressure treatment. A few years later Wilson (1974) found a synergistic effect of high pressures and high temperatures on the inactivation rate of bacterial endospores, which was later confirmed by Mallidis and Drizou (1991), and Seyderhelm and Knorr (1992) for *Bacillus stearothermophilus* spores. Since then many authors have confirmed this finding for different types of spore-forming bacteria (Margosch et al., 2006; Rajan et al., 2006; Bull et al., 2009; Mathys et al., 2009; Olivier et al., 2011; Reineke et al., 2012). A detailed overview of literature on high pressure-treated spore strains, applied process conditions and spore inactivation levels achieved, until 2008, was given by Heinz and Knorr (2002) and Mathys (2008a), which was updated until September 2012 and can be found in the annex.
Further, Heinz and Knorr (2002) suggested a system to compare the effect of high pressure on the heat resistance of endospores that is shown in Figure 2. 19.

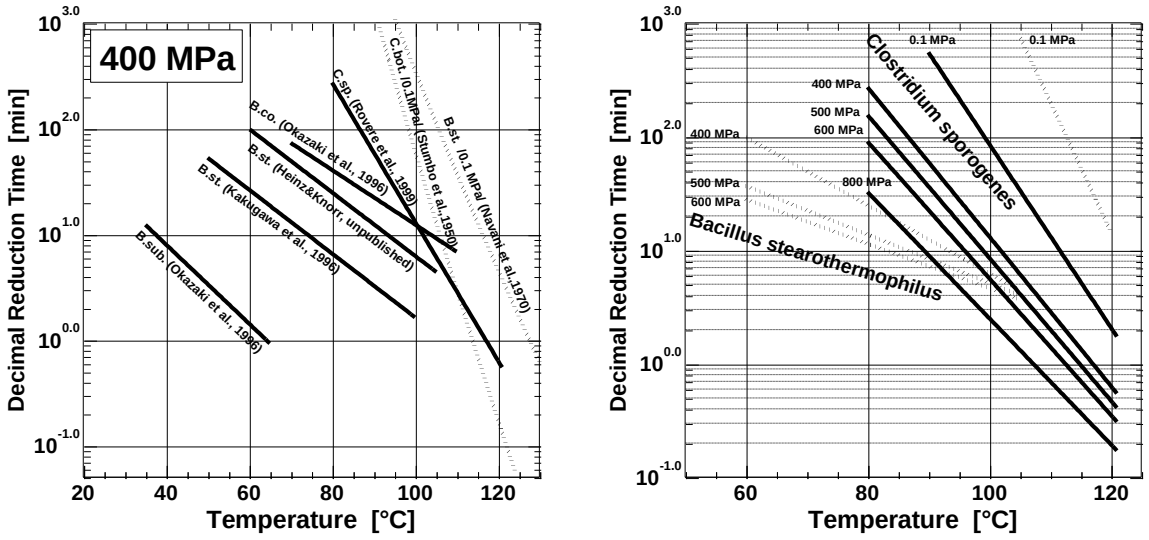

Figure 2. 19. a) Thermal resistances of endospores at 400 MPa [B.sub. (*Bacillus subtilis*); B.st (*Bacillus stearothermophilus*); B.co. (*Bacillus coagulans*); C.sp. (*Clostridium sporogenes*)] compared to ambient pressure [B.st (*Bacillus stearothermophilus*); C.bot (*Clostridium botulinum*): dotted lines] and b) thermal resistances of *Clostridium sporogenes* in beef broth (Rovere et al., 1999) and *Bacillus stearothermophilus* in mashed broccoli in dependence of the applied pressure (Heinz & Knorr, 2002).

In contrast to the often reported synergistic effect of pressure and temperature, certain spore strains, such as *Bacillus subtilis* or *Geobacillus stearothermophilus*, are actually more resistant under certain pressure–temperature combinations (Wuytack et al., 1998; Margosch et al., 2006), which includes high variations in the z-value under pressure (Zhu et al., 2008; Mathys et al., 2009; Ramaswamy et al., 2010). For other spore-formers, such as the proteolytic Type B *Clostridium botulinum* TMW 2.357, an inactivation behavior nearly

independent of pressure has been reported for pressures below 1.2 GPa (Margosch et al., 2006). Furthermore, the resistance against pressure and heat of spore strains like *Bacillus coagulans* also varies with the pH of the suspension media or the food matrix used (Olivier et al., 2011; Vercammen et al., 2012). A significant spore inactivation could also be achieved through the combination of high pressure and ambient temperature (Sale et al., 1970; Wuytack et al., 1998; Reineke et al., 2012), but this process is insufficient for food sterilization, due to pressure-resistant spore populations that cannot be germinated by pressure.

It has been well established that spore inactivation at high pressures and at least moderate treatment temperatures (T < 60°C) proceeds as a minimum two-step process. In this the endospores are first induced to germinate by high pressure, and the germinated spores are subsequently killed by the combination of pressure and heat, due to their increased overall stress sensitivity (Sale et al., 1970; Wuytack et al., 1998; Paidhungat et al., 2002; Reineke et al., 2011b; Reineke et al., 2012). Whereas germination can by strongly accelerated with increasing the treatment temperature (Gould, 1970; Reineke et al., 2012), a temperature decrease to room temperature strongly retards or even inhibits the germination progress (Sale et al., 1970; Nakayama et al., 1996). In addition to the treatment temperature, the applied pressure level has a significant impact on the germination pathway of at least all *Bacillus* and *Geobacillus* spore strains (Paredes-Sabja et al., 2011), which is shown for *Bacillus subtilis* in Figure 2. 20. At pressures between 150 MPa and 400 MPa germination is induced without the presence of nutrients (Gould, 1970) by triggering the spores´ nutrient receptors (Setlow, 2003). The activation of the nutrient receptors causes opening of the spores´ dipicolinic acid channels and the release of Ca-DPA. The release of Ca-DPA is accompanied by activation of the spores´ CLEs (Paidhungat et al., 2001), which degrade the cortex and cause a loss of the optical density (OD) of the spore. At moderate pressures (< 150 MPa), spores proceed through stage II of germination (Setlow, 2003) and degrade their SASPs during the pressure treatment (Wuytack et al., 1998).

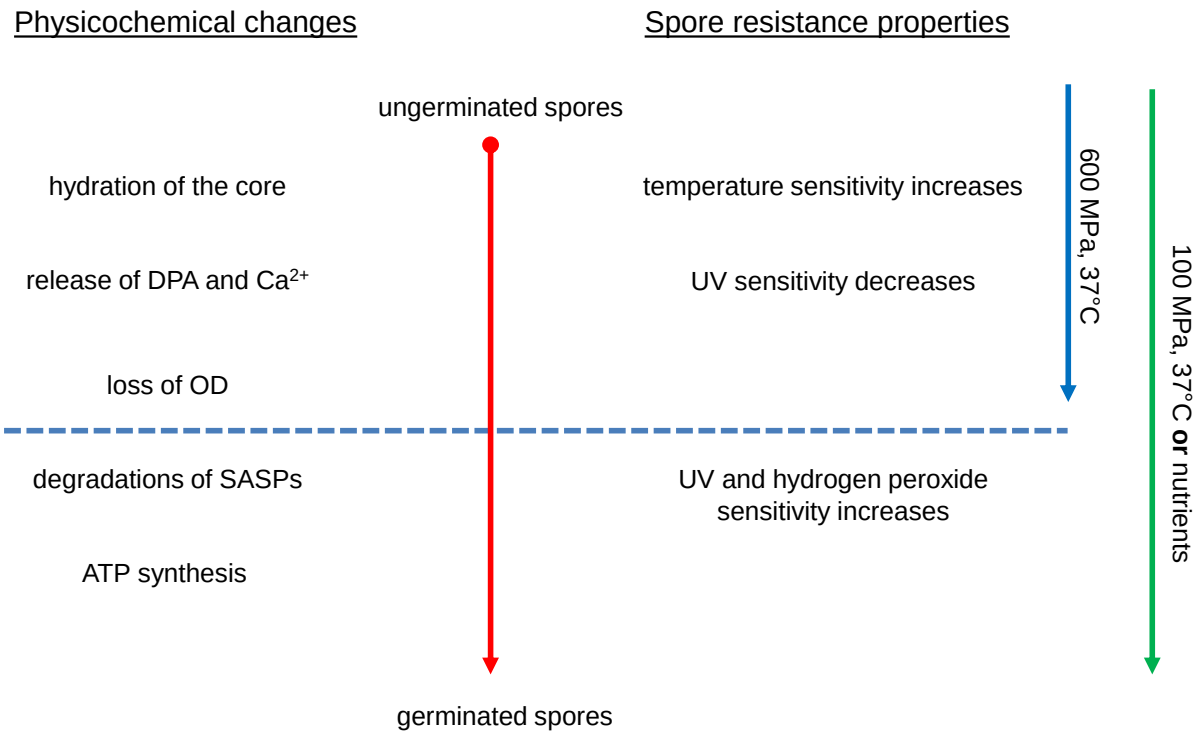

Figure 2. 20. Pressure-induced germination mechanism for *Bacillus subtilis* treated at 37 °C according to Wuytack et al. (1998).

Pressures above 200 MPa could directly open the spores' DPA-channels (Reineke et al., 2012), whereas this mechanism dominates spore germination at pressures above 500 MPa. Pressure levels above 500 MPa are more relevant for food processing, since a germination of the entire spore population is possible (Heinz & Knorr, 1998; Wuytack et al., 1998). However, after the release of Ca-DPA the germination process seams to abort, at least for *Bacillus subtilis*, at an early stage of germination (Wuytack et al., 1998; Reineke et al., 2013a). Nevertheless, several studies showed that the heat resistance of various spore strains does not correlate with high resistance to pressure (Margosch et al., 2004; Margosch et al., 2004b; Olivier et al., 2011). Furthermore, Margosch et al. (2004) supposed that the resistance of spores to combined pressure and temperature treatments depends on their ability to retain Ca-DPA.

For the combined treatment of very high pressures (> 600 MPa) and temperatures (> 60°C), Ardia (2004) supposed an inactivation mechanism for *Geobacillus stearothermophilus*, and assumed that the CLEs are inactivated in a certain range of pressure–temperature conditions. At this stage, only a further temperature increase can accelerate spore inactivation, and consequently a further pressure increase would have no effect. With inactive CLEs, the spore cortex can only be rehydrated by ultra high pressure, and hence pressures higher than 800 MPa could squeeze water inside of the spore cortex (Ardia, 2004) causing an increased heat sensitivity of the spore. By contrast, Margosch et al. (2006)

proposed that bacterial spores are very complex, and that a single protein or enzyme can only be responsible for stabilization under pressure if it is required for a vital function of the vegetative cell (e.g. as a structural or protective component).

Based on Setlow's (2003) germination model for *Bacillus subtilis,* Mathys et al. (2009) proposed an extended inactivation mechanism of *Geobacillus stearothermophilus* spores, based on data derived under isothermal and isobaric pressure–temperature conditions. The mechanism includes a stable and sensitive domain for the spores' inactivation in the pressure and temperature landscape (Figure 2. 21) and hence covers the diverse inactivation behaviors reported by other research groups.

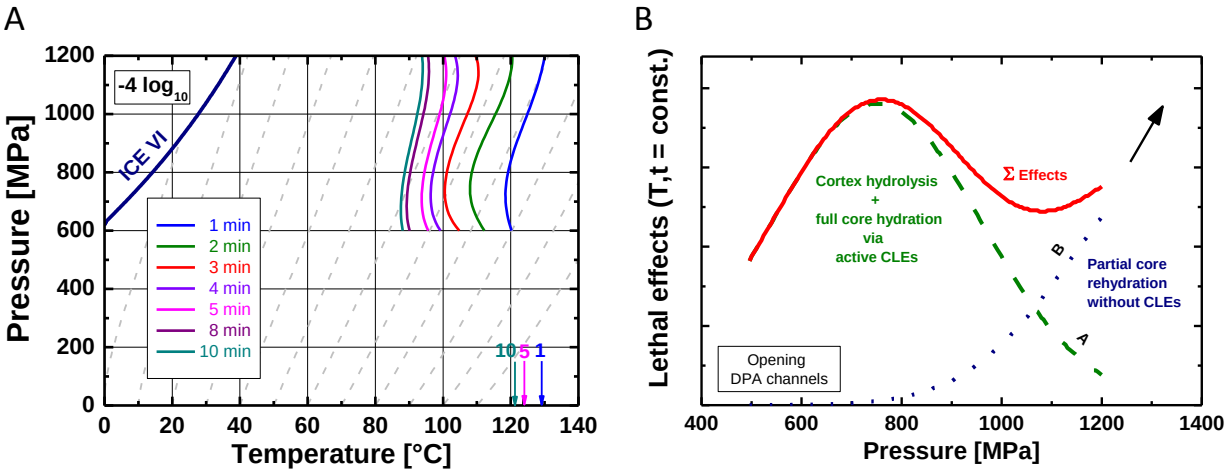

Figure 2. 21. A) 4 log inactivation of *Geobacillus stearothermophilus* in pressure stable 0.05 M ACES buffer (pH 7) after 1-10 min treatment at isothermal/isobaric conditions with N (1 s) as initial population N_0. Data for thermal inactivation are shown as arrows with holding times in minutes. Dashed lines indicate the adiabatic heating of water. B) Suggested summed lethal effects at different pressure levels, with two generated lethal effect distributions A (green dashes) and B (blue dots), showing different germination reactions at given process intensity. All lethal effects resulted in the cumulative distribution (∑ Effects) with a sensitive and stabilized zone adapted from Mathys et al. (2009).

The germination mechanism is initiated by an opening of the Ca-DPA–protein channels at pressures higher than 500 MPa, which enable spore germination in the absence of nutrients (Paidhungat et al., 2002). At these HP conditions spore germination is limited in buffer solutions. The key factor for full cortex hydrolysis and core hydration is the activation of the main CLEs. These enzymes probably show different activities or stabilities under specific pressure and temperature conditions, generating different pathways of inactivation. Heinz and Knorr (1998) assumed that pressure and temperature initially trigger the CLEs, but inactivate the same enzymes at a later stage. Hence, CLE activity is dependent on pressure, temperature and time. At ultra-high pressures a partial core rehydration without any CLE activity could induce an additional lethal effect. Summarizing all competing reactions, a cumulative lethal effect distribution as a function of the applied pressure level can be

generated (∑ Effects, Figure 2. 21B). However, the mechanisms of spore inactivation under HPTS conditions are still not fully understood, and further research is needed to identify the components in bacterial endospores that are most sensitive to pressure and thus provide a fast and complete inactivation, which will be discussed for recent data on *Bacillus subtilis* in chapter 8. conclusions and perspectives.

2.3 Modeling of inactivation kinetics

In microbiology, and especially in food microbiology, models of inactivation kinetics are useful for describing the growth or destruction of microbial populations. In food preservation, knowledge about the quantity of surviving microorganisms is essential to guarantee food quality, and therefore also to ensure consumer health. In recent years different inactivation theories have been developed, which can be divided into mechanistic and vitalistic models (Watson, 1908; Lee & Gilbert, 1918).

2.3.1 The vitalistic approach

Based on the assumption that all individuals of a population are different, the vitalistic conception was developed (Lee & Gilbert, 1918). It considers the biological diversity of microbial resistance against different kinds of external stress, such as temperature, radiation, chemical agents or pressure. It therefore assumes that the survival curve of the microorganisms represents the distribution of lethal events dependent on time. This allowed a better description of the often-occurring shoulder and tailing in the inactivation kinetics of bacterial spores than with the first order approach (Van Boekel, 2002). The reasons for such concave or convex curve shapes are given in chapter 2.1.6.

To describe these non-ideal curve progressions as accurately as possible, several model approaches have been formulated for different food ingredients. In particular, Dogan et al. (2009), Peleg et al. (1998) and Van Boekel (2009) give a list of different non-linear kinetic models. Van Boekel (2002; 2009) suggests the use of the Weibull model, derived from reliability engineering, as an adjustable, universal tool to fit both linear and non-linear inactivation curves of various food ingredients and decontaminating processes with great accuracy. This is described in Eqn. 17,

$$log_{10} S(t) = -b \cdot t^n \qquad \text{Eqn. 17}$$

with b as the scale parameter, n as the shape parameter and S(t) as $N(t)/N_0$.

The shape parameter, n, determines the curve progression of the inactivation curve, and simultaneously gives information about the dying behavior of the respective microorganisms. For values of n smaller than 1 the decreasing curve becomes increasingly flatter. This

implies that the microorganisms that have not yet been killed are more resistant to the treatment than the microorganisms that have already been killed. If n equals 1 the graph is linear and therefore corresponds to a first order inactivation. Consequently, all cells have the same, time-independent resistance to the treatment. For values of n greater than 1 the inactivation curve decreases progressively. From such a curve shape it can be deduced that the remaining cells exhibit increasing sensitivity against the applied treatment (Van Boekel, 2002; 2009). Furthermore, the Weibull model provides parameters analogous to the D- and z-values of first order kinetics.

However, the inactivation of bacterial endospores also involves additional difficulties regarding kinetic modeling, since shoulders and/or tailings occur relatively frequently. Van Boekel (2009) presents four commonly used approaches to describe the inactivation of spores: the Shull, Sapru, double Weibullian and Peleg models. The first two models mentioned are based on first order kinetics, whereas the last two describe nonlinear curve shapes.

In order to describe nonlinear inactivation of bacterial spores, the so-called double Weibullian model is often applied. Its equation consists of two parts, whereby the minuend describes the shoulder and the subtrahend characterizes the rest of the inactivation graph. This model is depicted in Eqn. 18, where b_1 and b_2 are the scale parameters, t is the treatment time and n_1 and n_2 are the shape parameters. In the case of a shoulder-free graph the parameter b_1 equals 0 (Van Boekel, 2009).

$$\log S(t) = b_1 \cdot t^{n_1} - b_2 \cdot t^{n_2} \qquad \text{Eqn. 18}$$

This model was also used by Reineke et al. (Reineke et al., 2011a) to model isorate lines for the high pressure inactivation of *Bacillus subtilis* spores. However, despite a good individual fit of each individual kinetic, no systematic variation of b_1, b_2, n_1, and n_2 depending on pressure and temperature was found, even for fixed n_1 and n_2 (Figure 2. 22).

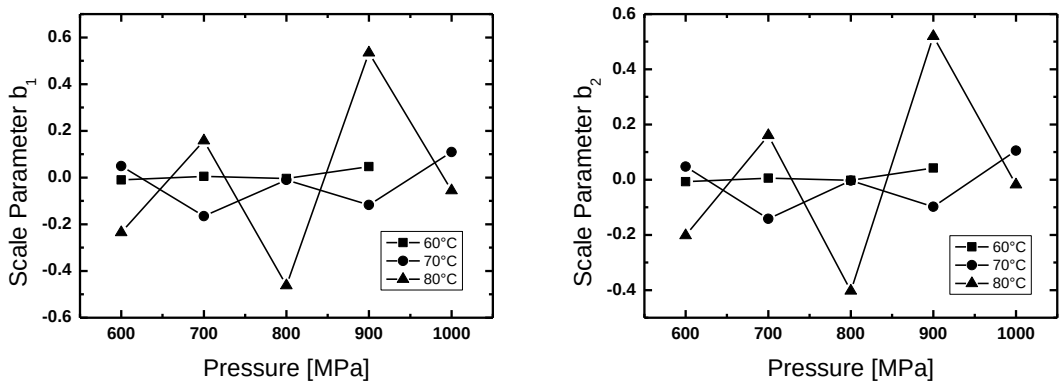

Figure 2. 22. Pressure dependence of the scale parameters b_1 and b_2 for fixed shape parameters for n_1=1.12 and n_2= 1.14 from (Reineke et al., 2011a).

Hence, this model failed in this case to calculate inactivation isorate lines dependent on pressure and temperature for this set of kinetic data.

2.3.2 The mechanistic approach

The root idea of the mechanistic conception is that the destruction of microorganisms is close to a chemical reaction and depends on time (Lee & Gilbert, 1918). Hence, the inactivation of bacterial spores would be defined as a pseudo first-order molecular transformation. The mechanistic concept assumes the discrete and random action of energy-transferring collisions, which increases with increasing temperature (van 't Hoff equation). Consequently, the probability of collisions also increases with the number of possible targets/microorganisms. Based on the assumption that a general similarity of resistance between the different individuals of a population exists (Lee & Gilbert, 1918), an equation is available that describes the logarithmic reduction of bacterial cells in response to chemical agents or heat as a linear function of time t (Chick, 1910).

$$ln\left(\frac{N}{N_0}\right) = k \cdot t \; or \; log\left(\frac{N}{N_0}\right) = \frac{-k \cdot t}{2.303} \qquad \text{Eqn. 19}$$

The number of surviving microorganisms (N/N_0) is logarithmically plotted versus time, and the exponential inactivation curve is transformed into a linear function with the slope k. This equation is the basis for evaluating thermal processes (i.e. canning) with variable treatment temperatures, as in the 12 D-concept. Derived from integrating Eqn. 19, the linear first order differential equation is:

$$\frac{dN}{dt} = -k \cdot N \qquad \text{Eqn. 20}$$

As a result, the number of surviving microorganisms (N) is proportional to the decrease in survivor count with time (dN/dt). Eqn. 20 is useful for detailed investigations of spore inactivation, including additional parameters affecting the inactivation of bacterial spores. To describe the temperature dependency of the corresponding rate constant (k), the Arrhenius equation (Eqn. 21) is a useful tool:

$$\frac{\partial lnk}{\partial T} = -\frac{E_a}{R_m T^2} \qquad \text{Eqn. 21}$$

where E_a represents the activation energy [kJ mol^{-1}] and R_m the molar gas constant, 8.3145 cm^3 MPa K^{-1} mol^{-1}. This remains the theoretical basis of the industrial sterilization process in, for example, the canning industry (Ball, 1923). Eyring (1935a; 1935b) derived a similar expression (Eqn. 22) for the pressure-dependence of k:

$$\frac{\partial lnk}{\partial p} = -\frac{\Delta V^{\#}}{R_m T}$$ Eqn. 22

with the activation volume of the reaction as $\Delta V^{\#}$ [$cm^3\ mol^{-1}$]. Due to the pressure-dependence of $\Delta V^{\#}$ and the order of reaction, Eqn. 22 might vary at different pressure levels. In any case the early observed experimental deviations from the first order approach (Esty & Meyer, 1922; Aiba & Toda, 1966), led to a modification of Eqn. 20. Many authors have described deviations from this log-linear inactivation shape (Casolari, 1994). They found an initial lag phase or "shoulder", as well as a longer leveling-off at longer treatment times, or "tailing". These phenomena cannot be accurately described by Eqn. 20. The use of an empirical reaction order, n, in Eqn. 20 leads to Eqn. 23, the nth-order reaction model (Kessler, 2002a):

$$\frac{dN}{dt} = -k \cdot N^n$$ Eqn. 23

With the nth-order reaction model non-linear $\log_{10}$ reductions could be described. To integrate the dependence of the corresponding rate constant, k, on temperature and pressure, the Arrhenius equation (Eqn. 21) and Eryings equation (Eqn. 22) can be used. The reaction order, n, has no mechanistic background and can be regressively determined. To create isorate lines in the pressure and temperature landscape, a functional relationship for k (p,T) can be used (Mathys, 2008a). However, the capability of the nth order model is limited to fit a tailing, but cannot accurately describe the often occurring shoulder formation (Van Boekel, 2009). This is depicted in Figure 2. 23 A), in which the residuals are not normally distributed, indicating a heteroscedastic error. Therefore, this model predicts an overestimated inactivation for low inactivation levels (< -2.5 $\log_{10}$) and an underestimation for higher inactivation levels (Reineke et al., 2011a).

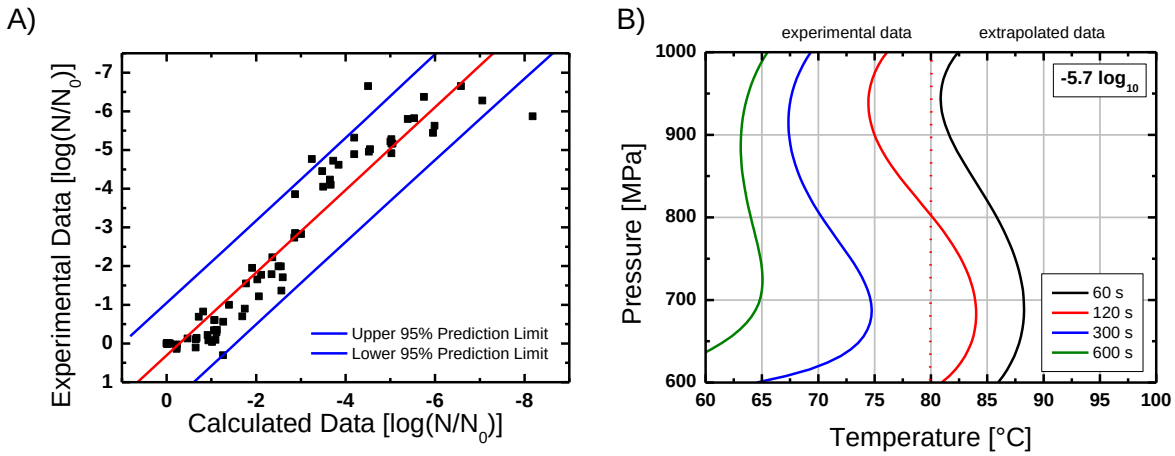

Figure 2. 23. A) Comparison between calculated data and experimental $\log_{10}$ reduction of *Bacillus subtilis* (PS832) with n = 1.2. B) Isorate lines (n=1.2) for a 5.7 $\log_{10}$ inactivation of heat sensitive *Bacillus subtilis* (PS832) spores after pressure treatment in 0.05 M ACES buffer solution (pH 7) derived under isobaric/ isothermal conditions with N (1 s) as initial population N_0; from (Reineke et al., 2011a).

To overcome this problem, multiresponse kinetic modeling could be a useful tool. This approach is based on simple chemical differential equations and describes quantitatively how reactants and products are linked. This model requires mechanistic insights to propose a reaction network based on simple chemical differential equations. Such a network of differential equations (Eqn. 24-26) for the germination and inactivation of bacterial spores was used by Reineke et al. (2012).

$$\frac{d[\text{dormant}]}{dt} = -k_a(p,T)[\text{dormant}] \qquad \text{Eqn. 24}$$

$$\frac{d[\text{germinated}]}{dt} = k_a(p,T)[\text{dormant}] - k_b(p,T)[\text{germinated}] \qquad \text{Eqn. 25}$$

$$\frac{d[\text{inactivated}]}{dt} = k_b(p,T)[\text{germinated}] \qquad \text{Eqn. 26}$$

These coupled ordinary differential equations can be solved by numerical integration, which enables simultaneous calculation of each rate constant k. Hence, in contrast to uniresponse models, several responses are calculated at the same time, which enables, for example, the calculation of non-linear chemical reaction in food (Van Boekel, 2002)

3. Shift of pH-value during thermal treatments in buffer solutions and selected foods

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SHIFT OF PH-VALUE DURING THERMAL TREATMENTS IN BUFFER SOLUTIONS AND SELECTED FOODS

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The pH value is one of the most important process parameters during thermal treatments, regardless if the medium is a simple buffer solution or a complex food matrix. When the temperature increases after an initial measurement of the pH at ambient temperature (25°C), a significant pH shift could occur, which could produce incomparable results in different buffer solutions or lead to side reactions during food preservation. Consequently, a measurement cell was constructed to record online the pH-value and temperature up to 130°C. By applying the Nernst equation, it was possible to exclude the temperature-dependent influence of the pH glass electrode on the total pH value. The pH shift was measured over a wide temperature range (ΔT 20–130°C) in the most commonly used buffer solutions and some selected food matrices. The ΔpH of certain buffer solutions, namely TRIS and ACES, showed a significant pH decrease of −2.01 ± 0.08 (ΔT 20–130°C) and −1.27 ± 0.1 (ΔT 20–130°C), respectively, whereas the pH of PBS buffer solution was nearly independent of temperature. The ΔpH decrease recorded in milk (−0.89 ± 0.6, ΔT 20–130°C) as well as commercial and self-made baby food (−0.56 ± 0.05, ΔT 20–130°C) is of special interest for the food industry to get a deeper insight in occurring reactions during thermal preservation processes.

Keywords: ***pH Shift, Temperature, Buffer solutions, Food, Milk, Spore inactivation***

INTRODUCTION

Microorganisms, such as bacteria, bacterial endospores, yeast, and molds, are sensitive to pH shifts in their environment. During thermal processes, such as pasteurization, sterilization, or freezing, the dissociation equilibrium of water and buffer solutions varies with pressure and temperature.[1–7] This change of the pK_a-value (decadic logarithm of acid dissociation constant K_a) may play an important role in different pH-sensitive reactions, but its behavior has rarely been investigated.

For basic inactivation studies of microorganisms, buffer solutions are commonly used to achieve constant pH-values and medium properties. In view of the dissociation equilibrium shift during heating, a biased error in the results of inactivation experiments occurs, which leads to incomparable results if they are not designed correctly. Several

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authors have shown that the thermal inactivation of bacterial spores,[8–12] vegetative microorganisms,[13] or protein and enzyme denaturation[14–16] strongly depends on the pH of the suspending media. According to Goldberg,[17] the known temperature dependence of the acid dissociation constant K_a (T) for different buffer systems could be interpolated up to 60°C.

Furthermore, the pH-value plays an important role in determining the amount of heat required to ensure a safe product during industrial food processing. Food could, therefore, be divided by its initial pH value into different classes. Foods with very low or high pH value will avert microbial growth and could be stored over long periods of time without undergoing further preservation processes. The majority of food, however, has a pH value that only offers some protection against microbial growth, or may even provide microorganisms with optimal conditions for reproduction. Few foods, like egg white, some bakery products, and sweet corn, have an alkaline pH value. Most foods have a pH value less than 7 and are, therefore, naturally acidic. Nevertheless, the shift of the pH value of a single food ingredient or additive may have a strong influence on the behavior during thermal processing of the whole product.

Common ways to reduce the risk of food spoilage include refrigerated or frozen storage, as well as drying or thermal processes. Aside from the process temperature, pressure, and time, the pH value of the treated food plays a key role in determining the extent of heat required during pasteurization or sterilization in food industry.[15,18] Extensive data about the pH value of various kinds of foods at ambient temperature are available,[19–21] and some data covers pH values for buffer solutions up to 80°C, or even 90°C for some foods,[15] however, there are limited data available about the shift of the pH values up to 130°C. In contrast to simple buffer solutions, these pH shifts are not calculable and need to be measured in complex matrices like foods. Currently, this measurement is only possible with a few pH glass electrodes.

The aim of this research was to develop a pressure-resistant measurement system that could record the pH value in dependence of the temperature up to 130°C. Furthermore, the pH shift of commonly used buffer solutions and selected foods was measured to extend the available data base for the pH shift from 90°C up to 130°C. These data are of key interest for different research and industrial applications, such as the inactivation of microorganisms (e.g., up to 130°C) and the sterilization of food during canning (e.g., up to 121.1°C). Hence, these data could give a deeper insight how chemical, biochemical, and biological reactions are influenced by the actual pH value under the applied temperature.

MATERIALS AND METHODS

pH Measurement System

In order to measure the pH shift during heating, it was necessary to construct a pressure resistant cell with an adequate sample volume. The developed cell had a maximum filling volume of 92 ml and a maximum working pressure of 10 bar (the limit of the pH electrode). All measurements were performed with a Jumo Tecline pH glass electrode (Jumo GmbH & Co. KG, Fulda, Germany), equipped with a high temperature pH glass (max. 130°C), high temperature gel electrolyte, and a double hole diaphragm. The glass electrode was plugged in to a pH meter (Digital-Labor-pH-Meter CG 811, Schott AG, Hofheim, Germany). The sample temperature was measured with a Pt100 temperature element integrated into the pH electrode. All pH values and temperatures were acquired with

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a USB data acquisition system (OMB-DAQ-56, Omega Engineering, Inc., Stamford, CT, USA) (1 Hz acquisition rate) and the data were documented with self written software created in LabVIEW 6.5.1 (National Instruments, Austin, TX, USA). The cell was equipped with a magnetic stirring device to guarantee accuracy and homogenous temperature distribution inside the measurement cell. For heating up, the measurement system was immersed into preheated silicon oil (SilOil M40.165.10, Huber GmbH, Offenburg, Germany) with a fixed temperature of 140°C.

The pH electrode was calibrated every experimental day at 20°C by a two-point calibration procedure with pH reference buffer solutions at pH 7 (Certipur buffer solution pH 7, Merck KGaA, Darmstadt, Germany) and pH 4 (Certipur buffer solution pH 4, Merck KGaA, Darmstadt, Germany). During the measurements the automatic temperature compensation of the pH meter was switched off and adjusted to 20°C.

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Buffer Solutions

Standard commercial pH buffer (Tampon pH 7.413, Fisher Bioblock Scientific, Illkirch, France) was used with certified data (in accordance with U.S. National Institute of Standards and Technology SRM 186-I-g potassium dihydrogen phosphate and SRM 186-II-g disodium hydrogen phosphate) for the pH shift up to 90°C (uncertainty of measurement ± 0.010 pH at $p = 0.05$).

N-(2-Acetamido)-2-aminoethanesulfonic acid buffer (ACES) (A-9758, Sigma-Aldrich-Chemie GmbH, Steinheim, Germany) pH 7, phosphate buffer system (PBS) pH 7, tris(hydroxymethyl)aminomethane buffer (TRIS also kwon as THAM) pH 8, acetate buffer pH 4.1 and citrate buffer pH 5.6 were used. All buffers had a molarity of 0.05 M. The pH was adjusted with 1 M HCl solution or 1 M NaOH solution, respectively.

Selected Food Systems

The shift of the pH value for raw milk (Demeter e.V., Darmstadt, Germany) and ultra heat treated milk (UHT-milk, EUCO GmbH, Hamburg, Germany) with a fat content of 3.5% was measured. Furthermore, the pH change in the different milk fractions was investigated. To obtain these fractions, whey proteins were isolated by adding rennet to the raw milk (32°C). After 30 min, the coagulated casein were cut and the curd was removed. Isolation of the casein fraction from raw milk was accomplished first via a centrifugation step (20 min, 2500 g, Megafuge 1.0 R, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 5°C to remove the fat phase, and afterwards the residue was ultra-centrifuged (80,000 g; 60 min; Beckmann Optima CE-80K, Beckmann Coulter Inc., Fullerton, CA, USA). The supernatant was removed and the casein pellet was again suspended in a salt solution that simulates the milk ultrafiltrate (SMUF solution).[22]

To measure the pH variation in the lactose fraction of milk, lactose (Oxoid, Hampshire, GB) with a final concentration of 4.5% was suspended in a SMUF solution. The solvation of lactose in the SMUF solution was necessary to reach a conductivity in the solution >100 $\mu S\ cm^{-1}$, and to, therefore, get a stable pH value in a batch measurement cell.[20] The pH was measured in accordance with pH measurements in fresh milk and iso-standards.[20,23] As a possible canning product, the in-situ pH shift in baby mashed carrots with potatoes was investigated. The pH was measured in a commercial baby food (Carrots with Potatoes, HIPP, Pfaffenhofen, Germany) and in fresh cooked baby food (78% carrots, 10% potatoes, 0.9% rape oil, and 11% water).

All measurements were performed at least in triplicates. The functional relationship between pressure, temperature, and filling volume was solved with MathCAD 2001i Professional (MathSoft Engineering & Education, Inc., USA). The presentation of the calculated and measured data was arranged by the Origin 7SR1 software (OriginLab Corporation, USA).

Thermal Inactivation of Bacterial Endospores

Especially the inactivation kinetics of bacterial endospores, which have to be fully inactivated during a sterilization process, could be strongly affected if the thermal treatment is carried out in a buffer solution where the pH value is temperature dependent or not. To proof the impact of the pH shift on the inactivation of bacterial spores, *Bacillus subtils* spore suspension (PS832) was centrifuged for 5 min at 3424 g (Biofuge pico, Kendro Laboratory Products GmbH, Langenselbold, Germany) and the spore pellet was resuspended in phosphate buffer system (PBS) or in acid buffer (ACES). Both buffers had a molarity of 0.05 M and a pH of 7. The spore suspension was filled into thin glass capillaries with a volume of 60 μL and hermetically sealed to prevent evaporation of the suspension due to temperatures above 100°C. The spores were inactivated in a thermostatic bath, filled with silicon oil at 100°C, which corresponds to a pH shift of -1 ± 0.1 in ACES and 0.02 ± 0.03 in PBS. After thermal treatment the glass capillaries were immediately stored at 4°C, the treated spore solution was serially diluted with 1/4 Ringer solution (TP887925 712, Merck KGaA, Darmstadt, Germany) on micro plates (Carl Roth GmbH, Karlsruhe, Germany) and two 50-μl samples of every dilution were drop-plated in petri dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37°C for 2 days and the colonies were counted.

RESULTS AND DISCUSSION

During the heat-up phase, the volume of the sample increases, which results in a pressure increase. The pressure has to remain below 10 bar, to avoid a leakage of the measurement cell or a rupture of the pH electrode. To calculate the pressure increase during heating, Eq. (1) was used[24]:

$$\Delta p = \Delta p_s + \Delta p_{A,} \tag{1}$$

where Δp denotes the total pressure, Δp_S the partials vapor pressure, and Δp_A the air pressure. A temperature increase will result in an increase of the saturated vapor pressure and air pressure inside the measurement cell. Heating a vessel with the volume V_v, a headspace volume V_{H1} and pressure p_{L1} from temperature T_1 to T_2, will lead to the final pressure p_{L2}[24]:

$$p_{L2} = p_{L1} \frac{V_{H2} T_2}{V_{H2} T_2}, \tag{2}$$

The volume of the headspace V_{H2} at the temperature T_2 leads to the final pressure p_{L2}, which is largely determined by the heat expansion of the processed fluid. To calculate the expansion of the measured fluids, data for water was assumed, taken from the NIST Database.[25] The results are presented in Fig. 1A. Based on the calculation for water, a

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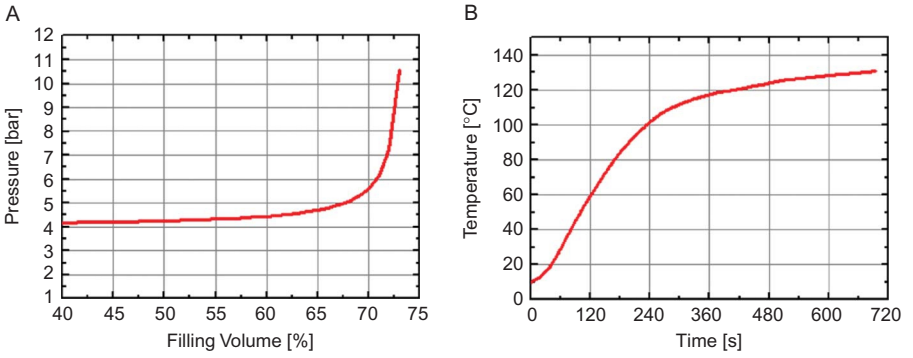

Figure 1 (a) Calculated increase of pressure inside the measurement cell as filling volume increases for a temperature increase of ΔT 20–130°C. (b) Temperature profile for UHT-Milk measured inside the treatment chamber (fixed silicon oil temperature of 140°C) (color figure available online).

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maximum filling volume of 70% (66 ml) was used in all experiments to avoid disrupting the pH electrode. Figure 1B illustrates the temperature profile during the pH measurement, which is of special interest for the pH measurement in milk and milk fractions.

The response of a pH glass electrode is on an interfacial equilibrium between functional glass surface groups and oxonium and/or alkali ions (M^+) in the contacting solution. Depending on the relative oxonium (pH) and alkali (pM) ion concentration, the pH glass surface may be covered, which would result in a negative charge density at the glass surface. This would generate a difference of potential between the inner and outer surface of the glass membrane. Due to the known pH of the inner electrolyte, the pH of the unknown solution could be measured as a voltage shift. If both solutions have the same pH (normally pH 7), the voltage difference is 0 (isotherms intersection in Fig. 2B).[20,26,27] The response of the pH glass membrane to media with different pH values is a linear shift of the membrane potential, which could be calculated with the Nernst equation.[28] The slope (m) of this straight line is dependent on the temperature and has the theoretical value of -59 mV at 25°C. Kratz [29] and Baucke[30] found a deviation of 0.3% from this ideal slope and traced this unique effect of glass electrodes back to the thermodynamic effects at the glass's surface.

To evaluate the developed measurement system up to 90°C, certified standard buffer solutions were used to determine the temperature-dependant slope shift of the used glass electrode. The automatic temperature compensation of the pH-meter (feasible up to 100°C) was switched off and manually adjusted to 20°C. The measured data up to 90°C (Fig. 2A) were in excellent agreement with literature data, and thus enabled the possibility to calculate the proportion in the pH shift caused by the pH electrode at higher temperatures. Figure 2B illustrates the glass electrode response under different isothermal conditions (ΔT 20–120°C). The slope of each graph is determined by the construction of the glass electrode. Whereas the temperature influence on the offset of the pH glass electrode increases under alkaline or acid conditions, it has a marginal influence under neutral conditions (pH 7 = 0 mV), which is pictured as the isotherms intersection in Fig. 2B. These basic and essential relations as well as calculations enabled the determination and measurement of the real change of the pH value in the processed fluid.

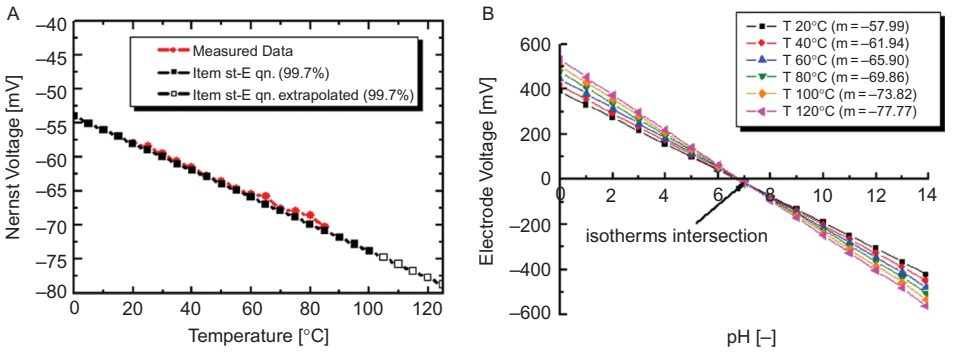

Figure 2 (a) Measured slope shift in a certified temperature-stable standard buffer solution versus 99.7% of Nernst-slope up to 100°C[20], data up to 130°C were extrapolated. (b) Linear function of the electrode response from 20°C to 120°C in correlation with pH (color figure available online).

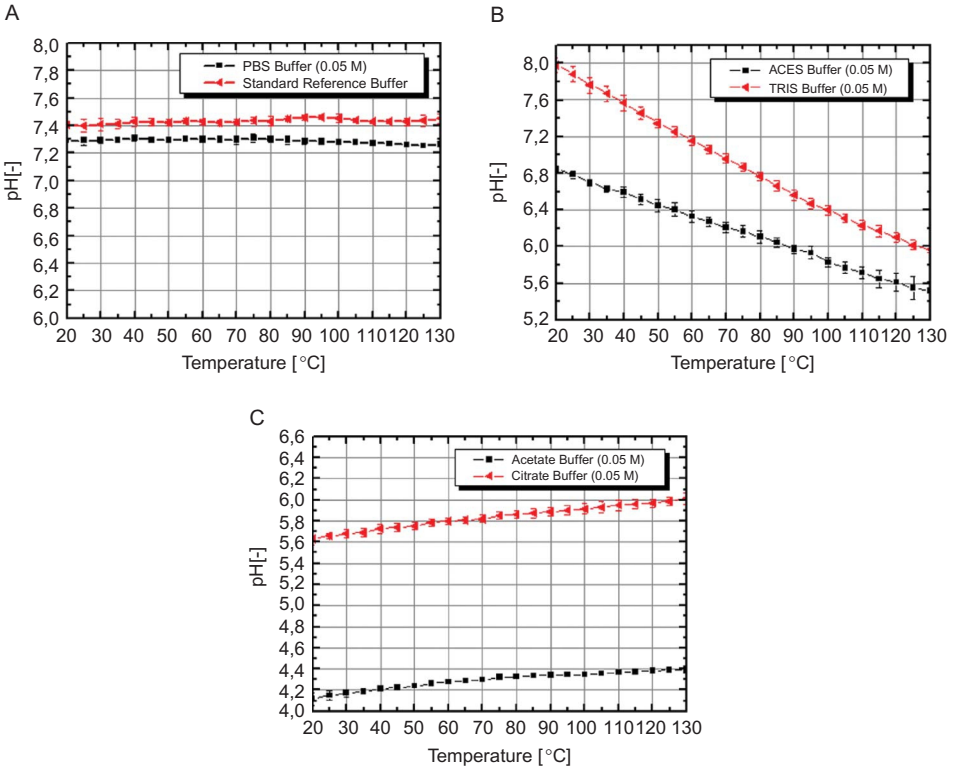

Figure 3 (a) pH shift in PBS buffer solution (0.05 M) and certified reference buffer-solution; (b) pH shift in ACES- (0.05 M) and TRIS buffer solution (0.05 M); C) pH shift in acetate- (0.05 M) and citrate buffer solution (0.05 M). Error bars indicate the standard error (color figure available online).

Consequently, this measurement system provided the possibility to measure the pH shift in different commonly used buffer solutions. The measured buffer solution could be divided into three categories: (i) temperature stable buffer solutions (Fig. 3A), (ii) buffer

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solutions with decreasing pH during heating (Fig. 3B), and (iii) buffer solutions with increasing pH during heating (Fig. 3C). TRIS and ACES buffer solution had the highest pH shift [-2.01 ± 0.08, (TRIS); -1.36 ± 0.1 (ACES); ΔT 20–130°C] of all tested buffer solutions, whereas PBS buffer solution showed almost no pH change (-0.03 ± 0.03, ΔT 20–130°C) during heating. The data measured up to 90°C are in accordance with the pH shifts published elsewhere.[20,31–33] The pH shifts in these buffer systems are of particular interest because they are commonly used for thermal inactivation experiments with microorganisms or enzymes up to 130°C. If this pH change is not considered during the experimental design, it could produce a much stronger inactivation of the used microorganism or enzyme in the TRIS or ACES buffer solution,[12] which, in turn, would produce incomparable results to for example the same inactivation in PBS buffer solution. Thermal treatments with bacterial spores were carried out in PBS and ACES buffer at 100°C due to their similar pKa values and, hence, equal buffer capacity,[12] to proof the influence of the pH shift. The initial pH of both buffer solutions was 7 (at 20°C) and remains stable for PBS (pH 7.01 ± 0.02 at 100°C) and decreased to 6.01 ± 0.05 (at 100°C) for ACES buffer. This shift of 1 pH unit had a strong effect on the thermal inactivation of *B. subtilis* PS832 (Fig. 4). During isothermal conditions, the *B. subtilis* spores showed a -1 to -1.5 $\log_{10}$ stronger inactivation in ACES buffer solution compared to PBS, which confirms the high impact of the pH shift during thermal treatments on biological and biochemical reactions.

The various behaviors of the different buffer solutions at the same temperature result from the different dissociation reactions and, consequently, the different molar enthalpies ($\Delta_r H^0$). In Table 1, the standard molar enthalpies with its pK_a values at 25°C are plotted, which are important for the dissociation equilibrium shift. While increasing the temperatures, the dissociation equilibrium shifts. Indeed, this relationship reflects the principle of Le Chatelier and Braun, which states that if a chemical system at equilibrium experiences a change, the equilibrium will shift in order to minimize that change. As a consequence, endothermic reactions dominate as temperatures increase. This leads to a 13 times more temperature-dependant dissociation of TRIS buffer in comparison to PBS buffer, which explains the high pH shift.

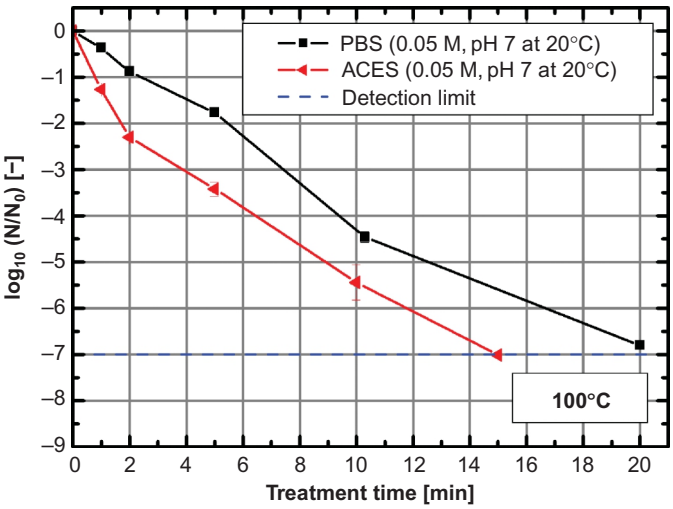

Figure 4 Thermal inactivation of *Bacillus subtilis* spores at 100°C in PBS and ACES buffer with an initial pH of 7 (0.05 M) at 20°C. Error bars indicate the standard error (color figure available online).

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Table 1 pK_A values and standard molar enthalpies ($\Delta_r H^0$) at 25°C[17].

Buffer system	pK_A at 25°C	$\Delta_r H^0$[kJ mol-1]
TRIS	8.07	+47.4
ACES	6.84	+30.4
PBS (2nd)	7.19	+03.6
Citrate (2nd)	4.76	+02.2
Acetate	4.75	−00.4

Buffer solutions with a stable or even an increasing pH value during heating exhibited a marginal positive or even negative molar enthalpy ($\Delta_r H^0$). These are, for example, PBS, acetate, or citrate buffer solution, which has a measured pH change of $+0.367 \pm 0.05$ (ΔT 20–130°C) for citrate and $+0.277 \pm 0.04$ (ΔT 20–130°C) for acetate buffer, respectively. Such buffer solutions are of special interest for different kinds of chemical and biochemical reactions, where nearly stable pH conditions have to be achieved at different temperature levels. In contrast to the relatively simple composition of buffer solutions, the change of the pH value in complex matrices (like food) during heating is hardly to predict and therefore a direct measurement of the pH has to be performed. These data are of industrial relevance for food pasteurization or sterilization to increase the knowledge about side reactions and interactions of food components or added ingredients like enzymes, food dyes, or antioxidants that are often pH sensitive and are not singularly inactivated by the applied temperatures. Furthermore, estimations about the impact of the pH shift on the total microbial or enzymatic inactivation could be possible, if inactivation data in buffer solutions with an analogue pH shift to the thermal treated food are compared with inactivation data observed in the thermal treated food.

As an example for a liquid food, the pH shift for the thermal treatment of milk and its components during pasteurization (62–85°C) or sterilization (109–120°C) is presented in Fig. 5A and 5B. The observed pH shift of -0.9 ± 0.06 (ΔT 20–130°C) in raw milk is in agreement with the literature (up to 90°C).[15,34–36] The reduction of the pH value during thermal treatment is due to the release of calcium phosphate from the casein micelles, hence, the generation of oxonium ions that decrease the pH.[15,34] Up to 50% of the pH decrease is caused by the formation of organic acids (mainly formic and levulinc acid) from the lactose fraction of the milk.[35] These reactions dominated the pH shift in milk, whereas the release of carbon dioxide, which would result in a pH increase, had a minor influence. The hydrolysis-reactions in the protein fractions of milk proceeded much slower[16] than the decomposition of lactose, which could explain the greater pH decrease above 100°C in the lactose solved in SMUF solution.

Contrary to the measured pH shift in buffer solutions, a hysteresis of the pH value measured in milk could be observed. Despite using a pH electrode, where the reference-element was equipped with a cartridge filled with sliver chloride to realize an adequate electrolyte outflow. Hence the observed hysteresis in milk could be induced by a partial or complete blockage of both diaphragms of the electrode, caused by protein aggregates. However, since the thermal treated milk reaches its initial pH-value (-0.07 ± 0.03) after 3 h storage at 20°C, the observed hysteresis is probably caused by a retarded uptake of Ca^{2+} ions into the casein micelles.

In contrast to the different behavior of the single milk fractions, the direct comparison of the total pH shift in raw milk versus a short time high temperature treated milk (UHT-milk) showed only marginal deviation in the pH value over the measured

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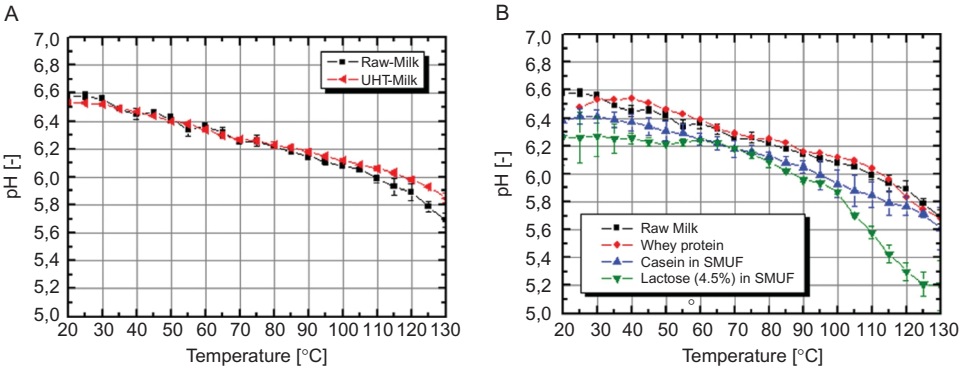

Figure 5 (a) pH shift in raw milk and ultra heat treated milk (UHT-Milk) as well as; (b) pH shift in raw milk and its components (whey protein, casein and lactose [4.5%]) in dependence of temperature. Error bars indicate the standard error (color figure available online).

temperature range. This leads to the assumption, that a short time (2–8 s) high temperature (135–150°C) preservation process has only a minor influence on the single milk components and, hence, should be preferred for milk sterilization.

Due to the higher energy input into the treated product during sterilization (110–121°C, >10 min) a lot of temperature instable substances are decomposed or converted, which results in a different behavior concerning the pH value if a thermal untreated product is compared with a commercial sterilized product. Such a typical canning product is baby food, which is presented in Fig. 6. The initial pH value of the fresh prepared baby food was 5.7 in contrast to 5.2 of the canned one. While heating up both products, the pH value equalized continuously and reached nearly the same pH value at 120°C. This indicated that all temperatures in labile components are degraded at the first heat up and a second or a

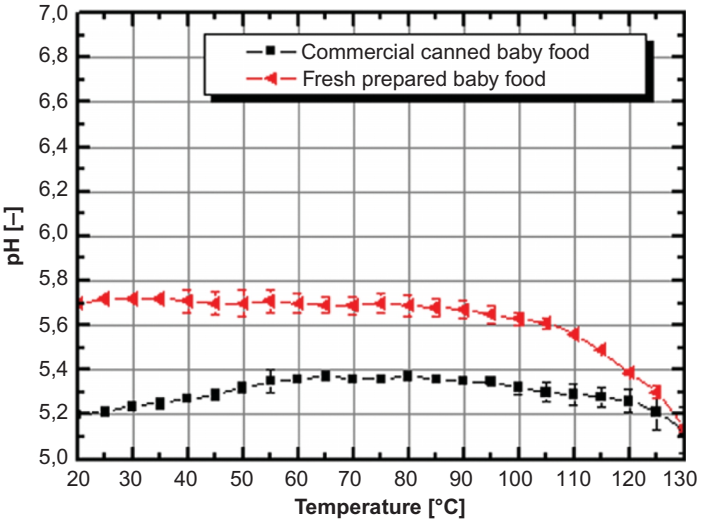

Figure 6 pH shift in commercial canned baby food versus pH shift in fresh prepared baby food by increase in temperature. Error bars indicate the standard error (color figure available online).

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further extended heating has only a minor influence on the total pH change (-0.07 ± 0.05, ΔT 20–130°C for commercial canned baby food during the second heat up, deviation for the second heat up of self made baby food was marginal). The slight decrease of the pH value (-0.56 ± 0.02) for the fresh prepared baby food could originate by various substances with buffer capability, like proteins in the product, or by matrix effects caused by nonpolar substances. These substances, like sugars, could induce a fine structure in aqueous solutions and, hence, influence the pH value.[37] Furthermore, the decrease of pH value could be induced by dispersed particles in the mash, which are polarizing the surrounding water molecules and, thus, increase the activity of oxonium and hydroxide ions.[38]

Changes in the pH values of foods play an import role during the preservation process because it influences every substance in the thermal-treated food matrix. This influence is not only on desired effects, like the unwanted inactivation of microorganisms, but also on unwanted denaturation of enzymes and proteins. Furthermore, the enlargement of available pH data at temperatures above 90°C for some commonly sterilized products enables a deviation between temperature effects and the influence of the actual pH under pasteurization or sterilization conditions on chemical and biochemical reactions. Thus, it could lead to an optimization of the whole hurdle concept.

CONCLUSION

The shift of pH values in different media over a wide temperature range (20–130°C) was measured due to its important influence during thermal treatments on microorganisms, enzymes and pH-sensitive additives. A measurement system was constructed to acquire the pH value and temperature up to 130°C with an acquisition rate of 1 Hz. The system was validated with a certified buffer reference solution up to 90°C, and the temperature-dependent influence of the pH glass electrode was excluded by using the Nernst equation. All measured data up to 90°C were in excellent agreement with literature data. The acquired data for the pH shift between 90°C and 130°C represents an important process window for thermal processing of different media. With the pH shifts measured in different buffer solutions, the results of microbial and enzymatic inactivation experiments can be compared more effectively and the influence of the temperature-dependent pH shift could be controlled by changing the buffer solution. The presented pH decreases in some food systems can be of special interest for the food industry to increase the understanding of occurring microbiological, biochemical, and chemical reactions in the whole food matrix or for single ingredients in the thermal-treated food. This knowledge would help in designing an optimized hurdle concept for thermal preservation processes or for a controlled modification of food properties (e.g., gelation properties of selected protein fractions or preservation of pH instable healthful food ingredients) during thermal preservation processes. In conclusion, the shift of pH value during thermal treatment (regardless if the temperature is increased or decreased) could not be neglected because of its strong influence on all kind of biological and chemical reactions.

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4. The Impact of High Pressure and Temperature on Bacterial Spores: Inactivation Mechanisms of *Bacillus subtilis* above 500 MPa

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The Impact of High Pressure and Temperature on Bacterial Spores: Inactivation Mechanisms of *Bacillus subtilis* above 500 MPa

Kai Reineke, Alexander Mathys, and Dietrich Knorr

Abstract: High-pressure thermal sterilization (HPTS) is an emerging technology to produce shelf stable low acid foods. Pressures below 300 MPa can induce spore germination by triggering germination receptors. Pressures above 500 MPa could directly induce a Ca^{+2}-dipicolinic acid (DPA) release, which triggers the cortex-lytic enzymes (CLEs). It has been argued that the activated CLEs could be inactivated under HPTS conditions. To test this claim, a wild-type strain and 2 strains of *Bacillus subtilis* spores lacking germinant receptors and one of 2 CLEs were treated simultaneously from 550 to 700 MPa and 37 to 80 C (slow compression) and at 60 to 80 C up to 1 GPa (fast compression). Besides, an additional heat treatment to determine the amount of germinated cells, we added $TbCl_3$ to detect the amount of DPA released from the spore core via fluorescent measurement. After pressure treatment for 120 min at 550 MPa and 37 °C, no inactivation was observed for the wild-type strain. The amount of released DPA correlated to the amount of germinated spores, but always higher compared to the belonging cell count after pressure treatment. The release of DPA and the increase of heat-sensitive spores confirm that the inactivation mechanism during HPTS passes through the physiological states: (1) dormancy, (2) activation, and (3) inactivation. As the intensity of treatment increased, inactivation of all spore strains also strongly increased (up to -5.7 $\log_{10}$), and we found only a slight increase in the inactivation of one of the CLE (*sleB*). Furthermore, above a certain threshold pressure, temperature became the dominant influence on germination rate.

Keywords: *Bacillus subtilis* spores, germination mechanisms, high pressure, inactivation mechanisms, sterilization

Practical Application: The continuous increase of high-pressure (HP) research over the last several decades has already generated an impressive number of commercially available HP pasteurized products. Furthermore, research helped to provoke the certification of a pressure-assisted thermal sterilization process by the U.S. FDA in February 2009. However, this promising sterilization technology has not yet been applied in industrial settings. An improved understanding of spore inactivation mechanisms and the ability to calculate desired inactivation levels will help to make this technology available for pilot studies and commercialization at an industrial scale. Moreover, if the synergy between pressure and elevated temperature on the inactivation rate could be identified, clarification of the underlying inactivation mechanism during HP thermal sterilization could help to further optimize the process of this emerging technology.

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Introduction

The application of isostatic high pressure (HP) is an emerging technology to produce shelf stable, minimally processed, fresh-like food products (Hendrickx and Knorr 2002) and is consequently a promising food processing alternative to classical heat treatment processes (Heinz and Knorr 1998). The pressures currently used in the food industry range from tens of MPa in common homogenizers or supercritical fluid extractors to up to 400 or 800 MPa in ultra HP homogenizers or HP pasteurization units, respectively.

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Isostatic HP in the food industry is primarily used for the pasteurization of foods at chilled and ambient temperatures and has been applied on a broad range of products. Hence, the number of industrial HP systems for food pasteurization has steadily increased during the past 10 y. At present, there are more than 160 industrial scale installations with a maximum individual volume of 687 L in use worldwide, producing 300000 t pasteurized products annually (Tonello Samson, C., 2010, NC Hyperbaric, Spain, personal communication).

However, HP must be combined with an elevated initial temperature (>80 °C) to sufficiently inactivate bacterial spores in "low acid foods" (such as meat products, baby food, or "ready-to-eat meals"). This combined HP and heat treatment can result in sterile food products with reduced thermal load, higher nutritional quality, and increased functionalities, rendering HP a promising improvement from traditional heat sterilization (Heinz and Knorr 2002; Mathys and others 2009). One benefit of HP thermal sterilization (HPTS) is that, during compression, adiabatic heating instigates a rapid temperature increase in the product and in the

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pressure-transmitting fluid (Heinz 1997; Ting and others 2002; De Heij and others 2003). Hence, an initial product temperature from 60 to 90 °C, results in a final product temperature of 90 to 130 °C after treatment with 600 MPa (De Heij and others 2003; Ardia and others 2004b; Juliano and others 2006; Barbosa-Canovas and Juliano 2008). Consequently, one of the main advantages of HPTS is that adiabatic heat instantaneously produces high product temperatures throughout food packages if relatively high compression rates are applied. Moreover, cooling is very fast during decompression, which could help to reduce process time and enhance product quality (Toepfl and others 2006).

For HPTS treatment, 2 different approaches have been suggested: 1st involves a single pressure pulse (Heinz 1997; Wilson and Baker 2000) and the 2nd involves several pulses (Hayakawa and others 1994; Meyer 2000). However, additional cycles decrease the lifetime of HP equipment and increase maintenance costs, so the multipulse approach is not recommended for technical and economic reasons.

Nonetheless, inactivation kinetics and the mechanisms of bacterial spores must be explored to guarantee that the sterilization process is safe (Heinz and Knorr 1996; Ananta and others 2001; De Heij and others 2005; Margosch and others 2006; Rajan and others 2006; Bull and others 2009; Mathys and others 2009). At ambient temperatures, ungerminated bacterial spores are highly resistant to pressure. They need at least 800 to 1700 MPa to be inactivated, which is far more than what is commercially feasible. However, for various *Bacillus* ssp. spores, inactivation has proven more efficient at ambient temperature and moderate pressure (200 $< p >$ 500 MPa) than at higher pressures ($p >$ 500 MPa) (Heinz and Knorr 1998; Wuytack and others 1998). These scholars have argued that pressure-induced nonnutrient germination may explain this increase in efficiency as it activates the germination receptors of the spores, making them more vulnerable to pressure and heat (Setlow 2003). This HPST treatment is unlikely for the commercial treatment of low acid foods; however, as the extension of spore germination is strongly time- and temperature-dependent, and more importantly, because full germination of the whole entire spore population is not possible.

An additional increase in pressure ($p >$ 500 MPa) activates spore germination by instigating changes in the spore's inner membrane. This process triggers the spores to release both the large depots of dipicolinic acid (DPA) and the associated divalent cations (predominantly Ca^{2+}) from their core via an opening in the spores' Ca^{2+}-DPA channels (Setlow 2003). Hence, spores that lack their nutrient receptors could also germinate at these very HPs (Paidhungat and others 2002); however, the complete inactivation mechanism of bacterial spores is still not fully understood. Inactivation studies of *Geobacillus stearothermophilus* spores yielded the highest heat sensitivity at approximately 700 MPa; higher pressures stabilized the spores, inhibiting them from heat-induced inactivation under pressure (Mathys and others 2009). Similar results were found for *B. amyloliquefaciens* (Margosch and others 2006; Rajan and others 2006) and other strains of *G. stearothermophilus* spores (Ardia 2004a). One possible explanation for the observed behavior would be that pressure at constant temperature and holding time triggers the mobilization of cortex-lytic enzyme (CLE) activities, and inactivates the same enzymes via structural unfolding. Together, these reactions would completely inhibit the CLE activity.

To test this claim, 2 different strains of *Bacillus subtilis* spores lacking germinant receptors and one of 2 CLEs, along with a wild-type strain, were treated in a pressure-stable buffer solution at pressure above 500 MPa under elevated process temperatures.

Materials and Methods

Bacillus subtilis strains and spore preparation

The *B. subtilis* strains used in this study (Table 1) were all isogenic and derivates of strain 168. In the FB strains, all 5 genes encoding the germinant receptors and one of the genes responsible for encoding one of the 2 CLEs (*sleB* and *CwlJ*) were inactivated [FB114 ΔsleB::tet, FB115 ΔcwlJ::tet].

Using a method described elsewhere (Nicholson and Setlow 1990; Paidhungat and Setlow 2000), sporulation was induced at 37 °C on solid 2× SG medium agar plates without antibiotics. The spore suspension was cleaned by repeated centrifugation (3-fold at 5000 g), washed with cold distilled water (4 °C), and was treated occasionally with sonication for 1 min. The cleaned spore suspensions contained ≥95% phase bright spores and nearly no spore agglomerates, as was verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.), and was stored in the dark at 4 °C.

Thermal inactivation of spores

The prepared *B. subtilis* PS832 spore suspension was diluted to an initial concentration of approximately 1×10^8 CFU/mL in a phosphate buffer system (PBS) (pH 7, 0.05 M), due to its stable pH at temperatures above 100 °C (Reineke and others 2010). In addition, the suspension was diluted in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (A-9758, Sigma-Aldrich-Chemie GmbH, Steinheim, Germany), which was used as buffer for the pressure treatments due to its stable pH at HPs (Mathys 2008).

The spore suspension was poured into thin glass capillaries with a volume of 60 μL and was hermitically sealed to prevent evaporation according to a method described elsewhere (Mathys 2008). The inactivation was carried out in a thermostatic bath (cc2, Huber GmbH, Offenburg, Germany) filled with silicon oil (M40.165.10, Huber GmbH) at temperatures between 100 and 110 °C. Following thermal treatment, the samples were immediately transferred into an ice bath. Afterwards, they were serially diluted with $\frac{1}{4}$ Ringer solution (TP887925 712, Merck KGaA, Darmstadt, Germany) in 96-well microtiter plates (Carl Roth GmbH, Karlsruhe, Germany), and 2 50-μL samples of every dilution were spread-plated in petri dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37 °C for 2 d and the colonies were counted.

Due to the strains' inability to germinate on nutrient agar plates without pressure treatment, a counting chamber (Thoma counting chamber, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) was used to determine the total cell count of strains FB114 and FB115.

Pressure treatment and analysis of spore inactivation

For pressure treatment, spores were diluted in ACES buffer solution (pH 7, 0.05 M) with an initial cell count of approximately 1.0×10^8 CFU/mL and split into small aliquots of 1.6 mL. These samples were then filled into plastic tubes (CryoTube Vials, Nunc

Table 1–*Bacillus subtilis* strains used in this study.

Strain	Genotype and phenotype	Source
PS832	Wild type	Paidhungat and Setlow (2000)
FB114	FB72* Δ*sleB*::*tet*	Paidhungat and others (2001)
FB115	FB72* Δ*cwlJ*::*tet*	Paidhungat and others (2001)

*Strain FB 72 Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*tet* (Paidhungat and Setlow 2000).

Brand Products, Rosklide, Denmark) or split again into 300 μL and pipetted into shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany) with an inner diameter of 3 mm and an outer diameter of 3.6 mm. Finally, the tubes were hermitically sealed with a soldering iron. All samples were stored on ice prior to, and following pressure treatment.

An HP sterilization unit (0101–7000-S, Sitec Sieber Engineering AG, Zurich, Switzerland), with a maximum vessel volume of 100 mL and a compression rate of 4 MPa s^{-1}, was used for pressure treatment of the plastic tubes (1.6 mL), and water served as the pressure-transmitting media. The HP vessel was preheated to the designated treatment temperature with a thermostatic bath (Huber GmbH). Water served here as the heating medium. Samples of all 3 spore strains were treated simultaneously under isothermal and isobaric conditions during dwell time (Figure 1). Another plastic tube was used as a dummy container—equipped with a thermocouple in the geometrical center of the tube—to determine the heat of compression during pressure build-up. The cooled samples and the dummy sample (approximately 2 °C) were preheated to the empirically determined starting temperature in the HP vessel. Treatment pressure and temperature were in the range of 550 to 700 MPa and 37 to 80 °C, respectively.

To reduce the influence of the compression time and to further examine the inactivation kinetics at higher treatment pressures (600 to 1000 MPa), a 2nd HP unit (U111 Unipress, Warsaw, Poland) was used. The vessel volume of this pressure apparatus was 3.7 mL, with a compression rate of 25 MPa s^{-1}. Here, Di- 2-ethyl- hexyl- sebacate served as the pressure-transmitting media. The higher adiabatic heat of compression (Reineke and others 2008) compared to water could be neglected, due to the direct measurement of the temperature in a dummy sample. To reach the designated treatment temperature, the pressure vessel was immersed into a thermostatic bath (cc2, Huber GmbH) filled with silicon oil (M40.165.10, Huber GmbH). Due to the size of the smaller HP vessel, the sample volume was reduced to 300 μL. Nonetheless, all 3 spore samples and a dummy sample for the temperature control were treated under isothermal and isobaric conditions (Figure 1) as previously described. After pressure treatment, aliquots of each sample were treated for 20 min at 80 °C to determine the number of germinated and therefore heat-sensitive spores (Wuytack and others 1998).

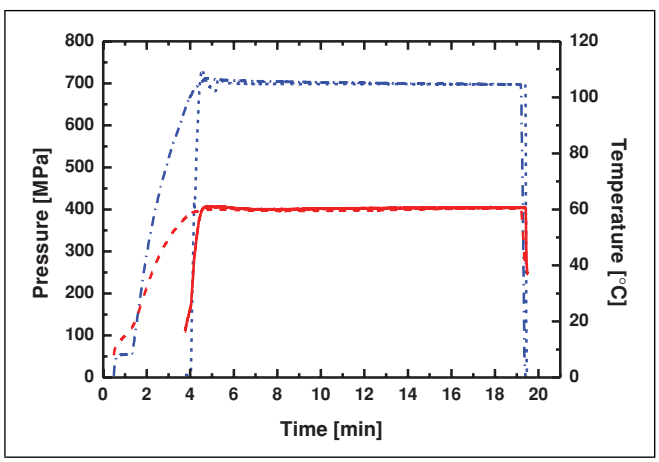

Figure 1–Pressure and sample temperature profiles for SITEC-HP unit pressure (blue, dashed-dotted), temperature (red, dashed) and Unipressp- HP unit pressure (blue, short dashed), temperature (red, solid) for 700 MPa, for a 60 °C treatment and 15 min dwell time.

Quantification of DPA release after pressure treatment

Given the release of the spore's large depot of DPA (approximately 10 % of spore dry weight) during the 1st stage of germination (Setlow 2003), the amount of DPA released could be a good indicator of the amount of germinated spores under pressure immediately after treatment.

To rapidly quantify the DPA, a terbium (Tb)—fluorescent method was used (Rosen 1997; Pellegrino and others 1998). After pressure treatment, 1-mL spore suspension was mixed 1:10 in filter-sterilized TRIS-HCl-$TbCl_3$ solution, which consisted of 50 mM TRIS-HCl buffer (Sigma-Aldrich Chemie GmbH) and 30 μM $TbCl_3$ (Sigma-Aldrich Chemie GmbH). Analytic grade water (Merck KGaA) was used for the preparation of the buffer solution. Fluorescent intensities were measured with a Perkin-Elmer 650–10s fluorescence spectrophotometer (Perkin-Elmer, Waltham, Mass., U.S.A.). The excitation wavelength was set to 270 nm (maximum absorption), and the emission wavelength was scanned with a slide width of 2 nm and a scan speed of 60 nm/min. The amount of released DPA after pressure treatment was determined by the amount of DPA stock solutions prepared in ACES buffer (pH 7, 0.05 M). The peak area of the fluorescence spectra was calculated with OriginPro (Version 7SR1, OriginLab Corp., Northampton, Mass., U.S.A.). A thermal treatment of a part of the sample, for 20 min at 121.1 °C was used to determine the maximum amount of DPA in the spore suspensions (Janssen and others 1958).

Results and Discussion

The results of the thermal inactivation of *B. subtilis* PS832 spores suspended in both of the buffer solutions used in this study are presented in Figure 2. Thermal resistance was only determined for strain PS832 due to the lack of nutrient receptors from the other strains and the accompanying cultivation problems. A nearly linear inactivation in both buffer systems was observed and hence fit with a 1st-order approach, which enabled the calculation of D and *z*-values. Furthermore, a higher inactivation in ACES buffer (pH 7, 0.05 M) compared with PBS buffer (pH 7, 0.05 M) was detected for all tested temperatures. The thermal inactivation in both buffer solutions differed about approximately 2 $\log_{10}$ at 100 °C and approximately 3 $\log_{10}$ at 105 °C and correspondingly the D values in PBS buffer ($D_{100°C}$ = 2.83 min and $D_{105°C}$ = 29.2 s) were significantly higher than in ACES buffer ($D_{100°C}$ = 2.3 min and $D_{105°C}$ = 17.1 s)

This impact of suspending media on the thermal resistance properties of spores was also reported by other authors (Palop and others 1997; Leguerinel and Mafart 2001; Moussa-Boudjemaa and others 2006; Mathys 2008; Reineke and others 2010). Leguerinel and Marfart (2001) attributed the impact of different acids with a same molarity to a shift between dissociated and undissociated forms, occurring under different treatment temperatures. This shift modifies the thermal spore resistance in addition to the amount of oxonium ions. However, they did not consider the impact of the temperature-dependent shift of the pH value in buffer solutions during the heat up. This shift was measured by Reineke and others (2010); however, who found that the thermal pH-shift for PBS was nearly independent of the treatment temperature (ΔT 20 to 130 °C), whereas for ACES a shift of 1.27 pH units (ΔT 20 to 130 °C) was observed. This strong decrease of the pH value in ACES might promote the formation of H-spores with a higher thermal sensitivity (Gould and Dring 1975). Furthermore, this change might explain the wide range of reported D values in the

literature for *B. subtilis* spores in different buffer solution with the same initial pH at 20 °C.

In this study, results of thermal inactivation indicated an absence of biphasic behavior or inactivation at low temperatures (80 °C, 20 min), whereby the spore suspension was suitable for both determining its pressure resistance and a thermal posttreatment. To assess the potential to rapidly quantify ($t < 5$ min after pressure treatment) the amount of spore germination immediately after pressure treatment, the authors tested their ability to measure released DPA via the addition of terbium. Consequently, *B. subtilis* PS832 spores were treated at 550 MPa and 37 °C under isothermal and isobaric conditions during dwell time.

These conditions induced no inactivation in the spore population (Figure 3), which confirmed the findings of other authors (Sale and others 1970; Wuytack and others 1998; Paidhungat and others 2002). However, the subsequent heat treatment demonstrated a continuous increase in heat-sensitive spores with extended dwell time, which indicates a loss of spore dormancy (Wuytack and others 1998). After a lag time of approximately 15 min, the amount of heat-sensitive spores increased up to 3.8 $\log_{10}$ after 120 min dwell, which denotes a germination of almost 99.99 %. Similarly, the quantitative fluorescent-based Tb-DPA method also indicated an enhancement of germinated spores with increasing dwell time (Figure 3).

The continuous rise in DPA concentration correlated with an increase in heat-sensitive spores. This increase was triggered by the release of DPA from the spore core in the 1st stage of germination, resulting in a loss of thermal resistance (Setlow 2000). After 120-min dwell time, the DPA concentration reached its maximum value of 40 μM DPA as was determined by thermal spore disintegration. Much like Heinz (1997), who reported a concentration 4.75×10^{-16} M DPA per spore after mechanical spore destruction, this study yielded 4×10^{-16} M DPA per spore.

Consequently, the Tb-DPA method has proven to be suitable for rapidly detecting ($t < 5$ min) spore germination in a buffer solution immediately after pressure treatment. However, to be able to quantify the data, this method demands a high quantity of spores (approximately 1×10^8 CFU/mL) and a large amount of germinated spores ($>1 \times 10^6$ CFU/mL). Hence, this method could be used in mechanistic studies of spore germination or to rapidly screen treatment conditions. It should not be used to determine spore inactivation; however, as the release of DPA is only an indicator for spore germination (Figure 3), and not for subsequent spore inactivation.

Instead, genetically modified spores were used to investigate the inactivation the behavior of *B. subtilis* PS832 under pressure and to prove that one or both CLEs are inactivated under a certain pressure and temperature range. In these spore strains (FB114 and

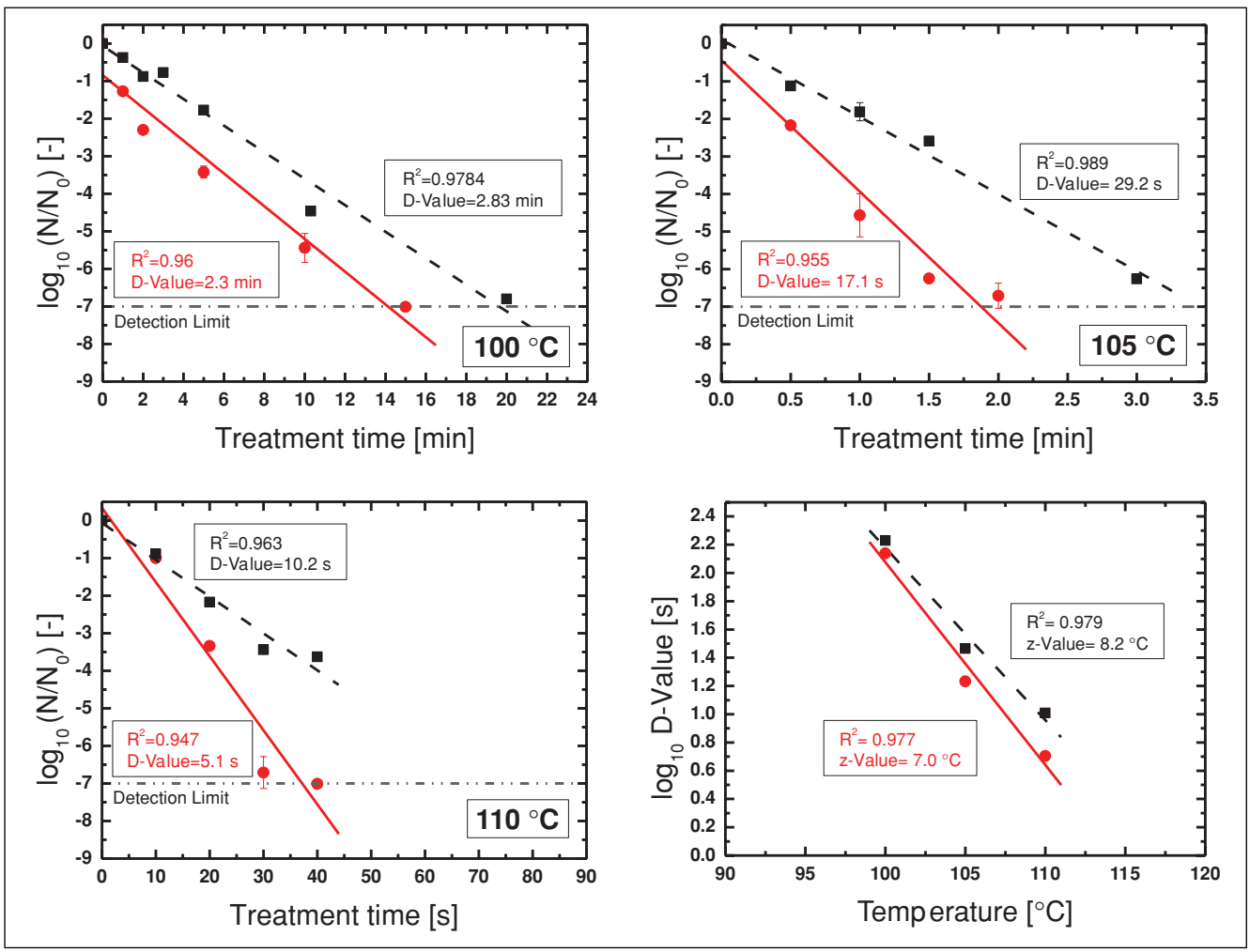

Figure 2–Thermal inactivation of *Bacillus subtilis* PS832 spores in PBS buffer (pH7, 0.05 M) (black, dashed; experimental inactivation [■]) and in ACES buffer (pH 7, 0.05 M) (red, solid; experimental inactivation [●]) at various temperatures and there corresponding D- and *z*-values.

FB115), all genes for encoding the germinant receptors were inactivated as well as the gene responsible for encoding one of the CLEs. To assess inactivation (Figure 4), all 3 strains were treated simultaneously under isothermal and isobaric conditions.

As pressure and temperature increased, an accelerated inactivation was detected for all strains as well as a shoulder and tailing formation. These changes diminished at higher temperatures. Furthermore, the authors observed a clear synergism between pressure and temperature (Figure 2 and 4). For the FB114 and FB115 strains, the CFU/mL on agar plates never reached the total concentration of spores determined by a counting chamber for short dwell times (<5 min) at 600 MPa, 60 to 80 °C and 700 MPa, 60 °C, but steadily increased as the process intensity increased. However, the total amount of spore germination was reached after 5 min (600 MPa, 60 °C), 1 min (600 MPa, 80 °C), or even under dynamic pressure and temperature conditions during compression (1 s dwell time) at 700 MPa, 70 °C. Hence, these findings confirm that at very HPs (p > 500 MPa), spore germination is possible without germinant receptors (Paidhungat and others 2002; Black and others 2005). Moreover, these data underline the increased ability of treatment temperature compared to treatment pressure to induce germination in the whole spore population.

The small amount of germinated spores without pressure treatment (not shown)—instigated by stochastic spontaneous germination (Paidhungat and Setlow 2000)—totaled less than 0.1% of the initial concentration of 1×10^8 spores/mL. Once the maximum CFU/mL on agar plates was reached, both FB strains were inactivated and both kinetics showed the same inactivation rate, indicating that none of the 2 CLEs were inactivated under these pressure and temperature conditions. The wild-type strain PS832 demonstrated similar inactivation behaviors, with germinant receptors and both CLEs intact under 700 MPa at all tested temperatures. Conversely, despite an increase in CFU/mL after dwell times between 1 s and 1 min, inactivation increased under 600 MPa at all treatment temperatures. The germination of superdormant spores might account for the increase in cell counts at short dwell times. However, this concerns only a small percentage of spores in each spore population—those that germinate extremely slowly from nutrients alone, but could be triggered to germinate under HPs (Wei and others 2010). The greatest difference in the level of inactivation, with 2.43 $\log_{10}$, was at 600 MPa and 60 °C after 45 min dwell time, and diminished with increasing process temperature.

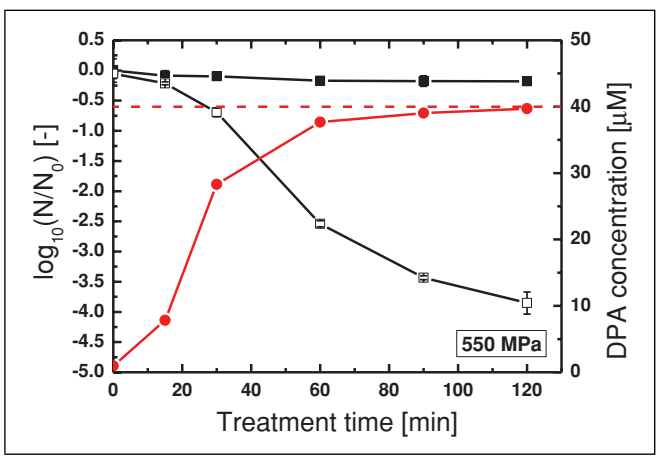

Figure 3–Inactivation kinetic for *Bacillus subtilis* PS832 spores (1 s to 120 min) in ACES (pH7, 0.05 M) treated at 550 MPa, 37 °C (■), with subsequent heat treatment at 80 °C, 20 min (□) after pressure release and its correlating amount of released DPA (●). The dashed line denotes the maximum concentration of DPA in the used spore suspension.

The only difference between strains FB114, FB115, and PS832, aside from the fact that only one CLE is active in strains FB114 and FB115, is the lack of germinant receptors. These findings, therefore, suggest that both pressure-induced germination mechanisms interfere with each other. During compression, germinant receptors were activated at pressures below 300 MPa. At higher pressures (p > 500 MPa), the spore's Ca^{2+}-DPA channels are also directly opened (Setlow 2003). Consequently, when they reach isothermal isobaric conditions at 600 MPa, spores with germinant receptors are already at an advanced stage in the germination process. A pressure increase to 700 MPa diminishes this advance in germination for the PS832 strain more than in both FB strains, due to the increase in the rate of germination under dynamic pressure temperature conditions (Figure 4) and the increase in time needed to compress to 700 MPa. Hence, the shoulder formation found for both FB strains at 600 MPa, 60 to 70 °C and 700 MPa, 60 °C, decreased at higher pressures and temperatures.

To determine the impact of compression time, especially at pressures levels between 600 and 700 MPa, inactivation experiments in an HP unit with a faster compression rate were carried out. At low-pressure levels, where primarily the germinant receptors are triggered, high compression rates ($p \geq$ 300 MPa in less than 12 s) should decrease the impact, and, consequently, the difference in inactivation levels between the PS832 and the FB strains at 600 MPa should reduce. In addition, the pressure range was extended to 1 GPa to investigate the influence of higher pressures on the inactivation behavior of the CLEs, and, afterwards, aliquots of the pressurized samples were treated with heat to determine the amount of germinated spores.

Table 2 lists the impact on spore germination and inactivation under dynamic pressure and temperature conditions during compression (1 s dwell time). Due to the superimposition of the adiabatic heat of compression (approximately 30 °C) and the heat flux of the preheated pressure vessel during compression time (approximately 40 s), no isothermal conditions during the dwell time could be achieved for 1 GPa and 60 °C. Consequently, data for those conditions are missing in the table.

For the wild-type *B. subtilis* (PS832), no significant inactivation was detected after 1 s dwell time, but the quantity of inactivated spores was slightly higher at a slower rate of compression. Furthermore, only a small population of pressurized spores was sensitive to thermal posttreatment. Consequently, these findings suggest that the compression rate influences the increase in inactivation of *B. subtilis* PS832 up to 600 MPa for treatments with longer compression time. Regarding the microbial counts for the strains FB114 and FB115 in table 2, accelerated spore germination (cell count without pressure treatment N_0 approximately 4×10^4 CFU/mL on agar plates) was detected for pressure levels up to 800 MPa (approximately 0.28 $\log_{10}$ for FB114 and approximately 0.18 $\log_{10}$ for FB115 at 60 °C). Again, an increase in the process temperature further accelerated spore germination (for example, approximately 1.04 $\log_{10}$ for FB114 and approximately 1.02 $\log_{10}$ for FB115 between 60 and 80 °C at 800 MPa). Consequently, within a 1 s dwell time at 800 MPa and 80 °C, germination was triggered in the whole spore population for both strains. Alternatively, only 10% of the spores germinated at 800 MPa and 60 °C. An additional increase in pressure ($p \geq$ 900 MPa) reduced the microbial count, indicating the onset of subsequent inactivation of the germinated spores during compression. There were fewer heat-sensitive spores

(approximately 1% to 2 % of the spore population) in the FB114 and FB115 strains compared to the PS832 strain, presumably due to a progressed germination for the strain with germinant receptors.

For extended dwell times under isobaric and isothermal conditions, the amount of heat-sensitive spores for all strains significantly increased, and full inactivation ($\geq$5.7 $\log_{10}$—detection limit) for all pressure and temperature levels could be achieved (Figure 5). These findings indicate the complete germination of the spore population. The increase in heat-sensitive spores accelerated within the first minutes of dwell time and decelerated afterwards. However, all spores were heat-sensitive in 17 % (600 MPa, 60 °C) to 50 % (700 MPa, 80 °C) of the dwell time needed to achieve an inactivation of $\geq$5.7 $\log_{10}$ under pressure.

The short lag phase is most likely due to the release of a sufficient amount of DPA from the first spores in which germination was induced. The acceleration following the lag phase may be attributed to an interaction of spore germination, induced by the continuous release of DPA, and a subsequent triggering of the germination receptors by the already released DPA (Setlow 2003). In addition, the observed tailing may be due to biological diversity in the spore population.

The increase in spore germination as temperature and pressure increased also correlated with the CFU/mL detected with the

Table 2–Microbial counts ($\log_{10}N/N_0$) after 1 s dwell time for *Bacillus subtilis* PS832, FB114, and FB115 with its corresponding amount of thermal sensitive spores*

Temperature	Strain	600 MPa	700 MPa	800 MPa	900 MPa	1 GPa
60 °C	*PS832*	−0.19 ± 0.11	0.1 ± 0.17	0.06 ± 0.12	0.24 ± 0.05	
		−0.17 ± 0.36*	−0.47 ± 0.14*	0.05 ± 0.06*	0.06 ± 0.03*	
	FB114	1.76 ± 0.03	1.95 ± 0.07	2.04 ± 0.08	1.1 ± 0.009	
		−1.05 ± 0.12*	−1.13 ± 0.04*	−1.02 ± 0.08*	−1.06 ± 0.17*	
	FB115	1.77 ± 0.03	1.94 ± 0.07	1.84 ± 0.19	1.76 ± 0.21	
		−0.95 ± 0.09*	−1.29 ± 0.04*	−1.07 ± 0.21*	−0.86 ± 0.23*	
70 °C	*PS832*	0.13 ± 0.10	0.08 ± 0.03	0.04 ± 0.02	0 ± 0.02	0.18 ± 0.01
		−0.09 ± 0.04*	−0.24 ± 0.00*	−0.33 ± 0.00*	−0.12 ± 0.15*	0.05 ± 0.15*
	FB114	01.71 ± 0.14	1.68 ± 0.02	2.65 ± 0.03	2.2 ± 0.1	2.19 ± 0.08
		−0.91 ± 0.27*	−1.04 ± 0.04*	−1.48 ± 0.08*	−1.09 ± 0.14*	−1.25 ± 0.08*
	FB115	1.81 ± 0.11	2.12 ± 0.17	2.7 ± 0.11	2.2 ± 0.06	2.16 ± 0.06
		−0.93 ± 0.10*	−1.69 ± 0.15*	−0.95 ± 0.12*	−0.81 ± 0.11*	−1.28 ± 0.13*
80 °C	*PS832*	−0.08 ± 0.00	−0.02 ± 0.02	0.11 ± 0.02	0.68 ± 0.04	0.16 ± 0.11
		−0.09 ± 0.05*	−0.87 ± 0.11*	−0.15 ± 0.06*	−0.05 ± 0.09*	−0.43 ± 0.07*
	FB114	2.01 ± 0.15	3.01 ± 0.21	3.06 ± 0.08	2.97 ± 0.1	3.04 ± 0.19
		−1.08 ± 0.20*	−1.87 ± 0.04*	−1.37 ± 0.15*	−2.13 ± 0.08*	−2.5 ± 0.25*
	FB115	2.25 ± 0.06	2.51 ± 0.09	2.88 ± 0.11	2.1 ± 0.27	2.18 ± 0.17
		−1.25 ± 0.10*	−1.83 ± 0.06*	−1.93 ± 0.1*	−2.54 ± 0.38*	−2.79 ± 0.11*

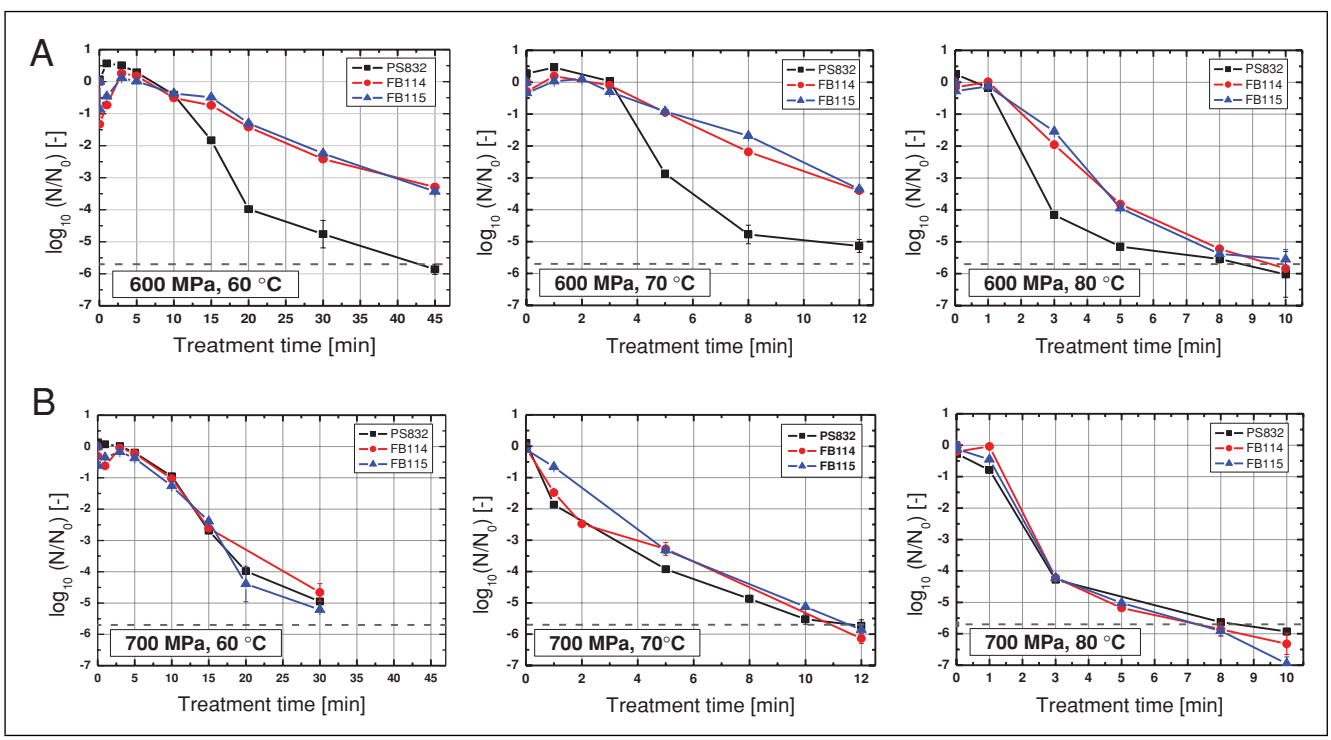

Figure 4–Inactivation kinetics for *Bacillus subtilis* PS832 (wild type) (■), FB114 (Δ*ger*, Δ*sleB*) (●) and FB115 (Δ*ger*, Δ*cwlJ*) (▲) spores in ACES (pH 7, 0.05 M) treated at: A) 600 MPa 60 °C –80 °C and B) 700 MPa 60 °C –80 °C, detection limit (dashed). Including kinetic data for 1 s dwell time.

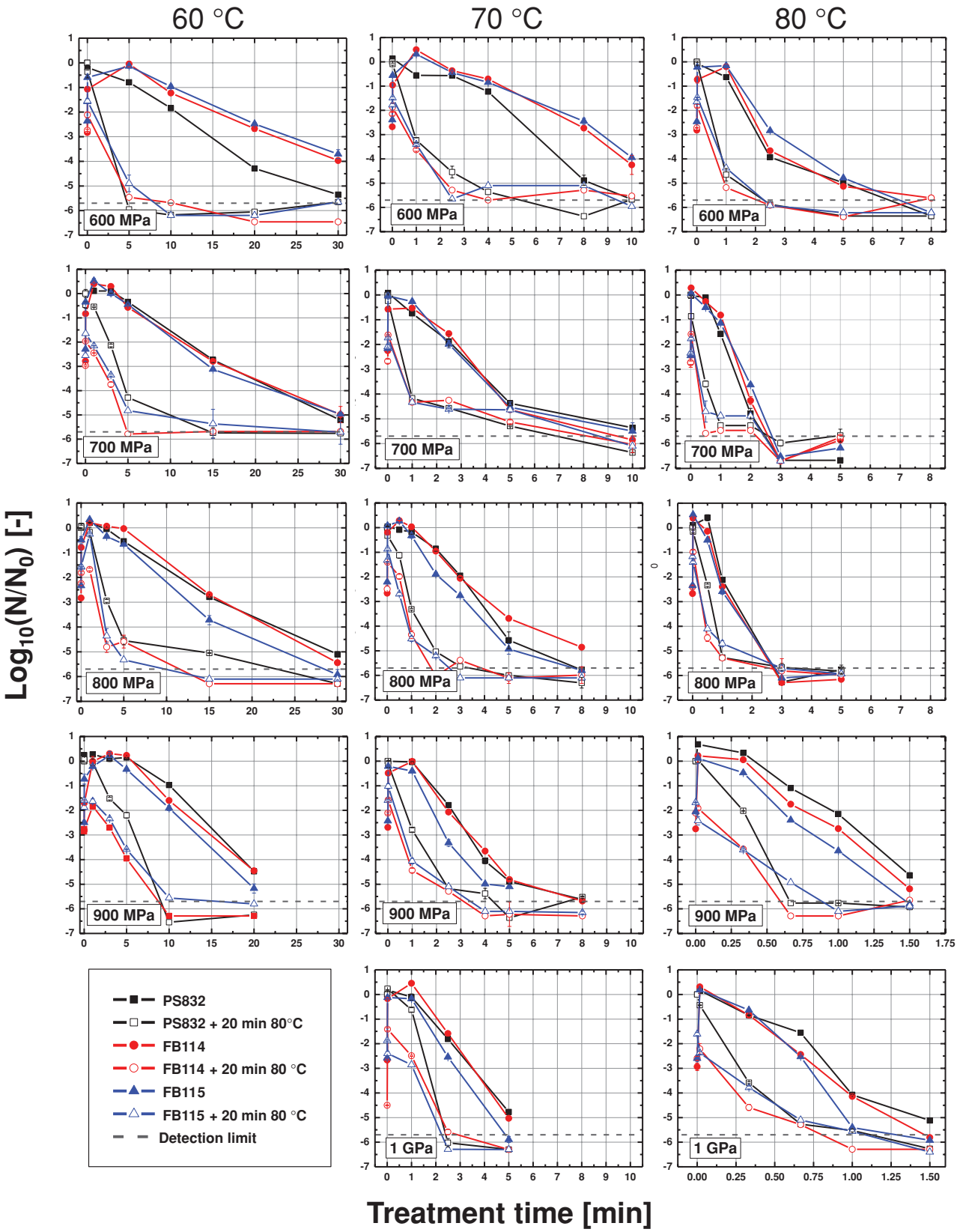

Figure 5–Inactivation kinetics for *Bacillus subtilis* PS832 (wild type) (■), FB114 (Δ*ger*, Δ*sleB*) (●) and FB115 (Δ*ger*, Δ*cwlJ*) (▲) spores in ACES (pH 7, 0.05 M) treated at 600 MPa –1 GPa and 60 °C – 80 °C with subsequent heat treatment at 80 °C, 20 min for PS832 (□), FB114 (○) and FB115 (Δ), detection limit (dashed). Including kinetic data for 1 s dwell time.

FB114 and FB115 strains. Whereas 5 min of dwell time were needed to germinate the whole spore population at 60 °C and 600 MPa, and 2.5 min were needed at 900 MPa, complete germination occurred at 80 °C with pressures ≥700 MPa during the compression phase. Contrary to the observations under slower compression, germination of the whole spore population was possible at 700 MPa and 70 °C (Figure 4), which denote that compression time influenced spore germination under pressure. In addition, treatment temperature had a more pronounced effect on germination than applied pressure.

Moreover, these findings confirm that spore germination is the first step toward spore inactivation under pressure (Knorr 1999; Margosch and others 2006; Mathys and others 2007). At a minimum, this process includes the release of a large portion of DPA from the spore core (Figure 3) in addition to the subsequent partial core hydration, and, therefore, a completion of stage I of the germination process.

When comparing the inactivation of strains PS832, FB114, and FB115 at 600 MPa with inactivation at 700 MPa, a similar trend occurred between the fast (20 s to 600 MPa and 27 s to 700 MPa) and slow (130 s to 600 MPa and 188 s to 700 MPa) rate of compression. The inactivation level of PS832 (wild-type) at 600 MPa was again higher than for both FB strains without germinant receptors, whereas all 3 strains showed the same inactivation behavior at 700 MPa.

Wuytack and others (1998) reported that germinant receptors can be triggered at pressures below 300 MPa (Gould and Sale 1970) and that the germination rate decreases at higher pressures (>500 MPa). Hence, one can assume that in the pressure temperature range used in this investigation, germination is induced by the activation of germination receptors during compression and by a pressure induced opening of the Ca^{2+}-DPA channels at pressures above 500 MPa (Paidhungat and others 2002). The instigation of germinant receptors, however, is slowed down but still active at 600 MPa during dwell time. In that both mechanisms interfere with each other during dwell time, germination of the PS832 spores is faster when under pressure than for strains lacking germinant receptors.

An additional increase in pressure (≥700 MPa) and temperature accelerated the germination of the FB114 and FB115 strains during compression (Table 2). Consequently, the germination triggered by the opening of the Ca^{2+}-DPA channels became the prevalent source of germination, rendering the inactivation kinetics of all 3 spore strains equal. Conversely was the behavior of the inactivation rate, here for instance, a constant 3 $\log_{10}$ inactivation was achieved under 600 to 900 MPa within 15 min at 60 °C for PS832, whereas a temperature increase from 60 to 80 °C under 600 MPa reduced the dwell time to 2 min, which indicates that after the germination was triggered the treatment temperature exerts a dominating influence on the inactivation rate.

For the inactivation of *G. stearothermophilus* at higher pressures (p > 600 MPa), Mathys and others (2009) proposed an extended inactivation mechanism based on Setlow's (2003) germination model for *B. subtilis*. The authors assumed that full cortex hydrolysis (end of stage 2 of spore germination) during pressure dwell time is needed to achieve spore inactivation. Crucial to both cortex hydrolysis and spore core hydration is the activation of the main CLEs. These enzymes most likely exhibit different activities or stabilities under specific pressure and temperature conditions, generating different pathways of inactivation. Heinz and Knorr (1998) argued that pressure and temperature initially trigger the CLEs, but then inactivate them at a later stage. Hence, CLE activity is dependent on pressure, temperature, and time. At ultra HPs, partial core rehydration without any CLE activity may induce additional lethal effects (Ardia 2004a), which could explain the observed pressure stability of some spore strains (Margosch and others 2006; Rajan and others 2006; Mathys and others 2009).

Alternatively, Margosch and others (2006) proposed that bacterial spores are very complex and that, although they stabilize under pressure, the behavior of a single protein or enzyme can hardly be described as determining inactivation unless it is required for a vital function (for example, structural or protective).

As some authors have suggested that pressure at constant temperature and holding time can trigger CLE activities and can later inactivate the same enzymes, it is clear that the 2 CLEs (*sleB* and *cwlJ*) in *B. subtilis* have different pressure temperature stability. Thus, the inactivation rate should be higher for either FB114 (Δ*sleB*) or FB115 (Δ*cwlJ*), and that rate should also be higher than for the PS832 strain. Based on the inactivation kinetics in Figure 5, no further inactivation of one of the FB strains could be observed at pressures up to 700 MPa or treatment temperatures below 80 °C. Nonetheless, between 60 and 70 °C, the degree of inactivation was slightly higher for the FB115 strain (up to 1.2 $\log_{10}$ at pressures ≥800 MPa and 70 °C), indicating that CLE *sleB* may be inactive in this pressure temperature range. At higher treatment temperatures, the effects diminished perhaps due to the high impact of the treatment temperature on the inactivation rate at different pressure levels. However, given that all 3 strains showed the same inactivation behavior, especially at 80 °C, the observed kinetics suggest that the CLEs may not be responsible for spore inactivation under HP and at elevated temperatures. Instead, perhaps HP and high temperatures are inactivating a different target structure responsible for spore germination or outgrow. For the HPST process of *B. subtilis* spores, but most likely also for other spore strains, this means that, the formation of spores with an intact and supplementally hydrated spore core—those are only limited in outgrow by inactive CLEs—could be excluded. Setlow and others (2002) found that spores with inactive CLEs could germinate normally after the addition of lysozyme, which may indicate a latent risk of food spoilage, if such spore would be present in a HPST-treated food.

However, to prove if the CLEs are active under pressure and full cortex degradation occurs, flow cytometry (Black and others 2006; Mathys and others 2007) or transmission electron micrographs of HP-treated spores may be used.

Conclusion

During this study, it was confirmed that spores treated with HP (Figure 3) complete stage I of germination. Furthermore, no inactivation was observed for moderate treatment temperatures under HP (550 MPa, 37 °C, 120 min), and spore germination accelerated with increasing pressure and temperature. Above a certain threshold pressure ($p \geq$ 600 MPa), however, the treatment temperature plays the primary role. It could be assumed, therefore, that for HPST on an industrial scale, once this threshold pressure has been reached, a simple increase in process temperature would reduce the processing time. However, this threshold pressure is presumably spore strain specific and must be determined individually for the target spore strain in the matrix for the food treated.

Nevertheless, for higher treatment temperatures, especially at 80 °C, a synergism between pressure and temperature was detected, where lethal effect increased with rising pressure. Furthermore, it was assumed that HP and temperature could inactivate

the CLEs, which could explain the observed stability under pressure for some spore strains. Despite a slight increase in inactivation of FB115 at 60 and 70 °C, the equal inactivation of all 3 tested spore strains fails to support the hypothesis that CLE are responsible for the reported pressure sensitivity and stabilization at certain pressure range. Consequently, HP and elevated temperature may be inactivating a different target structure responsible for spore germination or outgrow.

However, further investigation of this assumption and a clarification of the underlying inactivation mechanism of bacterial spores during the HPST are needed. Furthermore, if a synergism between pressure and elevated temperature on the inactivation rate is identified; future research could facilitate optimization of this emerging technology.

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5. The different pathways of spore germination and inactivation in dependence of pressure and temperature

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The different pathways of spore germination and inactivation in dependence of pressure and temperature

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ABSTRACT

Bacillus subtilis spores (PS832) and isogenic strains, which lack the germinant receptors (FB114 and FB115), were treated up to 24 h between 100 and 700 MPa and 30–80 °C to improve the understanding of spore inactivation during the high pressure thermal sterilization. Flow cytometry was used to analyze the physiological state of the spores after pressure-treatment. A two step kinetic model was capable to derive a global model with an acceptable fit for all strains.

The kinetic data demonstrated that spore germination without germination receptors is possible even at 300 MPa and no inactivation occurred up to 700 MPa and 50 °C. Contrary for PS832, a spore inactivation of more than 3 $\log_{10}$ was detected at 300 MPa, whereas no inactivation was observed at 550 MPa and moderate temperature.

Possibly, spores with nutrient receptors could proceed through stage II of germination at low pressure levels, whereas this step is retarded at higher pressures.

Industrial relevance: The high pressure thermal sterilization (HPTS) is an emerging technology to reduce the thermal load of a sterilized food product, which consequently increases its sensorial and nutritional quality. However, an industrial installation of this promising technology has not been implemented, yet. An improved understanding of the underlying inactivation mechanisms of bacterial spores may contribute to an optimization of this process.

The kinetic data of the current manuscript proves that a significant spore inactivation is possible at moderate treatment pressures and temperatures for long pressure dwell times. However, for a full spore inactivation in an economical treatment time, high pressures and elevated temperatures are needed. For *B. subtilis* a threshold pressure of 600 MPa was identified, whereas above this pressure the process temperature dominates the inactivation rate.

Furthermore, the used two step kinetic model, applied for different kinetic data sets showed an excellent fit in the shared pressure–temperature range, which confirms that inactivation data derived under isothermal isobaric conditions could be transferred to other HP units.

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1. Introduction

High pressure thermal sterilization (HPTS) is a promising alternative process strategy to produce a sterile food product with reduced thermal load and hence, with a higher nutritional value (Hendrickx & Knorr, 2002; Knorr, Froehling, Jaeger, et al., 2011). In general HPTS preserves quality attributes like vitamins, color, texture and taste of sterilized food much better than the classical retorting (Lau & Turek, 2007). Consequently, the HPTS process is rapidly gaining industrial interest, especially due to the acceptance of the pressure assisted thermal sterilization (PATS) by the US Food and Drug Administration in 2009. However, to successfully implement this promising technology into the food industry, further information about the germination and inactivation mechanisms of bacterial endospores under high pressure is needed. In this regard bacterial endospores require special attention, because of their high resistance to wet and dry heat, freezing and thawing, chemical agents, disinfectants, irradiation and high pressure (Setlow, 2003). These high resistance properties are attributed to a number of unique features of endospores, like the thick peptidoglycan layer called the cortex and the inner membrane, which has a relatively low permeability to hydrophilic molecules. Further to the high level of dipicolinic acid (DPA) associated with Ca^{2+}-Ions (Setlow, 2003) in the spore core, which is responsible to the core's low water content, in which water is supposed to be in a free but highly viscous state (de Vries, 2006; Sunde, Setlow, Hederstedt, et al., 2009).

As long as a spore stays in the dormant state, it causes no problems in the food, due to its metabolic dormancy. However, a germination followed by an outgrowth of the spore, could cause food spoilage

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during storage with a high risk for food born diseases after consumption of these foods (Collins & East, 1998). Consequently, low acid canned foods are extensively thermal processed to eliminate 12 $\log_{10}$ cycles (12D) of *Clostridium botulinum*, that requires at least 2.52 min at 121.1 °C (Odlaug & Pflug, 1977). Besides the physical and chemical changes that occur in the food at this high temperature, the retorting process is accompanied by long heat up and cooling down times.

To reduce this unwanted over processing, a combination of a preheating step to e.g. 90 °C and the adiabatic heat of compression could be used to rapidly achieve 121.1 °C, which is one of the advantages of isostatic high pressure treatment (Toepfl, Mathys, Heinz, et al., 2006) and used in the PATS process. Further, the combination of high pressure and elevated temperatures could significantly reduce the treatment temperature to inactivate target spore formers for the sterilization process like e.g. *C. botulinum* (Margosch, Ehrmann, Buckow, et al., 2006), *Bacillus amyloliquefaciens* (Rajan, Ahn, Balasubramaniam, et al., 2006) or *Geobacillus stearothermophilus* (Mathys, Reineke, Heinz, et al., 2009).

In dependence of the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, which could induce spore germination or a subsequent inactivation during the treatment. At pressures between 150 MPa and 400 MPa and ambient temperatures spore germination is induced without the presence of nutrients (Gould & Sale, 1970), by triggering of the spores' nutrient receptors (Setlow, 2003).

The activation of the nutrient receptors causes a subsequent release of DPA, which triggers the activation of the cortex lytic enzymes (CLEs) that degrade the spore cortex. In the case for *Bacillus subtilis*, there are two CLEs, CwlJ and SleB, either of which alone is sufficient for spore germination. In regard to CwlJ, a direct activation by the released DPA from the spore core or by exogenous DPA is possible. In contrast, SleB could not be directly activated by DPA and the precise activation mechanism is still under discussion (Paidhungat et al., 2002).

Moreover, for various *Bacillus* ssp. spores, the following inactivation under pressure has proven more efficient at ambient temperature and moderate pressure (200 MPa$<p>$500 MPa) than at higher pressures ($p>$500 MPa) (Heinz & Knorr, 1998; Wuytack, Boven, & Michiels, 1998). However, as full germination of the whole entire spore population is not possible (Heinz & Knorr, 1998; Wuytack et al., 1998), this tyndallization approach failed to achieve a commercial sterile product.

A treatment pressure above 500 MPa combined with elevated temperatures ($T>$60 °C), could induce a rapid spore germination via an opening of the spores' Ca^{2+}-DPA channels (Paidhungat, Setlow, Daniels, et al., 2002), which is accompanied by the release of the large depots of DPA and the associated divalent cations (predominantly Ca^{2+}) from their core. Hence, spores that lack their nutrient receptors could also germinate at these very high pressures (Paidhungat et al., 2002; Reineke, Mathys, & Knorr, 2011).

However, the complete inactivation mechanism of bacterial spores is still not fully understood, but has to be explored to guarantee, that the sterilization process is safe (Heinz & Knorr, 1996; Margosch et al., 2006; Mathys et al., 2009) and optimized.

The aim of this study was to investigate the first rate determining step for the HPTS process in detail, which is the spore germination. Therefore *B. subtilis* and isogenic strains, which lack their nutrient receptors, were treated in a broad pressure–temperature range and the germination as well as inactivation kinetics were modeled as isorate lines in pressure–temperature diagrams.

2. Materials and methods

2.1. Sporulation of B. subtilis

All *B. subtilis* strains used in this study (Table 1) were isogenic and derivates of the strain 168. Further, the strains FB114 and FB115 lack all genes, which are responsible for the encoding of their germinant receptors as well as one gene responsible for the encoding of one of the two CLEs (SleB and CwlJ).

Table 1
Bacillus subtilis strains used in this study.

Strain	Genotype and phenotype	Source
PS832	Wild-type	Paidhungat and Setlow (2000)
FB114	FB72[a] Δ*sleB*::*tet*	Paidhungat et al. (2001)
FB115	FB72[a] Δ*cwlJ*::*tet*	Paidhungat et al. (2001)

[a] Strain FB 72 Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*tet* (Paidhungat & Setlow, 2000)

To induce a sporulation in the three used spore strains, a method described elsewhere (Nicholson & Setlow, 1990) was used. Cell suspensions of vegetative cells were spread plated on 2×SG medium agar plates without antibiotics. After sporulation the spores were harvested and the suspension was cleaned by repeated centrifugation (3-fold at 5000 *g*), washed with cold distilled water (4 °C), and sonicated for 1 min. The cleaned spore suspension contained approximately 1.0×10^9 spores/mL (≥95% phase bright) and nearly no spore agglomerates, as was verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.).

2.2. Pressure treatment and analysis of spore germination and inactivation

For pressure treatment, each spore suspension was diluted in pressure stable N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer solution (Mathys, Kallmeyer, Heinz, & Knorr, 2008) (pH 7, 0.05 M) with an initial cell count of approximately 1.0×10^8 spores/mL. 1.6 mL of each sample was filled into plastic tubes with screw caps (CryoTube Vials, Nunc Brand Products, Roskilde, Denmark). Prior and after the pressure treatment, all samples were stored on ice.

For the pressure trials a HP sterilization unit (0101-7000-S, Sitec Sieber Engineering AG, Zurich, Switzerland), with a compression rate of 4 MPa/s and 100 mL vessel volume was used, where water served as pressure transmitting media. The double jacket HP vessel was preheated to the designated treatment temperature with a thermostatic bath (Huber ministat, Huber GmbH, Offenburg, Germany). The ice cooled samples of all three strains were treated simultaneously under isothermal and isobaric conditions during dwell time. To reach this isothermal conditions, the individual starting temperature for each temperature level was determined empirical prior the pressure trails, whereas the temperature profiles were recorded in geometrical center of a dummy container (1.6 mL, CryoTube Vials, Nunc Brand Products, Roskilde, Denmark), equipped with a J-Type thermocouple (Fig. 1).

After pressure treatment, aliquots of each spore sample (500 μL) were mild heat treated (20 min at 80 °C) to determine the number of germinated and thus heat-sensitive spores (Wuytack et al., 1998) for the wild-type spore strain PS832. Afterwards, all samples were serially diluted with 1/4 Ringer solution (TP887925 712, Merck KGaA, Darmstadt, Germany) in 96-well microtiter plates (Carl Roth GmbH, Karlsruhe, Germany) and 50 μL of each sample of every dilution was spread-plated in duplicates in petri dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37 °C for 2 days and the colony forming units (CFU) were counted. The determination of surviving cells was done at least in duplicates.

The total cell count of the strains FB114 and FB115 (N_0) was determined via a counting chamber (Thoma counting chamber, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), because of the inability of these strains to germinate on nutrient agar plates without e.g. a pressure treatment. Further, this attribute of FB114 and FB115 was used to determine the amount of germinated spores after pressure treatment.

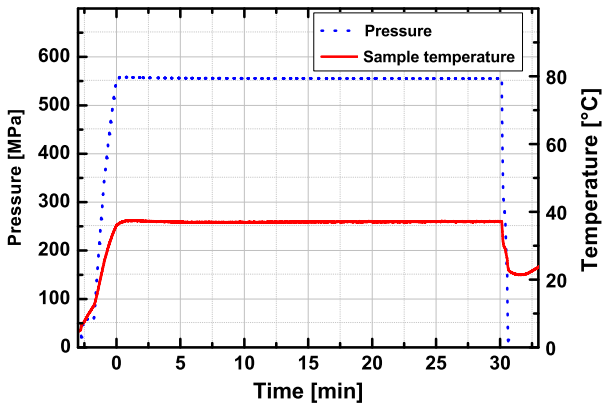

Fig. 1. Pressure (blue, dotted) and sample temperature (red, solid) for a 550 MPa, 37 °C treatment with 30 min pressure dwell time.

2.3. Flow cytometry analysis

For flow cytometry analysis, a double staining with SYTO16 (Invitrogen, Carlsbad, CA, USA) and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA), according to the method of Mathys, Chapman, Bull, et al. (2007), was used. Both fluorescent dyes are able to stain DNA, whereas the membrane permeant SYTO16 acts as an indicator for spore germination and the membrane impermeant PI as an indicator for membrane damage. The analysis was carried out by using a BD FACSCalibur flow cytometer (BD Biosince, San José, CA, USA) equipped with a 15 mW, 488 nm air-cooled argon ion laser. BD CellQuest Pro (Version 5.2, BD Biosciences) was used as operation and acquisition software. To achieve a clear determination of the different spore populations and to discriminate non-spore-particles, the amplification of the forward scatter signal (FSC) was set to E01 (10-fold) and FSC threshold was set to 72. The amplification of the side scatter signal (SSC) was set to 721 V and SSC threshold was set to 706. The treated spore suspensions were diluted with ACES buffer solution (0.05 M, pH 7) to achieve a flow rate of about 1000 events/s as well as a constant ratio of spores for the staining. The concentrations of the fluorescent dyes for staining, were 15 μM PI and 0.5 μM SYTO16 in the diluted spore suspension. Afterwards, the samples were stored in the dark at ambient temperature for 15 min. The analysis of the FCM data as well as the gating to identify germinated spores, was done according to Baier, Reineke, Doehner, et al. (2011). All analyses were done at least in duplicates.

2.4. Modeling approaches

For the analysis of germination and inactivation kinetics a multiresponse kinetic model was tested to derive a global model, in order to calculate a pressure–temperature isorate diagram.

Based on the germination and inactivation data for isobaric and isothermal conditions ($t_0 = 1$ s dwell time), a two step model (Eq. (1)) was suggested, based on a mechanistic model for chemical reactions, and solved with a non-linear multiresponse regression.

$$\text{dormant} \xrightarrow{k_a} \text{germinated} \xrightarrow{k_b} \text{inactivated}. \tag{1}$$

The set of the used differential rate equations was:

$$\frac{d[\text{dormant}]}{dt} = -k_a(p,T)[\text{dormant}] \tag{2}$$

$$\frac{d[\text{germinated}]}{dt} = k_a(p,T)[\text{dormant}] - k_b(p,T)[\text{germinated}] \tag{3}$$

$$\frac{d[\text{inactivated}]}{dt} = k_b(p,T)[\text{germinated}] \tag{4}$$

with k_a and k_b as rate constants.

Similar models have been previously developed and analyzed for high pressure inactivation kinetics of *B. subtilis* (Heinz & Knorr, 1996) and *Bacillus licheniformis* (Mathys, Chapman, Bull, et al., 2007) as well as for growth and death kinetics of *Staphylococcus aureus* (Taub, Feeherry, Ross, et al., 2003).

The set of differential equations (Eqs. (2)–(4)) was solved with the differential equation solver Berkeley Madonna (Version 8.0.1, R. I. Mackey & G.F. Oster, University of California at Berkeley, CA, USA).

To obtain a functional relationship of the rate constants k to pressure and temperature a surface fit with second order polynomial equations was done with TableCurve 3D version 3 (SPSS Inc., Chicago, IL, USA). The isokineticity lines were calculated with MathCAD 15 (Mathsoft Engineering & Education, Inc., USA) with the derived rate equations from Eqs. (2)–(4):

$$[\text{dormant}] = [\text{dormant}]_0 \exp(-k_a t) \tag{5}$$

$$[\text{germinated}] = [\text{germinated}]_0 \exp(-k_b t) + \frac{k_a[\text{dormant}]_0}{k_b - k_a}\left(\exp(k_a t) - \exp(-k_b t)\right) \tag{6}$$

$$[\text{inactivated}] = [\text{inactivated}]_0 + [\text{germinated}]_0 \times \left[1 - \exp(-k_b t) + [\text{dormant}]_0 \times \left[1 + \frac{k_a \exp(-k_2 t) - k_b \exp(-k_a t)}{k_b - k_a}\right]\right] \tag{7}$$

and were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

The aim of this study was to investigate the germination rate in detail and how it is influenced by different combinations of pressure and temperature, because of its important role as the first rate determine step in the HPTS process. The second goal was to develop isorate pressure–temperature diagrams for both spore germination and inactivation under isothermal isobaric conditions. Consequently, the germination rates under dynamic (compression and decompression) and under isothermal and isobaric conditions (pressure dwell time), have to be evaluated separately.

3.1. Germination under dynamic pressure–temperature conditions

For the wild-type *B. subtilis* strain PS832 the mild thermal post-treatment does not denote a significant increase in thermal sensitive and hence germinated spores for a compression to pressures and temperatures below 500 MPa and 50 °C (1 s pressure dwell time). However, pressure of 600–700 MPa and treatment temperatures of 50 °C and 60 °C, caused a spore germination in 0.3 $\log_{10}$ of the pressurized spore suspension (data not shown). This trend is consistent with previous reported data for higher pressures (600 MPa–1 GPa) and temperatures (60–80 °C), were a similar germination rate was reported for 700 MPa and 60 °C (Reineke, Mathys, & Knorr, 2011).

Moreover, for nearly all applied pressure ($p \leq 600$ MPa) and temperature combinations an increase of 0.3 $\log_{10}$ CFU compared to the untreated samples was detected (data not shown). This "shoulder" formation for moderate treatment temperatures under pressure is not unusual and was as well reported for thermal treatments. A possible explanation for this increase in CFU/mL after short pressure dwells might be the disaggregation of spore agglomerates (Mathys,

Table 2
Microbial counts ($\log_{10}N/N_0$) after 1 s dwell time for *Bacillus subtilis* FB114 and FB115, with 3.00 $\log_{10}$ denoting 100% germination of FB114 and 2.6 $\log_{10}$ denoting 100% germination of FB115, respectively.

Temperature	Strain	400 MPa	500 MPa	600 MPa	700 MPa
50 °C	FB114	0[a]	0[a]	1.76 ± 0.109	1.14 ± 0.166
	FB115	0[a]	0[a]	1.5 ± 0.36	1.21 ± 0.13
60 °C	FB114	0.936 ± 0.18	1.75 ± 0.213	2.4 ± 0.254	2.78 ± 0.199
	FB115	1.01 ± 0.16	1.44 ± 0.277	1.7 ± 0.37	2.6 ± 0.305

[a] No significant spore germination was detected.

Heinz, Schwartz, et al., 2007), which could be neglected in this study due to the very small amount of agglomerates in the used spore suspension or it could be caused by the germination of superdormant spores (Wei, Shah, Ghosh, et al., 2010).

Regarding the germination behavior of both FB-strains under dynamic pressure–temperature conditions, one has to take into account, that they are unable to germinate on nutrient agar plates, because of the lack of germinant receptors. However, a small amount of spores, which totaled less than 0.1% of the initial spore concentration of 1×10^8 spores/mL, germinated spontaneous, which decreased the detection limit from 6.3 $\log_{10}$ for the strain PS832 to 3 $\log_{10}$ for the FB-strains.

This stochastic spontaneous germination for the strains FB114 and FB115 was also previously reported by Paidhungat and Setlow (2000). The author also reported that both FB-strains are able to germinate, if pressures of 550 MPa are applied (Paidhungat et al., 2002). This finding could be confirmed for the germination behavior during the compression time for pressures below 500 MPa and 50 °C (Table 2). Further, at some pressure–temperature combinations germination, detected by plate counts, could be induced even during the compression (Table 2).

However, in this regard one has to mention that the amount of heat sensitive spores increased to a lower extent than the amount of germinated spores, detected as an increase in CFU/mL compared to the non-pressurized sample (N_0) (Fig. 2 and Supplementary Fig. 1).

Consequently, this finding points towards a retarded germination progress for both FB-strains compared to the wild-type strain PS832, which could be attributed to the lack of germinant receptors. Furthermore, an accelerated germination rate with increasing pressure and temperature could be identified (Table 2), which confirms previously reported data for these two strains treated at higher pressures and temperatures (Reineke, Mathys, & Knorr, 2011). The higher amount of germinated spores at 60 °C and 600–700 MPa reported in this study is probably attributed to the longer compression time needed to reach the final pressure (180 s vs. 28 s compression time to reach 700 MPa).

3.2. Germination under isothermal isobaric conditions

The germination behavior of *B. subtilis* FB114 under isothermal isobaric conditions is presented in Fig. 2. Kinetic data for 600–700 MPa at 30 °C are missing, due to the high adiabatic heat of compression, which made it impossible to realize isothermal conditions at these high pressures with the used compression rate.

Again, it could be confirmed that spore germination without the presence of nutrient receptors is possible above 500 MPa (Setlow, 2003). Moreover, even at 200 MPa at all tested temperature levels a significant (≥2 $\log_{10}$) germination was detected after 40 min dwell time. Extended dwell times (up to 2 h) further increased the amount of germinated cells at 30 °C and no spore inactivation was detected.

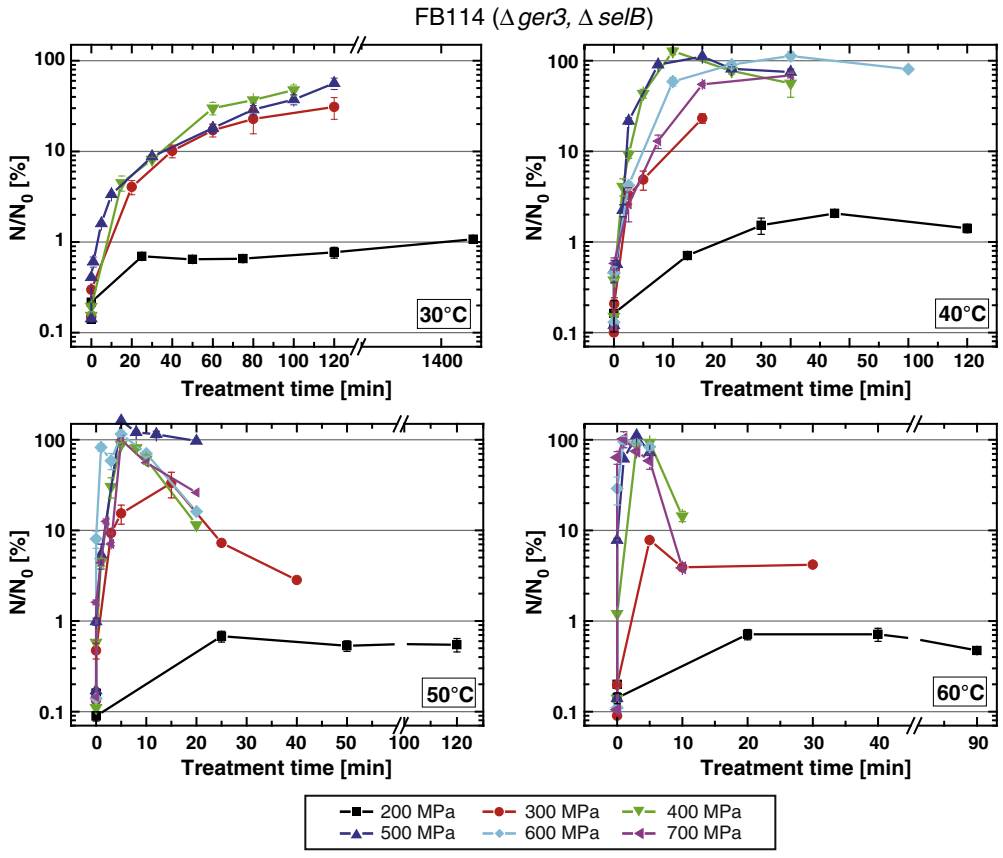

Fig. 2. Germination behavior of *Bacillus subtilis* FB114 (Δ*ger3*, Δ*selB*) under isothermal (30 °C–60 °C) and isobaric (200 MPa–700 MPa) conditions.

Consequently, the reported pressure level of 500 MPa (Black, Wei, Atluri, et al., 2006; Paidhungat et al., 2002; Setlow, 2003; Wuytack et al., 1998), in literature, which is needed for direct opening of the spores Ca^{2+}-DPA-channels and hence to induce a non-nutrient germination has to be lowered to 200 MPa.

However, especially at 30 °C the amount of thermal sensitive spores (post-treatment at 80 °C for 20 min) for both FB-strains increased to a lower extent than the amount of germinated spores, detected as an increase in CFU/mL compared to the non-pressurized sample (N_0) (data in the Supplementary material). This finding implies that the germination process is irreversibly triggered, but that a completion of stage I of germination, which is accompanied with an increased heat sensitivity of the spores, is not possible at 30 °C even after 2 h under pressure. With increasing temperatures (≥40 °C) and pressures (≥400 MPa), this effect is diminished, which will be discussed in Section 3.4.

Further, an increase of pressure and temperature highly accelerated the germination rate. Whereas only in 56% of the whole spore population a germination could be induced at 30 °C and 500 MPa after a 2 h treatment, a full germination (100%) was detected after 20 min at 40 °C, 5 min at 50 °C and 3 min at 60 °C at the same pressure level (Fig. 2). At temperatures above 50 °C additionally a spore inactivation was noticed for all pressure levels, whereas for all kinetics (despite at 300 MPa) prior to an inactivation a germination of approximately 100% was reached, which underlines that the spore germination is the first step of spore inactivation under pressure (Margosch et al., 2006; Mathys, Chapman, Bull, et al., 2007; Wuytack et al., 1998). However, a complete germination of the entire spore population at 300 MPa could not be excluded.

The germination and inactivation behaviors of the wild-type *B. subtilis* strain PS832 (Fig. 3) were different compared to both FB-strains (Fig. 2). For PS832 an inactivation of ≥2 $\log_{10}$ was achieved at 200–300 MPa after 2 h of pressure treatment at 30 °C, whereas no inactivation was detected at pressure above 300 MPa. Furthermore, the inactivation rate at 200 MPa was higher and additionally no lag-phase like at 300 MPa (first 50 min) occurred (Fig. 3a). This pronounced lag phase at 300 MPa was not detected during the thermal post-treatment (Fig. 3b), but the highest germination rate was found at 200 MPa, as well. Fig. 3b further confirms that prior to the inactivation germination took place, whereas the kinetic data points towards a different pathway of inactivation below 400 MPa in comparison to higher process pressures. Wuytack et al. (1998), reported that spores treated at 100 MPa were more sensitive to UV-light and hydrogen peroxide, which she attributed to a degradation of the small soluble acid proteins (SASPs) that occurs during stage II of spore germination (Setlow, 2003). Hence it could be assumed, that this SASP degradation and consequently the completion of stage II of germination could be possible up to 400 MPa, with an accompanying increased in pressure sensitivity of these spores.

The nearly first order inactivation tendency of PS832 during the first 2 h of pressure treatment does not continue for extended dwell times (24 h). After germination was triggered in the spore population (Fig. 3b) a subsequent inactivation of ~3 $\log_{10}$ or 0.1%, respectively, was detected (Fig. 3a). However, for extended dwell times the existence of a pressure resistant subpopulation, which was unable to germinate under pressure, was found.

An increase of the process temperature highly accelerated the germination and inactivation rates. Interestingly at 40 °C an inactivation up to 400 MPa was detected (data not shown), which was not the case for 500–700 MPa. Temperatures of 50 °C and above enabled a spore inactivation at all tested pressure levels, with highest germination and inactivation rates for pressures ≤500 MPa.

3.3. Model evaluation

In this study a two step kinetic model based on simple differential chemical equations was used to derive a second order or global model, respectively, from all generated data. Previously, other modeling approaches, like a double Weibullian model or an nth-order model were tested (Reineke, Doehner, Baier, et al., 2011). However, the double Weibullian model:

$$\log S(t) = b_1 \cdot t^{n_1} - b_2 \cdot t^{n_2} \tag{8}$$

with $\log S(t) = N/N_0$ (with N_0 as the initial spore count), b_1 and b_2 as scale parameters and n_1 and n_2 as the so called shape parameters (Van Boekel, 2009) as well as the nth-order model:

$$\log \frac{N}{N_0} = \frac{1}{1-n} \log\left(1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1)\right) \tag{9}$$

with N and N_0 = spore counts (CFU/mL) at time t and t_0, respectively, t = time, k = inactivation rate constant and n = reaction order, failed to derive a second order model with an acceptable fit, for spore inactivation kinetics after HPTS processing (Reineke, Doehner, Baier, et al., 2011).

The advantage of the used multiresponse models in this study, in comparison to uniresponse models (e.g. Eqs. (8), (9)), is that parameter-estimates become more precise, due to that the rate constants $k_a(p,T)$ and $k_b(p,T)$ can be calculated from multiple data (Van Boekel, 2009). Hence, by the use of the differential equation solver, it was possible to calculate a rate constant for the germination (k_a) and

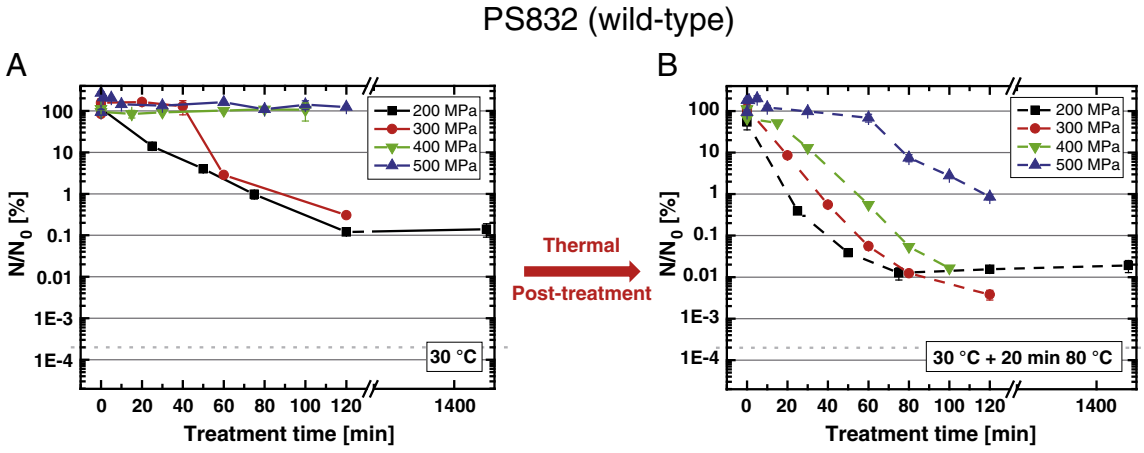

Fig. 3. A. Inactivation kinetics for *Bacillus subtilis* PS832 (wild-type), under isothermal (30 °C) and isobaric conditions (200–500 MPa) during dwell time. B. Inactivation kinetics for *B. subtilis* PS832 (wild-type), after a thermal post-treatment (20 min at 80 °C) as indicator for spore germination. The dotted line in dark gray represents the detection limit.

Table 3
Derived rate constants $\ln(k_a)$ and $\ln(k_b)$ in [1/s] from Eqs. (3) and (4) for the *Bacillus subtilis* strains PS832 (wild-type), FB114 (Δger, ΔsleB) and FB115 (Δger, ΔcwlJ).

Temperature [°C]	Pressure [MPa]	PS832 $\ln(k_a)$	PS832 $\ln(k_b)$	FB114 $\ln(k_a)$	FB115 $\ln(k_a)$
30	200	−8.634	−7.797	−9.916	−9.511
	300	−8.575	−8.297	−7.854	−7.651
	400	−9.007	−8.864	−7.202	−6.982
	500	−9.893	−9.667	−7.874	−7.815
40	200	−8.408	−6.333	−9.079	−9.673
	300	−7.478	−7.106	−6.420	−6.237
	400	−7.610	−8.653	−5.771	−5.572
	500	−7.839	−10.277	−5.904	−5.684
	600	−8.458	−11.971	−6.367	−6.610
	700	−9.463	−13.046	−6.997	−7.263
50	200	−8.143	−6.322	−9.336	−10.128
	300	−6.773	−6.75	−5.695	−6.259
	400	−6.730	−7.106	−4.903	−5.435
	500	−6.753	−9.972	−4.948	−5.337
	600	−7.470	−11.034	−3.418	−3.896
	700	−7.770	−11.894	−5.300	−5.472
60	200	−7.922	−6.802	−9.409	−10.709
	300	−6.451	−6.047	−5.612	−6.120
	400	−6.071	−6.093	−3.898	−3.763
	500	−5.465	−6.795	−3.793	−4.176
	600	−5.177	−7.297	−2.863	−3.840
	700	−5.773	−6.866	−2.751	−3.012

the inactivation (k_b) simultaneously, for each pressure–temperature combination. The individual rate constants k_a for the germination of all three investigated strains are presented in Table 3.

By the use of these rate constants it was possible to derive a second order model, which enabled the calculation of isorate lines for the germination behavior under pressure and temperature for various dwell times (Figs. 4 and 5). To display accuracy of fit, Fig. 4 also contains a comparison of calculated and experimental data. The residuals are nearly randomly distributed and hence, it is assumed that no heteroscedastic error is present. The good agreement of calculated and measured germination is as well found in the coefficient of determination of 0.975 for FB114 and 0.971 for FB115, respectively.

The isorate lines in Fig. 4a clearly depict that above 200 MPa a germination of both FB-strains is possible at all tested temperature conditions, whereas with increasing temperature the time needed to achieve a 2 $\log_{10}$ germination dramatically decreased. This finding underlines the prominent role of high treatment temperatures, if germination should by induced in a relatively short process time (Reineke, Mathys, & Knorr, 2011), which is relevant for the industrial HPTS process, due to the prominent role of spore germination as the first rate limiting step of the subsequent inactivation.

Further the optimum for the pressure induced germination under the tested conditions is present at around 300 MPa and 30 °C for a 60 min dwell time (Fig. 4a), which shift towards higher pressures (400 MPa) and temperatures (45 °C) for shorter pressure dwell times (5 min).

Such pressure sensitive zones, followed by a stabilization zone, were also previously reported by others (Ardia, 2004; Margosch et al., 2006; Mathys et al., 2009; Rajan et al., 2006), for the inactivation of spores by high pressure. However, due to the fact that spore inactivation kinetics are superimposed kinetics, which indirectly contain the germination kinetics, these observed pressure sensitive zones may represent pressure–temperature combinations for an optimal completion of the germination stage I, which resulted consequently in a faster inactivation.

The direct comparison of the isorate lines for both FB-strains (Fig. 4a) shows a quite equal behavior, which denotes that the rate

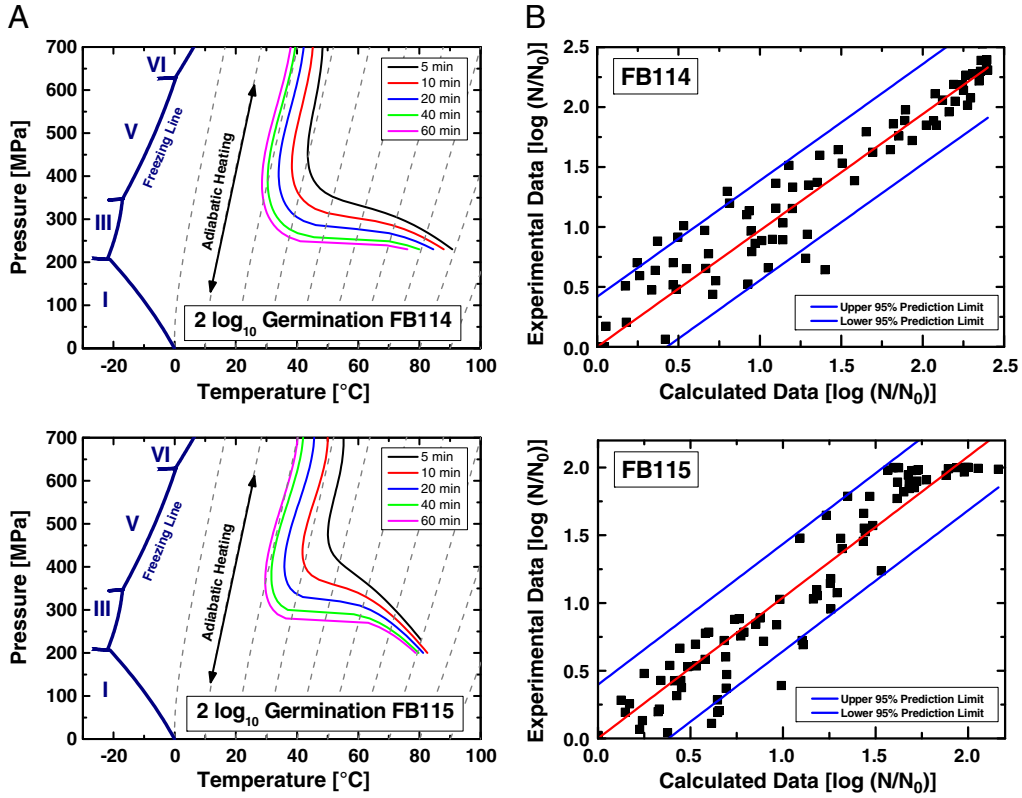

Fig. 4. A. Isorate lines for a 2 $\log_{10}$ germination of *Bacillus subtilis* FB114 (Δger, ΔsleB) and FB115 (Δger, ΔcwlJ) for isothermal isobaric conditions during pressure dwell time. Dashed lines represent the adiabatic heat of compression for water. The solid blue line represents the freezing line between liquid water and the different pressure- dependant ice modifications. B. Comparison among calculated and experimental data with 95% prediction limit lines (solid blue).

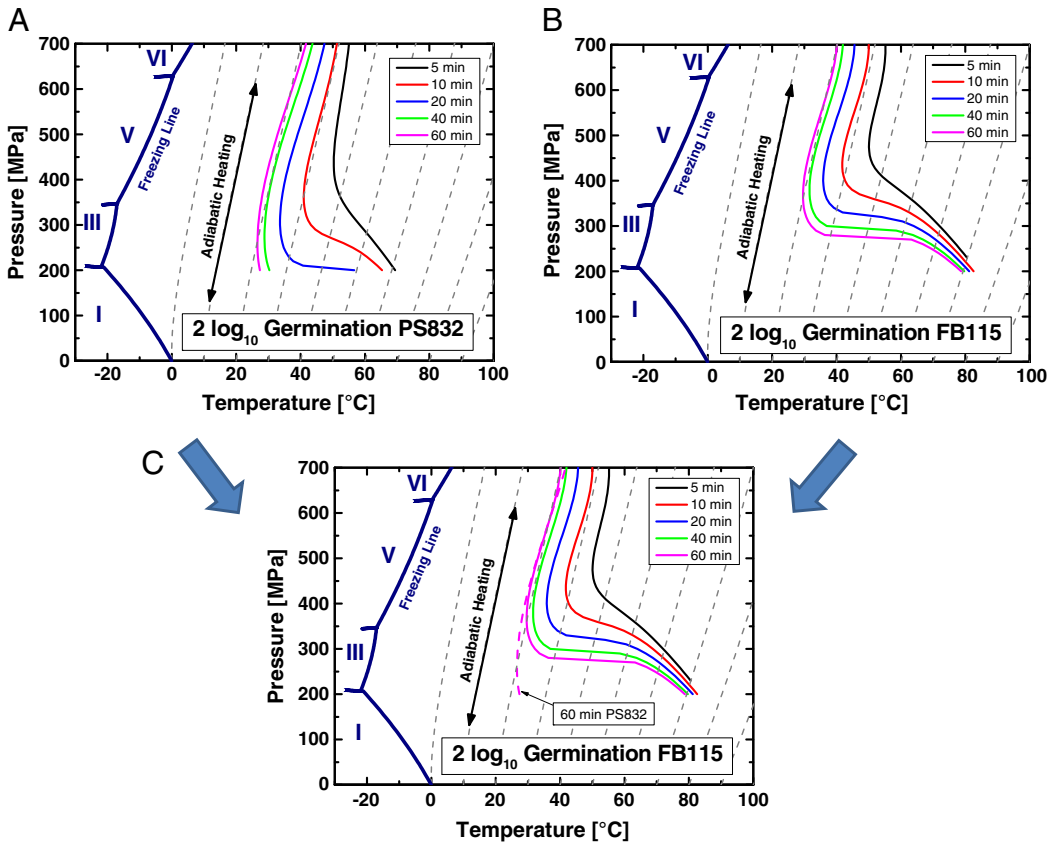

Fig. 5. A. Isorate lines for a 2 $\log_{10}$ germination of *Bacillus subtilis* PS832 (wild-type) and (B) FB115 (Δger, ΔcwlJ) for isothermal isobaric conditions during pressure dwell time. C. Isorate lines for FB115 and various pressure dwell times and 60 min isorate line for a 2 $\log_{10}$ germination of *B. subtilis* PS832 (dashed pink). Dashed gray lines represent the adiabatic heat of compression for water. The solid blue line represents the freezing line between liquid water and the different pressure-dependent ice modifications.

limiting step for spore germination (determined as CFU on agar plates) is not the activity of one of the two cortex lytic enzymes (SleB or CwlJ) and is hence probably attributed to the direct opening of the Ca^{2+}-DPA-channels. However, the direct analysis via FCM immediately after HP treatment demonstrated an accelerated germination under pressure for FB114, which will be discussed in Section 3.4.

For the wild type spore strain PS832 the isorate lines for a 2 $\log_{10}$ germination in Fig. 5a illustrate the dominant role of the germination receptors at low treatment pressures ($p \leq 300$ MPa). For a further increase in pressure and temperature the isorate lines for the germination of the strain FB115 (Fig. 5b) and PS832 after a 60 min pressure treatment converge (Fig. 5c) and run equal at pressures ≥400 MPa, which is attributed to the direct opening of the Ca^{2+}-DPA channels. Hence, the difference between these two isorate lines (dashed pink for PS832 and solid pink for FB115), depicts the impact of the germinant receptors on the germination rate for *B. subtilis*. However, in this regard one has to mention that the germination for PS832 was determined via thermal sensitive spores, whereas the germination for FB114 and FB115 was determined via an increase of CFU/mL on nutrient agar plates. Consequently, the spores of the strain PS832 (wild-type) are possibly more advanced in their germination state e.g. full completion of stage I under pressure (Wuytack et al., 1998) and hence the influence of the nutrient receptors is possibly underestimated, which will be discussed in Section 3.4.

The isorate lines for a 3 $\log_{10}$ inactivation of *B. subtilis* PS832 under isothermal and isobaric conditions are presented in Fig. 6. The optimum for spore inactivation is between 300 MPa and 400 MPa at moderate temperature and long pressure dwell times (≥40 min), which is probably coupled with the optimum of spore germination under these conditions. An increase of pressure and temperature diminished the effect between the different dwell times, which could be explained by the slight inactivation under these conditions (Fig. 3a). Further under these pressure–temperature conditions only an inactivation of 3 to 4 $\log_{10}$ cycles is possible, due to the pressure resistant subpopulation (Fig. 3), which could not be germinated or subsequently inactivated. Hence, these treatment conditions are not suitable for sterilization, but could be of interest if the overall spore load of a thermal sensitive food or food additive should be significantly reduced.

However, to achieve full spore germination (Black et al., 2006) and inactivation (Reineke, Mathys, & Knorr, 2011) of *B. subtilis* PS832 in an industrial relevant dwell time higher treatment temperature and pressure are needed. Hence, previously published data (Reineke, Mathys, & Knorr, 2011) for suitable HPTS process conditions, were independently modeled (Fig. 6b and Table 1 in the Supplementary material) and superimposed with the isorate lines derived in this study (Fig. 6c). In both models (Fig. 6a and b), the possibly occurring inactivation during the compression, was excluded and only the inactivation for isothermal isobaric conditions was modeled. Interestingly, for both models a perfect fit for the overlapping pressure (600 MPa–700 MPa), temperature (60 °C) and time (10 min) conditions, was found. Hence, this is the first report that kinetic data derived in two different HP-Units, with different compression rates (4 MPa/s vs. 25 MPa/s) and vessel volumes (100 mL vs. 3.75 mL) under similar experimental conditions are equal. Consequently, kinetic data from lab-scale HP equipment could possibly be transferred to industrial-scale equipment, if the matrix is the same and the entire inactivation under non isothermal isobaric conditions is excluded. The possible occurring inactivation during the compression time could be regarded as an additional safety factor. Furthermore, due to the high thermal sensitivity of the *B. subtilis* spores at treatment temperatures

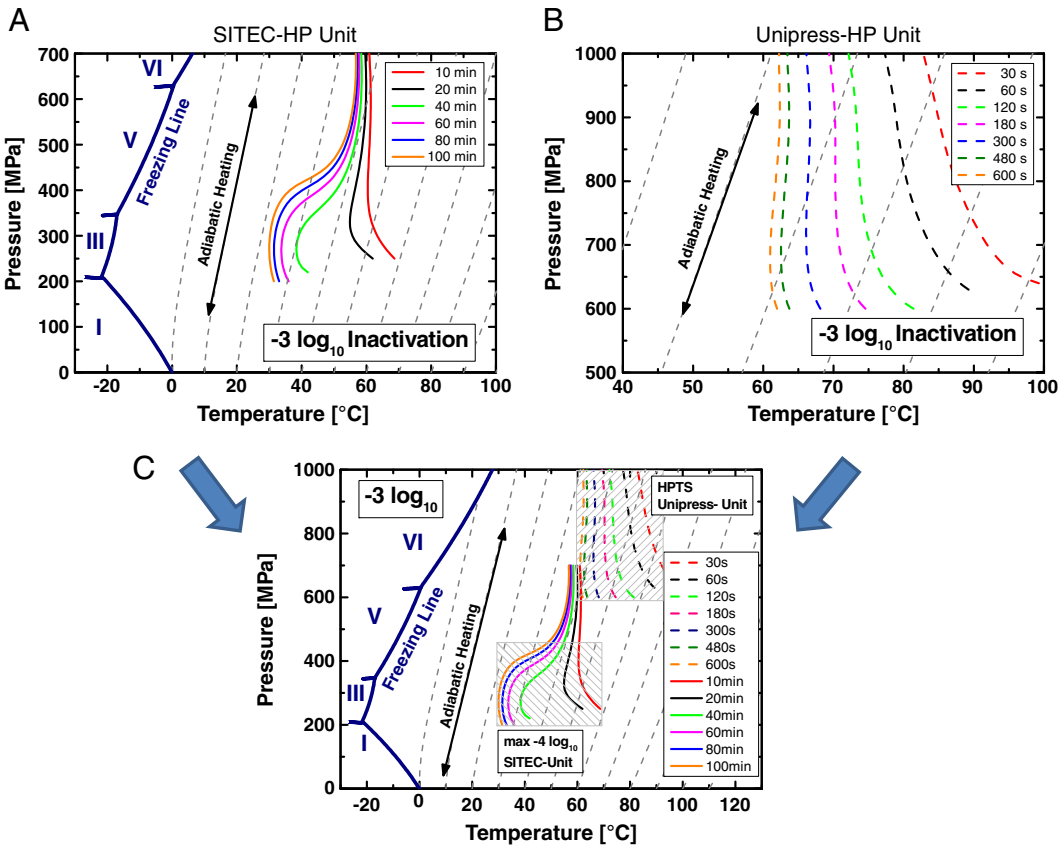

Fig. 6. Isorate lines for a 3 $\log_{10}$ inactivation of *Bacillus subtilis* PS832 (wild-type) for isothermal isobaric conditions derived in A) a SITEC-HP-Unit with a compression rate of 4 MPa/s and B) in a Unipress-HP-Unit with a compression rate of 25 MPa/s for kinetic data published elsewhere (Reineke, Mathys, & Knorr, 2011). C) Superimposition of both independently modeled kinetic data sets. Dashed gray lines represent the adiabatic heat of compression for water. The solid blue line represents the freezing line between liquid water and the different pressure-dependent ice modifications.

above 60 °C and the nearly pressure independent inactivation (Fig. 6b), these spores may also be used to map temperature inhomogeneities in industrial-scale HPTS-vessels.

3.4. Physiological state of spores immediately after HP treatment

To get a deeper insight into the proceeding spore germination during the high pressure treatment and to differentiate, what was the germination state immediately after the pressure treatment and what happened during the 48 h cultivation, FCM analysis was performed. According to Mathys, Chapman, Bull, et al. (2007a) a double staining with the DNA binding fluorescence dyes SYTO16 and propidium iodide (PI) was used. The membrane permeant dye SYTO 16 is used as an indicator for the germination because staining with this dye is not possible until a degradation of the spore's cortex occurred (Black, Koziol-Dube, Guan, et al., 2005). As PI is membrane impermeant, it was used as an indicator for a rupture of the inner membrane and, therefore, as an indicator for inactivation of the spores. Mathys, Chapman, Bull, et al. (2007a) found four physiological states located in different regions in density plots. Spores in region R1 are in the dormant state, spores in region R2 are germinated spores, spores in region R3 are in a state of unknown physiology and spores in region R4 are inactivated spores. These definitions are applied for the evaluation of the flow cytometric results in this work as well.

In Fig. 7 the result for the strains FB114 and FB115 is shown, for a 550 MPa treatment after 60 min dwell time at the optimal growth temperature of 37 °C. Under these treatment conditions a direct opening of the Ca^{+2}-DPA channels is assumed by several others (Black et al., 2006; Paidhungat et al., 2002; Reineke, Mathys, & Knorr, 2011; Wuytack et al., 1998) and further both strains showed nearly the same germination rate under these conditions, detected by plate counts. However, the FCM analyses clearly depict a difference between the germination behaviors of these two trains. The cortex degradation of FB114 occurs faster in comparison to FB115. Hence, we assume that this effect can be traced back to the different CLEs, which lack in the strains, respectively.

For triggering SleB, available in FB115, first of all water uptake is necessary (Paidhungat et al., 2002), which cannot occur until DPA is released. As release of DPA triggers directly *CwlJ* (Paidhungat, Ragkousi, & Setlow, 2001), enabled in FB114, there can be an advance in germination during the high pressure treatment.

For FB115 no spores were detected in the region of germinated spores (R2) after 60 min pressure dwell time. However, approximately 20% of the spore population was found in the unknown state (R3), which were also thermal sensitive (data not shown). According to the model of Mathys, Chapman, Bull, et al. (2007a) the unknown state could only be reached after germination (R2) and, therefore, in the case of FB115 a population in region R2 should have been detectable before or contemporaneously with a population in region R3. However, this was not the case, but according to Black et al. (2005) the degradation of the cortex is essential for every staining of parts inside the spore core. Hence, this effect is perhaps due to an uncompleted cortex degradation, which led to a decrease in the permeability of the cortex. Furthermore, the degradation of the SASPs could not have occurred, and, thus, the DNA is not already accessible for staining. In conclusion, this population is not comparable to the population in the unknown state, described by Mathys, Chapman, Bull, et al. (2007), because the origin of this population is not the same.

The detected germination rate by FCM analysis for the wild-type spore strain PS832 was significantly higher under equal pressure–

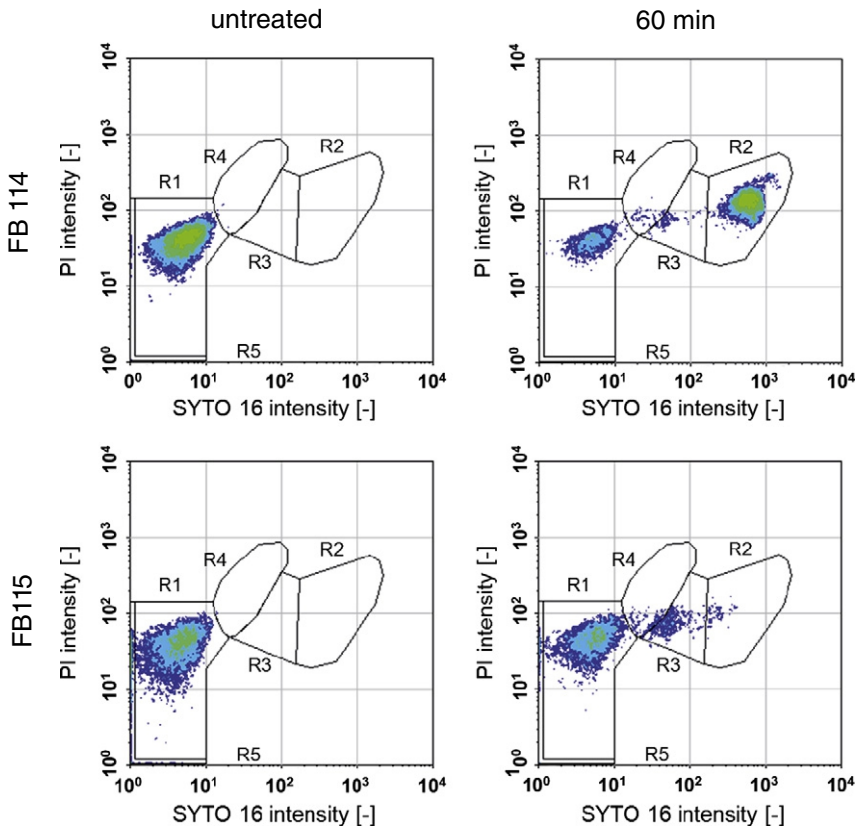

Fig. 7. Results of the flow cytometric analysis (density plots) for the spores strains FB114 (Δger, ΔsleB) and FB115 (Δger, ΔcwlJ) after high pressure treatment with a pressure of 550 MPa at 37 °C. Gating: R1 — dormant, R2 — germinated, R3 — unknown, R4 — inactivated and R5 – background signal.

temperature conditions, which is shown in Fig. 8 and in the Supplementary Fig. 2. The whole spore population passed through the region R2 (60 min) and after 120 min most of them were detected in R3.

According to Setlow (2003) a mixing of spores and germinants could trigger the germination process within seconds and once this process is induced, it will proceed even after the removal of the germinant. Hence, the accelerated germination of the wild-type strain PS832 in comparison to both FB-strains could be caused by triggering the nutrient receptors with pressures in the range of 100–200 MPa (Wuytack, Soons, Poschet, et al., 2000). This range is passed through during the first 20 s of pressure build up and hence, possibly sufficient to induce germination of a large amount of the spore population.

This assumption is confirmed, by the FCM analysis for lower treatment pressures (150 MPa), where the germination is further accelerated. Within 15 min pressure dwell time, all spores were germinated and had already reached the region R3 of the unknown state. Thus, for the wild-type spore strain of *B. subtilis* the model of Mathys, Chapman, Bull, et al. (2007) is confirmed as well as that this state (R3) is not culturable. However, no spores in the inactivated state (R4) could be identified by flow cytometry after a 120 min treatment even if the plate counts showed an inactivation up to 3.5 $\log_{10}$ cycles. Nevertheless, spores killed by a subsequent heat treatment could be detected in the region of inactivated spores (R4; Supplementary Fig. 3). Consequently, spores in the unknown state (R3) are possibly sublethaly injured cells, or cells that reached stage II of germination (Setlow, 2003). Hence, if the germinating spore is able to degrade existing RNA and to synthesis novo RNA (Setlow & Kornberg, 1970) or even to synthesize proteins needed for the outgrowth (Keijser, Ter Beek, Rauwerda, et al., 2007) under moderate pressure (≤300 MPa) defects during the syntheses may occur. Such a misfolding or incomplete ribosomal synthesis of proteins under sublethal pressure conditions ($p<150$ MPa) was reported for *Lactobacillus sanfranciscensis* (Hormann, Scheyhing, Behr, et al., 2006). Hence, it could be assumed that under moderate pressures (≤300 MPa) the germinating spore is possibly synthesizing lethal acting mis-folded proteins. Moreover, the inner spore membrane is highly expanded after cortex degradation at stage II of germination in comparison to stage I (after DPA release) and hence more sensitive to pressure (Smelt, Hellemons, & Patterson, 2001).

A treatment with 600 MPa at elevated temperature (60 °C) led to a very fast germination of spores whereas no inactivation in the first 2 min could be observed (Fig. 8 and Supplementary Figs. 2 and 3). Comparing the two treatments at higher pressures (>500 MPa) it stands out that regarding the first 60 min of the treatment at 37 °C and after 120 s at 60 °C an equal amount of germinated spores was detected by FCM. The amount of thermal sensitive spores increased up to almost 3 $\log_{10}$ cycles, denoting an amount of almost 99.9% of germinated spores in the sample after 2 min dwell time at 60 °C.

Consequently, the germination rate at 60 °C is about 30 times higher than the germination rate at 37 °C, even if the temperature is higher than their optimal growth temperature.

4. Conclusions

The HPTS process is a promising technology to reduce the thermal load of sterilized food and hence retain its quality. However, the detailed inactivation mechanisms of bacterial spores are still not fully understood. In this study we could confirm that the first step of spore inactivation under pressure is at least the partial germination (stage I). Furthermore, we confirmed that pressure induced spore germination without the presence of nutrient receptors is possible for very high pressure (>500 MPa). Moreover, the presumed direct opening of the Ca^{2+}-DPA-channels, which induces the germination, is even active at 200 MPa and moderate temperatures. However, at these conditions the triggering of the nutrient receptors in wild-

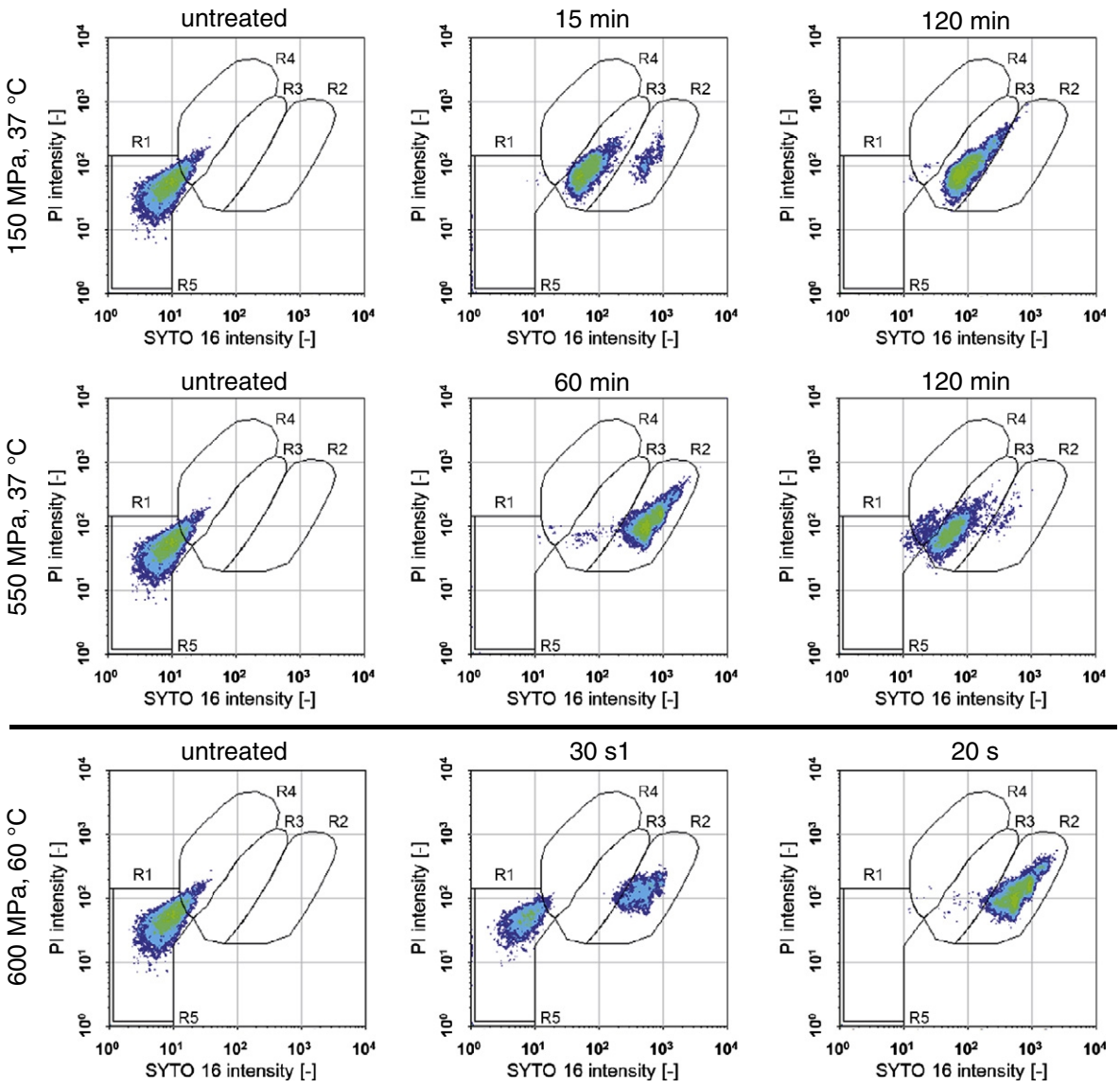

Fig. 8. Results of the flow cytometric analysis (density plots) for the spores of *Bacillus subtilis* spores (PS832) after high pressure treatment with different pressures. Gating: R1 — dormant, R2 – —germinated, R3 — unknown, R4 — inactivated and R5 - background signal.

type *B. subtilis* is the dominant factor influencing the germination rate.

A two step kinetic model based on simple differential chemical equations was suitable to derive a second order model for all investigated spore strains and enabled the calculation of isorate lines in the pressure-temperature landscape. By a superimposition of the isorate lines for FB115 and the wild-type spore strains PS832, it was possible to estimate the dominating influence of the germinant receptors for low pressure ($p \leq 400$ MPa) treatments. Under these pressure–temperature conditions and for long pressure dwell times only for the strain PS832 an inactivation of max. 3–4 $\log_{10}$ was detected by plate count, whereas under higher pressures and moderate temperatures (≤ 40 °C) the germination rate was retarded and no inactivation occurred. This finding, combined with FCM analysis, led to the assumption that spores treated at lower pressures possibly proceed through stage II of germination, wherein the fully hydrated inner spore membrane is more sensitive to pressure or novo RNA synthesis or protein syntheses is disabled, which could lead to lethal defects in the RNA sequence or mis-folded proteins.

An increase of the process temperature above 60 °C highly accelerated the germination as well as the inactivation rate for all tested strains. In this regard the calculated isorate lines for isothermal isobaric conditions during pressure dwell time for *B. subtilis* PS832 were superimposed with previously published data for higher treatment pressures and temperatures. These kinetic data were obtained in a different high pressure unit, with a sixfold higher compression rate and a smaller vessel volume, whereas the entire inactivation under non isothermal isobaric conditions was excluded. In the overlapping pressure (600–700 MPa) temperature (60–80 °C) range both isorate lines showed a perfect fit, which is the first report that kinetic data for spore inactivation under isothermal isobaric conditions could be transferred to other HP systems and possibly be used for up scaling. The additional inactivation during the compression time could be regarded as an additional safety factor. Furthermore, due to the high sensitivity of the *B. subtilis* spores at treatment temperatures above 60 °C and the nearly pressure independent inactivation, these spores may also be used to map temperature inhomogeneities in industrial-scale HPTS-vessels.

However, further investigations are needed to clarify for instance, if the SASPs are degraded during low pressure treatments (≤ 400 MPa), which could confirm that under these conditions the spores proceed trough stage II of germination. Furthermore, the

quantification of the released DPA after or in ideal case during the pressure treatment and its implementation into a germination model, could enable the possibility for a better discrimination under which pressure–temperature combinations triggering the germination receptors or the opening of the Ca^{2+}-DPA-channels are the dominant factors.

Supplementary materials related to this article can be found online at doi:10.1016/j.ifset.2011.09.006.

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6. The release of dipicolinic acid – the rate-limiting step of *Bacillus* endospore inactivation during the high pressure thermal sterilization process

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The release of dipicolinic acid — The rate-limiting step of *Bacillus* endospore inactivation during the high pressure thermal sterilization process

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ABSTRACT

High pressure combined with elevated temperatures can produce low acid, commercially sterile and shelf-stable foods. Depending on the temperature and pressure levels applied, bacterial endospores pass through different pathways, which can lead to a pressure-induced germination or inactivation. Regardless of the pathway, *Bacillus* endospores first release pyridine-2,6-dicarboxylic acid (DPA), which contributes to the low amount of free water in the spore core and is consequently responsible for the spore's high resistance against wet and dry heat. This is therefore the rate-limiting step in the high pressure sterilization process. To evaluate the impact of a broad pressure, temperature and time domain on the DPA release, *Bacillus subtilis* spores were pressure treated between 0.1 and 900 MPa at between 30 and 80 °C under isothermal isobaric conditions during dwell time. DPA quantification was assessed using HPLC, and samples were taken both immediately and 2 h after the pressure treatment. To obtain a release kinetic for some pressure–temperature conditions, samples were collected between 1 s and 60 min after decompression. A multiresponse kinetic model was then used to derive a model covering all kinetic data.

The isorate lines modeled for the DPA release in the chosen pressure–temperature landscape enabled the determination of three distinct zones. (I) For pressures <600 MPa and temperatures >50 °C, a 90% DPA release was achievable in less than 5 min and no difference in the amount of DPA was found immediately 2 h after pressurization. This may indicate irreversible damage to the inner spore membrane or membrane proteins. (II) Above 600 MPa the synergism between pressure and temperature diminished, and the treatment temperature alone dominated DPA release.

(III) Pressures <600 MPa and temperatures <50 °C resulted in a retarded release of DPA, with strong increased differences in the amount of DPA released after 2 h, which implies a pressure-induced physiological like germination with cortex degradation, which continues after pressure release. Furthermore, at 600 MPa and 40 °C, a linear relationship was found for the DPA release rate constants $\ln(k_{DPA})$ between 1 and 30 min.

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1. Introduction

Thermal retorting is the process commonly used for producing shelf-stable low acid food products. For a complete inactivation of all endospores, however, high levels of heat have to be applied. To reduce this undesirable amount of over-processing, an isostatic high pressure treatment combined with elevated starting temperatures (T_{init} > 70 °C) has been identified as a potential alternative to conventional thermal processing (Knorr et al., 2011; Lau and Turek, 2007; Oey et al., 2008). This high pressure thermal sterilization (HPTS) has certain key advantages over its thermal counterpart. Among these are the instantaneous pressure transmission and the rapid and uniform heating up of the entire processed food (if it is homogenous in its composition), due to the adiabatic heat of compression (Toepfl et al., 2006). Furthermore, several papers indicate a synergism between pressure and temperature with regards to spore inactivation (Bull et al., 2009; Margosch et al., 2006; Mathys et al., 2009; Olivier et al., 2011; Rajan et al., 2006; Reineke et al., 2012). Contrary to the synergistic effect of pressure and temperature, however, some spore strains, such as *Bacillus subtilis* or *Geobacillus stearothermophilus*, are more resistant under certain pressure–temperature combinations (Margosch et al., 2006; Mathys et al., 2009; Wuytack et al., 1998). For other spore formers, such as the proteolytic Type B *Clostridium botulinum* TMW 2.357, an inactivation behavior nearly independent of pressure has been reported for pressures below 1.2 GPa (Margosch et al., 2006).

It is possible to achieve a significant spore inactivation with the combination of high pressure (HP) and ambient temperature (Reineke et al., 2012; Sale et al., 1970; Shigeta et al., 2007; Wuytack et al., 1998), but this process is insufficient for food sterilization,

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due to some pressure-resistant spore populations that cannot be germinated by pressure. Consequently, higher treatment temperatures are needed. If the food is preheated to moderate treatment temperatures (T > 60 °C), the inactivation of spores proceeds as at least a two-step process (Heinz and Knorr, 1996; Margosch et al., 2004b; Mathys et al., 2007; Paidhungat et al., 2002; Reineke et al., 2012; Wuytack et al., 1998). In this process the endospores are first induced to release DPA by high pressure, and the germinated spores are subsequently killed by the combination of pressure and heat, owing to their increased overall sensitivity to stress (Paidhungat et al., 2002; Reineke et al., 2011a, 2012; Sale et al., 1970; Wuytack et al., 1998). Whereas pressure induced germination can be strongly accelerated by increasing the treatment temperature (Gould, 1970; Reineke et al., 2012), a temperature decrease to room temperature strongly retards or even inhibits the germination progress (Nakayama et al., 1996; Sale et al., 1970).

In addition to the treatment temperature, the applied pressure level has a significant impact on the germination pathway of all *Bacillus* and *Geobacillus* spore strains (Paredes-Sabja et al., 2011). At pressures between 150 MPa and 400 MPa, a nutrient-like physiological germination is induced without the presence of nutrients (Gould, 1970), through triggering the spore's nutrient receptors (Setlow, 2003). The activation of the nutrient receptors causes the release of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), by an assumed opening of the spore's dipicolinic acid channels. The existence of such DPA-channel proteins is unconfirmed, but it is likely that the SpoVAD protein is involved in $DPA_{2,6}$ movement into and out of the spore (Li et al., 2012). The release of DPA is accompanied by activation of the spore's cortex lytic enzymes, which are responsible for degradation of the cortex (Paidhungat et al., 2001).

A second pathway that hydrates the spore core and hence lowers the spore's thermal resistance, is a non-physiological DPA release at pressures above 200 MPa. This may directly open the spore's DPA-channels or induce structural changes in other membrane proteins (Reineke et al., 2012). This mechanism dominates the DPA release at pressures above 500 MPa. Such pressure levels are more relevant for food processing, since a DPA release in the entire spore population is possible (Heinz and Knorr, 1998; Wuytack et al., 1998). However, after the hydration of the spore core, at least for *B. subtilis*, it seems that the spore cannot proceed to its germination (Reineke et al., 2012; Wuytack et al., 1998).

Regarding spores' different resistance properties to combined pressure and temperature treatments, Margosch et al. (2004b) supposed that spore resistance depends on the ability to retain DPA. Consequently, spore strains with a high wet heat resistance do not necessarily have a high resistance to pressure (Margosch et al., 2004a, 2004b; Olivier et al., 2011).

An indicator spore strain for HPTS is not yet established and thus in this study we used *B. subtilis* to analyze systematically DPA release under various pressure and temperature conditions. This choice was made because it is the best understood spore-forming bacteria. The hydration of the spore core is the first indication of a loss of heat resistance and thus the first step of a successful spore inactivation. Investigating this process could therefore increase understanding of spore germination and/or inactivation pathways under pressure, and could also open up the possibility of guaranteeing a safe and optimized HP-based sterilization process.

2. Materials and methods

2.1. Sporulation and spore preparation

The strain *B. subtilis* PS832 (obtained from P. Setlow; University of Connecticut, Health Center) that was used in this study was sporulated according to a method described by Nicholson and Setlow (1990). To induce sporulation a liquid preparatory culture obtained from a single colony was grown in 50 mL LB-Lennox-broth (Molekula, Manchester, U.K.) at 37 °C under continuous shaking (120 rpm). After 24 h, 200 μL of the cell suspension was surface plated on 2× SG medium agar plates and incubated at 37 °C. After sporulation the spores were harvested and the suspension was cleaned by repeated centrifugation (3-fold at 5000 *g*). It was then washed with cold distilled water (4 °C) and sonicated for 1 min. The cleaned spore suspension contained approximately 1.0×10^9 spores/mL (≥95% phase bright) with nearly no spore agglomerates, as verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.).

2.2. Isostatic high pressure treatment

For pressure treatment, the *B. subtilis* spores were resuspended in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer solution (pH 7, 0.05 M), which has the most stable pK_a-value under the used pressure-temperature conditions (Mathys, 2008). In the buffer solution the cell count totaled approximately 1.0×10^8 spores/mL. From this spore suspension, samples of 1.6 mL were filled into plastic tubes with screw caps (CryoTube Vials, Nunc Brand Products, Roskilde, Denmark), and small aliquots of 300 μL were pipetted into shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany, inner diameter 3 mm, outer diameter 3.6 mm). Finally, the shrinking tubes were hermitically sealed with a soldering iron.

For the pressure trials two different HP systems were used. The first kinetic data set was derived in an HP unit (0101-7000-S, Sitec Sieber Engineering AG, Zurich, Switzerland) with a compression rate of 4 MPa/s and an 100 mL vessel volume, where water served as the pressure-transmitting medium. The double-jacket HP vessel was preheated to the designated treatment temperature with a thermostatic bath (Huber ministat, Huber GmbH, Offenburg, Germany). The ice-cooled samples were treated under isothermal and isobaric conditions during dwell time. To reach these isothermal conditions, the individual starting temperature for each temperature level was determined empirically prior to the pressure trials, whereas the temperature was measured in the geometrical center of a dummy container (1.6 mL, CryoTube Vials, Nunc Brand Products, Roskilde, Denmark) equipped with a J-Type thermocouple.

The second pressure system used in this study was a U111 unit (Unipress, Warsaw, Poland), with a 3.7 mL vessel volume and a compression rate of 25 MPa/s. Due to the smaller size of this pressure vessel, the sample volume was reduced to 4×300 μL. In this pressure equipment, Di-2-ethyl-hexyl-sebacate served as the pressure-transmitting medium. The higher adiabatic heat of compression (Reineke et al., 2008) compared to water could be neglected, owing to the direct measurement of the temperature in a dummy sample. All samples and a dummy sample for the temperature control were therefore treated under isothermal and isobaric conditions during pressure dwell time as previously described. To reach the designated treatment temperature, the pressure vessel was immersed in a thermostatic bath (cc2, Huber GmbH) filled with silicon oil (M40.165.10, Huber GmbH). A representative pressure temperature profile for both pressure systems is published elsewhere (Reineke et al., 2011a).

Prior to and after the pressure treatment all samples were stored on ice. Additionally, aliquots of each spore sample were mild-heat treated (20 min at 80 °C) after the pressure trials, to determine the number of heat-sensitive spores (Wuytack et al., 1998). Following this appropriate dilutions of each sample were surface-plated in duplicate in petri dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37 °C for 2 days, and the colony-forming units (CFU) were counted. Kinetic data for DPA release and viable spores in the SITEC HP unit were determined between 300 MPa and 600 MPa and between 30 °C and 60 °C. In a second set of experiments, the U111 unit was used to extend the pressure and temperature range from 200 MPa to 900 MPa and up to 80 °C, respectively.

2.3. Determination of released dipicolinic acid

Quantification of the DPA concentration in the samples was made using counterion HPLC (Tabor et al., 1976). A Dionex Ultimate 3000 system was used (Dionex Corporation, Sunnyvale, CA, USA), with a reversed phase separating column (RP 18—5 μm LiChroCART 124-4; Merck, KGaA, Darmstadt, DE) that was protected with a guard column (LichroCART 4-4; Merck KGaA, Darmstadt, DE). The DPA detection limit of this HPLC setup was 1 μM. Prior to analysis spore samples were filtered through a 0.2 μm nylon filter (Rotilabo-Spritzenfilter, Carl Roth GmbH, Karlsruhe, Germany) in order to remove spores and other particles. All samples were stored at −80 °C prior to the HPLC analysis.

To determine the total amount of DPA in the spore suspensions, 1 mL of each individual batch was thermally treated at 121 °C for 20 min (Janssen et al., 1958) and then analyzed. The total amount of DPA measured per spore batch was approximately 40 μM, which resulted in a measurement range from 0.5 μM to 40 μM. To evaluate the release of DPA after a thermal treatment, spores were suspended in PBS buffer (pH 7, 0.05 M), because of its stable pK_a at elevated temperatures and at pressures below 0.3 MPa (Reineke et al., 2011b). For the treatment, 120 μL of the spore suspension was filled into thin glass capillaries and hermetically sealed to prevent evaporation. The spores were treated in a thermostatic bath (Huber GmbH, Offenburg, Germany) which was filled with silicon oil (M40.165.10, Huber GmbH, Offenburg, Germany) at 95 °C, 100 °C and 105 °C.

The pressure-treated samples were filtered 2 h after the treatment, and additionally an aliquot was made immediately after decompression for all samples pressurized in the U111 system. In an independent test series, samples were treated at 200 MPa and 600 MPa at 40 °C, and were filtered immediately after the pressure treatment, and after 1 min, 5 min, 10 min, 20 min and 1 h. Between sampling they were stored at 4 °C and subjected to continuous shaking at 150 rpm (HLC Cooling-Thermo-Mixer MKR13, Ditabis, Pforzheim, Germany). Pressure-induced DPA release was calculated relative to the total DPA content of each individual spore batch, and all of the data are represented as the mean of at least two independent experiments.

2.4. Flow cytometry analysis

From each pressure trial in the U111 pressure system one shrinking tube was snap-frozen immediately after decompression and stored at −80 °C prior to flow cytometry (FCM) analysis.

For the sample preparation double staining was used, involving SYTO16 (Invitrogen, Carlsbad, CA, USA) and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA), according to the method of Mathys et al. (2007). Both fluorescent dyes are able to stain DNA, but the membrane permeant SYTO16 acts as an indicator for spore germination, whereas the membrane impermeant PI indicates membrane damage. The treated spore suspensions were diluted with ACES buffer solution (0.05 M, pH 7) to achieve a flow rate of about 1000 events/s, as well as a constant ratio of spores for the staining. The concentrations of the fluorescent dyes for staining were 15 μM PI and 0.5 μM SYTO16 in the diluted spore suspension. Afterwards, the samples were stored in the dark at ambient temperature for 15 min. The analyses were carried out using a BD FACSCalibur flow cytometer (BD Bioscience, San José, CA, USA) equipped with a 15 mW, 488 nm air-cooled argon ion laser. BD CellQuest Pro (Version 5.2, BD Biosciences) was used as operation and acquisition software. To achieve a clear determination of the different spore populations, and to discriminate non-spore particles, the amplification of the forward scatter (FSC) was set to E01 (10-fold) and the FSC threshold was set to 72. The amplification of the side scatter (SSC) was set to 721 V and the SSC threshold was set to 706. All analyses were performed to a minimum in duplicate.

2.5. Calculation of isokineticity lines

In order to calculate isokineticity lines to show the dependence of pressure and temperature, a set of simple differential chemical equations was used (Eqs. (1) & (3)–(5)).

Firstly:

$$\frac{d[c(DPA)]}{dt} = -k_{DPA}(p,T)[c(DPA)] \quad (1)$$

describes the release of DPA under isothermal isobaric conditions, with k_{DPA} as a rate constant. The amount of released DPA under dynamic pressure–temperature conditions ($t_0 = 1$ s dwell time) was excluded. This equation was also utilized to calculate the time-dependent release of DPA after decompression at 200 MPa and 600 MPa, at 40 °C.

For the calculation of germinated and inactivated spores under isothermal isobaric conditions, a two-step multiresponse kinetic model was used (as described in Reineke et al., 2012), which was then solved with a non-linear multiresponse regression.

$$dormant \xrightarrow{k_a} germinated \xrightarrow{k_b} inactivated. \quad (2)$$

The set of differential rate equations used in this case was:

$$\frac{d[dormant]}{dt} = -k_a(p,T)[dormant] \quad (3)$$

$$\frac{d[germinated]}{dt} = k_a(p,T)[dormant] - k_b(p,T)[germinated] \quad (4)$$

$$\frac{d[inactivated]}{dt} = k_b(p,T)[germinated] \quad (5)$$

with k_a and k_b as rate constants.

The set of differential equations (Eqs. (1) & (3)–(5)) was solved with the differential equation solver, Berkeley Madonna (Version 8.0.1, R. I. Mackey & G.F. Oster, University of California at Berkeley, CA, USA).

In order to obtain a functional relationship of the rate constants k to pressure and temperature, a surface fit with second order polynomial equations was carried out using TableCurve 3D version 3 (SPSS Inc., Chicago, IL, USA). The isokineticity lines were calculated using MathCAD 15 (Mathsoft Engineering & Education, Inc., USA), with the rate equations derived from Eqs. (3)–(5):

$$[dormant] = [dormant]_0 exp(-k_a t) \quad (6)$$

$$[germinated] = [germinated]_0 exp(-k_b t) + \frac{k_a[dormant]_0}{k_b - k_a}[exp(k_a t)] - exp(-k_b t) \quad (7)$$

$$[inactivated] = [inactivated]_0 + [germinated]_0 \times \left[\left[1 - exp(-k_b t) + [dormant]_0 \cdot \left[1 + \frac{k_a exp(-k_b t) - k_b exp(-k_a t)}{k_b - k_a}\right]\right]\right]. \quad (8)$$

The data calculated in this way were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA).

2.6. Reproducibility of results

All experiments were carried out at least in duplicate. The presented data are either the means taken from two replicate experiments or from single representative experiments. Generally, the cell counts of pressure-treated samples were reproducible within 0.5 $\log_{10}$. For kinetic modeling, at least 4 kinetic points for each pressure–temperature combination were used, excluding dynamic pressure temperature conditions during compression and decompression.

3. Results and discussion

3.1. Release kinetics of DPA between 300 MPa and 600 MPa

For the first set of experiments an HP system with a moderate compression rate of 4 MPa/s was used. To reduce the impact of a pressure-induced triggering on the nutrient receptors, only pressures above 300 MPa and moderate treatment temperatures (≤60 °C) were applied.

After fitting the measured amount of released DPA under isothermal isobaric conditions with Eq. (1), a surface fit was made of ln k_{DPA} (Table 1) versus pressure and temperature. The equation generated by this three-dimensional surface fit (adj $R^2 = 0.943$) enabled us to calculate isorate lines under isobaric and isothermal conditions for a 90% DPA release depending on temperature and pressure (Fig. 1). The isorate lines in Fig. 1 for treatment times between 3 min and 20 min show varying DPA release behavior under different pressure–temperature conditions. Whereas under moderate pressures and temperatures long pressure dwell times are needed, an increase in temperature strongly accelerated the DPA release rate. Furthermore, for pressures above 500 MPa and a 5 min pressure dwell time, the DPA release rate is mainly dependent on temperature and the impact of pressure diminishes.

By contrast, at treatment pressures below 500 MPa a trend towards lower temperatures is apparent. This indicates that under these conditions the mechanism for DPA release from the spore core is different to that at higher treatment temperatures under equal pressure levels (Fig. 1). Such a trend was also reported in the few existing studies on spores' DPA release after pressure treatment. Wuytack et al. (1998) measured DPA release after a 60 min treatment at 100 MPa and 600 MPa at 40 °C, finding that *B. subtilis* released 97.5% at 100 MPa, compared to 87.0% at 600 MPa. These findings were attributed to a nutrient-like physiological germination at 100 MPa, as opposed to a germination initiated at 600 MPa, which is arrested at some early stage.

Contrary to this Margosch et al. (2004b) and Paidhungat et al. (2002) stated that the pressure-induced release of DPA at 550 MPa or higher is due to a non-physiological effect. This could further trigger germination after pressure release, or could lead to an inactivation. This assumption, however, is based on DPA release data that was derived only at 800 MPa and 70 °C for *B. subtilis* and two other spore strains (Margosch et al., 2004b).

3.2. DPA release at pressure vs. after the pressure treatment

To discriminate between the two hypotheses introduced above, the amount of DPA released was measured immediately after the pressure treatment and was then compared with the data determined

Table 1
Calculated DPA release rate constants ln(kDPA) from Eq. (1) for *Bacillus subtilis* for isothermal isobaric conditions. Data for 600 MPa and 30 °C are missing, due to the unfeasibility of reaching isothermal conditions after compression.

Temperature [°C]	Pressure [MPa]	ln(k_{DPA})
30	300	−5.92
	400	−6.54
	500	−8.07
40	300	−5.38
	400	−5.81
	500	−6.48
	600	−7.22
50	300	−4.53
	400	−5.35
	500	−5.32
	600	−4.36
60	300	−5.80
	400	−4.56
	500	−3.60
	600	−3.51

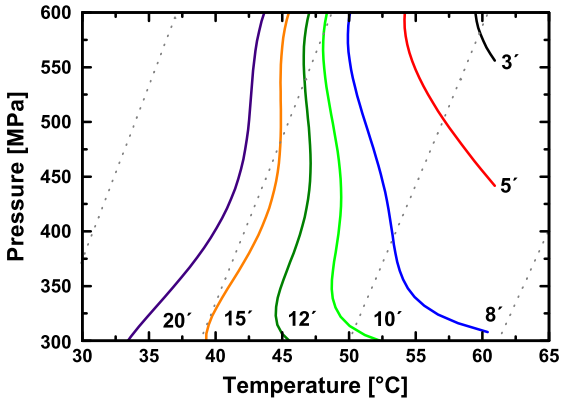

Fig. 1. Isorate lines from 3 to 20 min for a 90% release of the total spore DPA content (*B. subtilis* PS832) 2 h after isothermal isobaric pressure treatment in the SITEC HP system. Dotted lines represent the adiabatic heat of compression for water.

2 h later. This time period was chosen because it is sufficient to ensure that each individual spore in which germination was triggered had enough time for a complete DPA release. The pressure and temperature range was also extended from 200 MPa to 900 MPa and 40 °C to 80 °C, respectively, in order to cover p–T conditions suitable for triggering the nutrient receptors as well as for a possible non-physiological DPA release.

For all kinetic points the amount of DPA determined in the suspension medium, as well as the data for heat-sensitive and inactivated spores, can be found in the supplementary material. The rate constants calculated for the DPA release ln(k_{DPA}) are shown in Table 2, along with those for the formation of a heat-sensitive spore population after the pressure treatment [ln(k_a)] and for the amount of inactivated spores during the pressure treatment [ln(k_b)]. Table 2 further includes the rate constants ln(k_{DPA}) for thermally treated spores in thin glass capillaries. The corresponding D-values for the thermal treatment were 9.6 min, 1.7 min and 20.2 s, for the 95 °C, 100 °C and 105 °C treatments, respectively.

Using the rate constants from Table 2 for the measured amount of DPA, it was also possible to calculate isorate lines, which are depicted in Fig. 2. The isorate lines are in good agreement with the experimental data (see Fig. 2 in the supplementary material). A comparison of the calculated and experimental data resulted in an adj. R^2 of 0.85 and 0.94 for the model, based on the DPA data measured immediately after decompression and 2 h later. The residuals are nearly randomly distributed and hence it is assumed that no heteroscedastic error is present in both models. Furthermore, only 3 residuals from the model used for the calculation of the DPA release after 2 h were outside the 95% upper or lower prediction limit, and no residual for the DPA release after decompression was outside these limits.

A comparison of the isorate lines in Fig. 1 with those in Fig. 2B shows an equal characteristic between 300 and 600 MPa. This finding demonstrates that in addition to data for heat sensitive and inactivated spores (Reineke et al., 2012), the release rates for DPA can also be compared between different HP systems, if they are derived under isothermal isobaric conditions during pressure dwell time. Furthermore, it can be confirmed that, above a threshold pressure of 600 MPa, the release of DPA from the spore core is nearly independent of the pressure and can be accelerated by a factor of 10, if the temperature is increased from 50 °C to 70 °C. These findings are in agreement with published DPA release kinetics for pressure treatments of *B. subtilis* at 600 MPa and 40 °C and 800 MPa at 70 °C. Wuytack et al. (1998) reported an 87% DPA release after 1 h at 600 MPa, and a 96% release was measured by Margosch et al. (2004b) after 2 min at 800 MPa.

In addition to this the extended pressure and temperature range allowed the inclusion of data for a purely thermally induced DPA release in the model shown in Fig. 2B. For short treatment times

Table 2
DPA release rate constants ln(kDPA) immediately and 2 h after pressure treatment from Eq. (1). Rate constant ln(ka) depicts the formation of heat-sensitive spores and ln(kb) of inactivated spores under isothermal isobaric conditions. Data for 800 MPa–900 MPa and 40 °C are missing, due to the unfeasibility of reaching isothermal conditions after compression.

Temperature [°C]	Pressure [MPa]	U111 ln(k_{DPA}) after 0 s	U111 ln(k_{DPA}) after 2 h	U111 ln(k_a)	U111 ln(k_b)
40	200	−5.58	−4.31	−7.65	−6.93
	600	−7.72	−7.04	−10.23	−10.10
60	200	−5.10	−4.92	−6.64	−5.46
	600	−4.72	−4.63	−5.24	−7.22
	800	−4.69	−4.22	−6.04	−6.76
	900	−5.08	−4.28	−6.43	−7.92
80	200	−4.23	−4.00	−6.14	−3.84
	600	−2.97	−3.03	−3.72	−4.51
	800	−2.41	−2.28	−3.46	−3.80
	900	−3.02	−3.08	−3.73	−2.98
95	0.1	–	−5.40*	–	–
100	0.1	–	−4.48*	–	–
105	0.1	–	−3.57*	–	–

* Data from thermal inactivation in thin glass capillaries.

(2–3 min) and pressures below 600 MPa, the isorate line shifts towards higher temperatures such that decreasing the process pressure results in a purely thermal process. If one now takes into account the difference in measured DPA immediately after decompression (Fig. 2A) and 2 h later (Fig. 2B), a perfect fit of the isorate line below 600 MPa is apparent when the isorate lines for treatment times up to 8 min are compared.

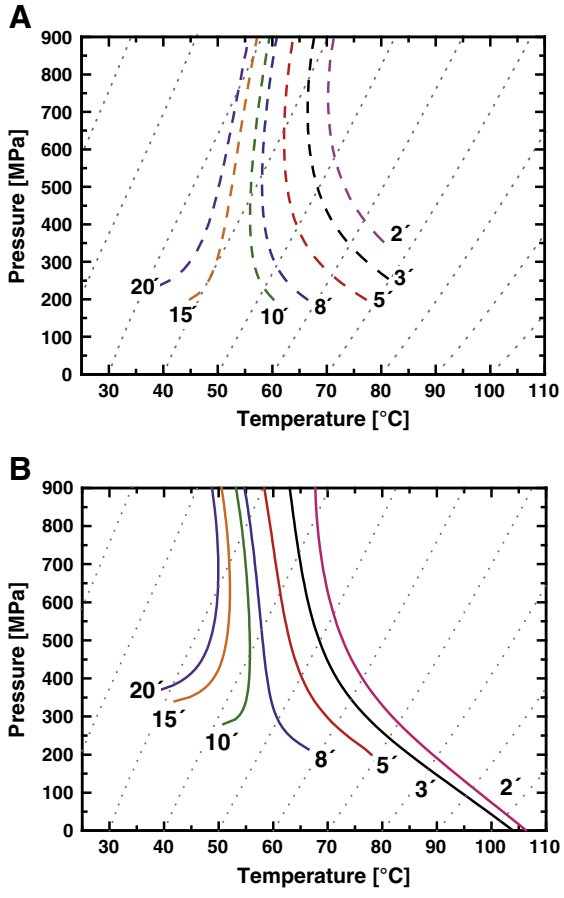

Fig. 2. Isorate lines from 2 to 20 min for a 90% DPA release immediately after the pressure treatment [(A) — dashed lines] and 2 h after decompression [(B) — solid lines] for isobaric and isothermal conditions. Dotted lines represent the adiabatic heat of compression for water.

The release of DPA from the spore core during a thermal treatment is still under discussion and has not been evaluated in full detail (Klijn et al., 1997). However, whereas the spore's exosporium or DNA damage have not been associated with wet heat resistance (Nicholson et al., 2000), the cortex and the spore coat are thought to contribute to this (Sanchez-Salas et al., 2011). Primarily, the high amount of DPA coupled with the low amount of water is responsible for the wet heat resistance. The most likely mechanisms for spore inactivation by heat are a rupture of the spore's inner membrane and the inactivation of core enzymes (Setlow, 2000; Warth, 1980). This therefore leads to the conclusion that if there is no difference between the DPA concentration detected immediately compared to 2 h after pressure treatment, a destruction of the inner spore membrane is likely for short pressure dwell times and high temperatures (zone I in Fig. 3).

The synergism between pressure and temperature above 50 °C and below 600 MPa could be explained by a pressure-induced crystallization of the inner spore membrane, which is likely to consist of a high proportion of gel phase lipids (Cowan et al., 2004). This phase transition of lipids under pressure makes biological membranes one of the most pressure-sensitive cellular components (Winter and Jeworrek, 2009). Another explanation of the synergism, however, could be that a ceasing of membrane protein function occurs above 200 MPa (Winter and Jeworrek, 2009), which might be attributed to a physiologically unacceptable overall ordered state of the spore's inner membrane. Hence, the assumed opening of the Ca^{2+}-DPA-channel proteins (Paidhungat et al., 2002) or a denaturation of other membrane proteins may also occur.

For higher pressures (>600 MPa), the impact of pressure diminishes and it is mainly temperature that accelerates the DPA release. This second zone (Fig. 3) also shows only a slight deviation of DPA release both immediately and 2 h after decompression. It can therefore be assumed that under these conditions full membrane destruction and/or denaturation of the membrane proteins takes place, and that the DPA release is mainly driven by diffusion.

A third zone (Fig. 3) can be identified for low pressures (<600 MPa) and moderate treatment temperatures (<50 °C). Under these conditions a significant DPA release after decompression was detected, which supports the assumption of Wuytack et al. (1998), that a physiological spore germination is triggered by pressure and continues after decompression. To further evaluate this assumption an independent test series at 200 MPa and 600 MPa at 40 °C was performed and the DPA release determined in smaller intervals.

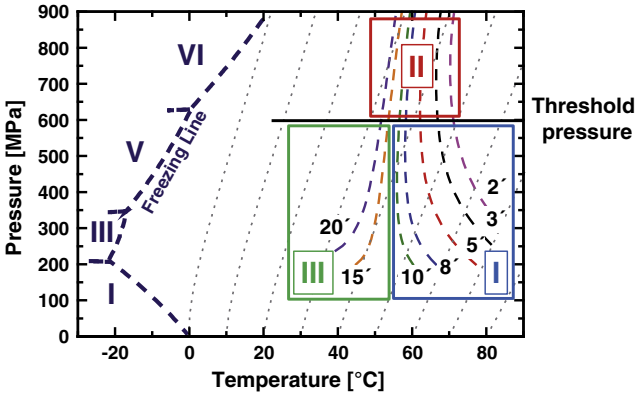

Fig. 3. Isorate lines from 2 to 20 min for a 90% DPA release immediately after the pressure for isobaric and isothermal conditions. Zone I–III; Assumed DPA release mechanisms: I) pressure dependent permeabilization of the inner spore membrane; II) full permeabilization of the inner spore membrane and temperature-dependent diffusion of DPA; and III) nutrient-like pressure induced germination. Dotted lines represent the adiabatic heat of compression for water. The dashed line displays the freezing line between solid and liquid water and the different pressure-dependent ice modifications.

3.3. Diffusion of DPA from the spore after pressurization

Based on the previous findings, pressure conditions suitable for a physiological (200 MPa) and a non-physiological (600 MPa) DPA releases were investigated in detail for a moderate treatment temperature of 40 °C. The time-dependent DPA release after decompression is shown in Fig. 4.

A treatment temperature of 40 °C was as chosen, firstly to enable an isothermal treatment for both pressure conditions, and secondly to retard the assumed temperature-dependent diffusion process as much as possible. Fig. 4a) and b) clearly depicts a faster release of DPA at 200 MPa as compared to 600 MPa. The finding that after 10 min at 200 MPa nearly a complete release of DPA occurred points towards a pressure-induced physiological DPA release. This release is comparable to a nutrient-induced germination, which is additionally verified by the fast release for shorter pressure dwell times. This is because a physiological germination will proceed even after removal of the germinant (Setlow, 2003). A similar period for a complete loss of refractility, coupled to the concentration of DPA in the spore core, was reported by Kong et al. (2010) for a nutrient-induced germination of *Bacillus cereus* spores.

When compared to the 200 MPa treatment, the DPA release at 600 MPa was strongly retarded. Whereas a ~100% DPA release was measured after 10 min at 200 MPa, more than 1 min was needed at 600 MPa. Furthermore, the DPA release after decompression was retarded as well, which enabled (through the use of Eq. (1)) the calculation of a DPA release rate k_{DPA} (Fig. 5). The calculated release rates

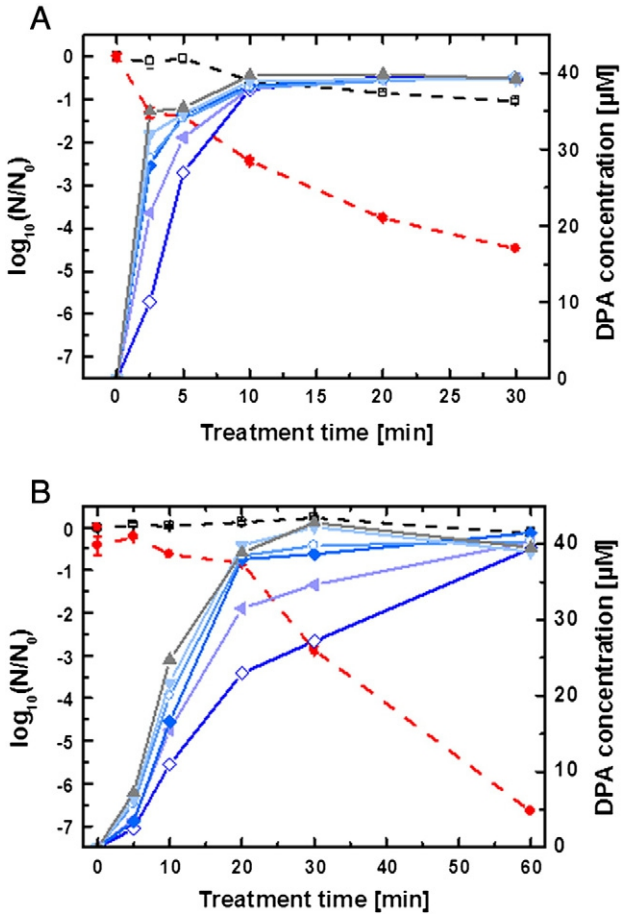

Fig. 4. Kinetic data for heat sensitive (dashed, •) and inactivated spores (dashed, □) under isothermal (40 °C) and isobaric conditions [A) 200 MPa; B) 600 MPa] during pressure dwell time. Solid lines represent the measured amount of released DPA after 1 s (◊) 1 min (◄), 5 min (♦), 10 min (○), 20 min (▼) and 60 min (▲) after decompression.

ln k_{DPA} clearly show that a compression to 200 MPa or 600 MPa and 40 °C (1 s dwell time) was insufficient to induce DPA release. Furthermore, nearly no further DPA release occurred after decompression for dwell times of ≥10 min at 200 MPa or ≥60 min at 600 MPa.

Interestingly, a linear increase for ln k_{DPA} was found for the pressure treatment times from 5 min to 30 min at 600 MPa (Fig. 5, dashed line). This finding indicates that after a 5 min pressure treatment at 600 MPa, diffusion of DPA through the inner spore membrane occurs, in contrast to the active physiological DPA release at 200 MPa. The retarded release of DPA under equal temperature conditions at 600 MPa, in comparison to that at 200 MPa, leads to the assumption that an irreversible structural change occurred at 600 MPa. This may have been either in the inner spore membrane or in membrane proteins, such as the SpoVAD protein, which might be responsible for the physiological DPA release (Li et al., 2012). Consequently, the barrier properties of the inner spore membrane are changed and diffusion then occurs due to the large concentration gradient between the high amount of DPA in the spore core and the low amount in the surrounding buffer solution.

3.4. Spore germination and inactivation

In order to enable correlation between the release of DPA and the loss of heat resistance, as well as the subsequent spore inactivation, 3 $\log_{10}$ isorate lines were calculated for each case (Fig. 6). A multiresponse kinetic model was used, which enabled simultaneous calculation of both rate constants (k_a and k_b) by using multiple data (Reineke et al., 2012). This increases the precision of the parameters (Van Boekel, 2009). The individual rate constants for the heat sensitive and inactivated spores are presented in Table 2. Comparison between the calculated and experimental data resulted in an adj. R^2 of 0.84 for spore germination and 0.96 for the inactivation. The residuals were randomly distributed and can be found in the supplementary material.

When the isorate lines for heat sensitive (Fig. 6A) spores are compared with those for inactivated spores (Fig. 6B), a shift is evident towards lower pressures and temperature for the heat sensitive spores. Furthermore, the shape of isorate lines follows the trend for the DPA release for isothermal isobaric dwell times up to 10 min. This therefore confirms that spore inactivation during high pressure processing is a stepwise inactivation process that is initiated by the release of DPA and followed by a spore core hydration, coupled with the subsequent loss of heat resistance. Hence, the resistance of spores to combined pressure and temperature treatments correlates with their ability to retain DPA (Margosch et al., 2004b).

For low pressures (≤600 MPa) and short pressure dwell times (≤10 min) the whole inactivation process shifts towards a purely thermal inactivation. However, especially under these conditions, a strong synergism between pressure and temperature is depicted in Fig. 6. These data therefore extend previously published kinetics

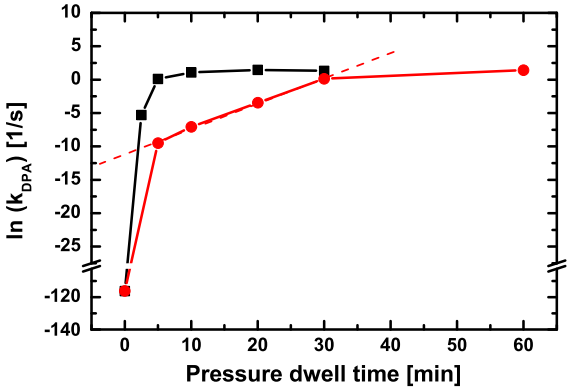

Fig. 5. Calculated release rate ln(k_{DPA}) [1/s] for an isothermal isobaric pressure treatment at 40 °C and 200 MPa (■) or 600 MPa (•).

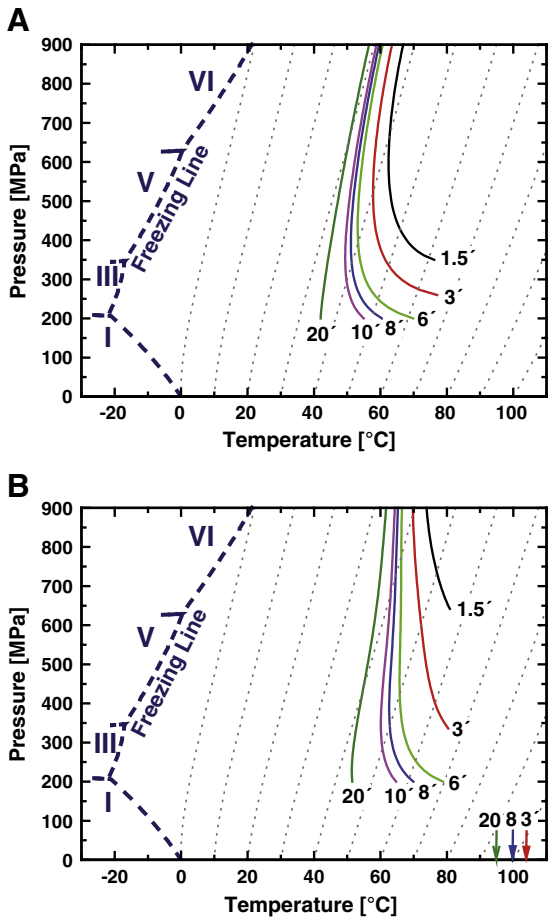

Fig. 6. Isorate lines from 1.5 to 20 min for 3 $\log_{10}$ heat sensitive (A) and inactivated (B) *Bacillus subtilis* spores under isothermal isobaric conditions during pressure dwell times. B) Arrows depict the time needed for a thermal 3 $\log_{10}$ inactivation. Dotted lines represent the adiabatic heat of compression for water. The dashed line represents the freezing line between water and the different pressure-dependent ice modifications.

(Reineke et al., 2012) to higher temperatures and lower pressures. Moreover, kinetic data from both studies are nearly identical under equal process conditions (e.g. 600 MPa and 3 min or 10 min).

For higher process pressures (≥600 MPa) and temperatures (≥60 °C), the synergism diminishes and the isorate lines for the heat sensitive (Fig. 6A) and inactivated spores (Fig. 6B) are mainly temperature dependent, similar to the 90% DPA release (Fig. 2). Margosch et al. (2004b) reported for a pressure treatment of *B. subtilis* spores (at 800 MPa for 2 min at 70 °C) that if more than 90% of DPA was released from the cells, the inactivation of spores was not further influenced by pressure.

In this study, the temperature needed to achieve 3 $\log_{10}$ heat sensitive spores at 800 MPa was 61.2 °C for a 3 min treatment and 56 °C for a 10 min treatment. However, to inactivate 3 $\log_{10}$ of spores at 800 MPa, temperatures of 70.2 °C and 63.7 °C were needed, respectively. It can therefore be seen that an impact of pressure and temperature is present at high treatment pressures, but is not so dominant as for lower pressures and high temperatures. Similar behavior has also been reported for *C. botulinum* (Margosch et al., 2004a), where a transient increase in temperature resulted in a strongly accelerated inactivation. Furthermore, this accelerated inactivation was reflected by an accelerated release of DPA.

For pressure dwell times above 10 min, the shape of the isorate lines for the DPA release and heat sensitive spores differs. Whereas the DPA release for pressures below 300 MPa strongly shifts towards lower treatment temperatures (Fig. 2A), this behavior was not found for the formation of heat-sensitive spores after pressurization (Fig. 6A) or for spore inactivation during the treatment (Fig. 6B). This suggests a different inactivation pathway under these conditions, which probably includes at least a successful pass through germination stage I. However, the temperature difference between a 3 $\log_{10}$ germination within 20 min (43.3 °C) at 300 MPa and a 3 $\log_{10}$ inactivation (52 °C) remained nearly constant, compared to higher process pressures and temperatures.

Wuytack et al. (1998) have stated that spores treated at 100 MPa and 40 °C were able to degrade their small soluble acid proteins (SASPs) and further to rapidly generate ATP, in contrast to spores treated at 600 MPa. When these findings are combined with inactivation data of the same *B. subtilis* spore strain (PS832) (Reineke et al., 2012), in which a 3 $\log_{10}$ inactivation was achieved for pressure dwell times exceeding 40 min, they lead to the assumption that a pressure-induced physiological spore germination is possible for pressures up to 600 MPa and ≤50 °C. To further evaluate this assumption flow cytometry analysis was performed.

3.5. Flow cytometry analysis

The results of the flow cytometry (FCM) analysis for each kinetic point are presented in Fig. 7. Due to the diverse behavior of the assumed spore DPA release mechanisms in this study, all stained spores were regarded as no longer in the dormant state. They were plotted against the calculated amount of heat sensitive spores based on Eq. (4). This plot was used because this population includes spores that are PI as well as SYTO16 stained. Fig. 7A shows the results for the FCM analysis at 40 °C and 60 °C. For the 40 °C treatment a good correlation of the residuals was found (adj. $R^2 = 0.964$) for all pressure dwell times, as well as an adequate fit (adj. $R^2 = 0.76$) at 60 °C and pressure dwell times below 3 min. Furthermore, under these conditions all treated samples showed a positive SYTO 16 staining, which indicates spore germination. This shifted to a positive PI staining for longer pressure dwell times, indicating an inactivation. The detection of SYTO 16 fluorescence suggests that spores are able to degrade their cortex (Black et al., 2005) under pressure, for pressure treatments up to 600 MPa and moderate treatment temperatures, and even for pressures up to 900 MPa with short pressure dwell times at 60 °C.

For pressure dwell times above 3 min (Fig. 7A) or at higher treatment temperatures (80 °C, Fig. 7B), the inactivation mechanism seems to change. As shown in Fig. 2, a rapid release of DPA was detected under these conditions. However, a defined SYTO 16 or PI staining of the spores was no longer possible. Whereas spores treated at 60 °C and dwell times ≥3 min showed an intermediate fluorescent signal between a positive SYTO 16 or PI staining (described as the unknown state; Mathys et al., 2007), or were even not stainable, all spores showed a positive PI staining after a thermal treatment at 80 °C for 20 min. This indicates that a rupture of the inner spore membrane occurred after an additional mild thermal heat treatment.

A further process temperature increase to 80 °C during the pressure treatment (Fig. 7B) resulted in spores with an intermediate or positive PI staining, if the pressure dwell time was below 60 s. Additionally, all of these spores showed positive PI staining behavior after an additional mild thermal treatment (20 min at 80 °C) after the decompression. No correlation was found, however, between the calculated amounts of heat sensitive or inactivated spores with the FCM results.

Interestingly, no staining with SYTO 16 or PI was possible for spores that were pressure treated for more than 1 min at 80 °C under all tested pressure conditions (200 MPa–900 MPa). Furthermore, these spores also showed no positive PI staining after a mild thermal treatment (20 min at 80 °C), even though under some treatment conditions a full spore inactivation (>5.7 $\log_{10}$) was achieved during the pressure treatment. There could be various explanations for these diverse staining behaviors, and only a few authors have used FCM analysis for

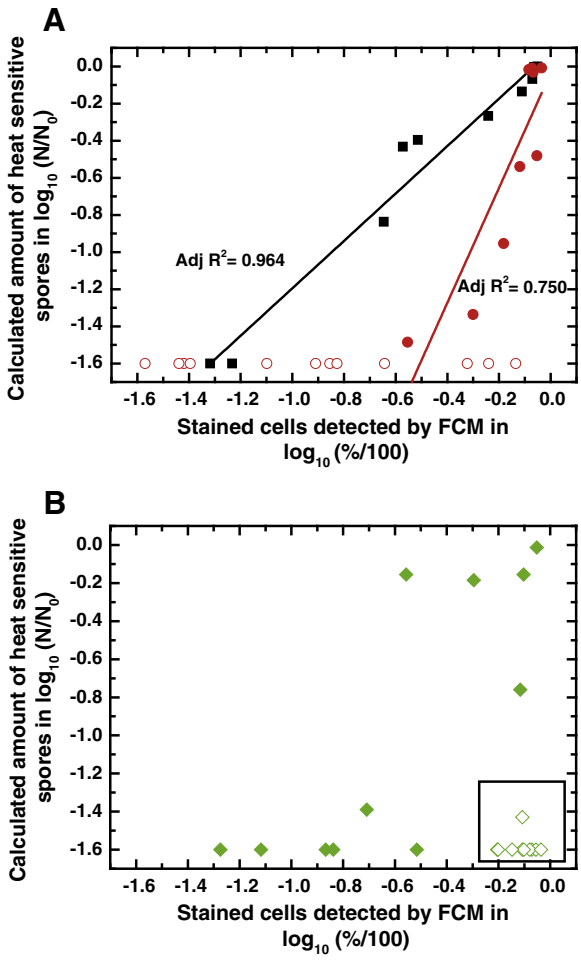

Fig. 7. Comparison of stained spores detected by FCM versus the calculated amount of heat sensitive spores. Residuals contain kinetic data from 200 MPa to 900 MPa arranged by the applied isothermal process temperature. A) Full symbols represent pressure dwell times below 3 min at 40 °C (■) and 60 °C (•); open dots represent pressure dwell times above 3 min at 60 °C (○). B) Full symbols represent pressure dwell times below 1 min and open symbols pressure dwell times above 1 min at 80 °C.

pressure-treated spores or for spores in general (Baier et al., 2011; Black et al., 2005, 2006; Laflamme et al., 2005; Mathys et al., 2007).

The positive SYTO 16 staining, as well as the good correlation of calculated heat sensitive and inactivated spores with the results from the FCM analysis, suggests that *B. subtilis* spores are able to degrade their cortex (Black et al., 2005) during pressure treatments up to 600 MPa and moderate treatment temperatures (Fig. 7A) and possibly also the SASPs (Kong et al., 2010). This finding supports the results from Wuytack et al. (1998) and Reineke et al. (2012), that under these treatment conditions spores are reaching stage II of germination (Setlow, 2003). Furthermore, the FCM analyses confirm the results for the DPA release, as well as for the spore germination and inactivation, in the sense that a change of the inactivation mechanisms occurs for pressure above 600 MPa and/or treatment temperatures above 50 °C.

The finding that spores treated at 80 °C, or for dwell times above 3 min at 60 °C, showed no positive SYTO 16 staining nearly independent of the applied pressure (200 MPa–900 MPa), may indicate that a cortex degradation (Black et al., 2005) under pressure is no longer possible, or that no or only a partial degradation of the SAPSs occurred (Kong et al., 2010). Even more interesting is that spores that were pressure treated at 80 °C for more than 60 s under all tested pressure levels showed no positive PI staining after an additional mild thermal treatment. In this case, inability for partial or full SASP degradation can be excluded because of a proper PI staining of autoclaved spores. A DNA disintegration, which is the target binding structure of SYTO 16 and PI, is also unlikely, since the applied pressure temperature conditions are not sufficient to cleave covalent bonds (Cheftel, 1995).

We therefore assume that for short pressure dwell times a partial cortex degradation may occur during the pressure dwell, the decompression phase (<10 s) and prior to the snap freezing (<1 min). This would enable a positive PI staining of the inactivated spore population. Pressure dwell times above 1 min at 80 °C may inactivate all cortex lytic enzymes and hence prevent the double-charged PI molecule from penetrating the spore cortex and binding at the DNA, even if the inner spore membrane is partly or fully ruptured.

4. Conclusions

In conclusion, the DPA release data presented in this study confirm that DPA release is the first indicator for pressure-induced loss of heat resistance, and the rate-limiting step for a successful spore inactivation in a high pressure thermal sterilization process. Furthermore, the isorate lines modeled for DPA release in the pressure–temperature landscape studied here enabled the determination of three distinct zones (Fig. 3). (I) For pressures <600 MPa and temperatures >50 °C, a 90% DPA release was feasible in less than 5 min and no difference in the amount of DPA was found both immediately after pressurization and 2 h later. When combined with the linear relationship for the DPA release rate constants $\ln(k_{DPA})$ between 1 and 30 min at 600 MPa treatment and 40 °C, these findings are an indication of irreversible damage of the inner spore membrane or membrane proteins in this region. (II) For pressures >600 MPa the synergism between pressure and temperature diminished and the treatment temperature dominated DPA release. (III) In the third region, for pressures <600 MPa and temperatures <50 °C, a retarded release of DPA was detected, with a strong difference in the amount of DPA released after 2 h. This indicates a pressure-induced germination that continues after pressure release.

The correlation of the kinetic data for the DPA release with the loss of heat resistance and the inactivation behavior of the spores suggests that spores' resistance to combined pressure and temperature treatments correlates with their ability to retain DPA. The results from flow cytometry analysis confirmed the assumption that a physiological pressure-induced germination occurs in zone (III), and that *B. subtilis* spores may reach stage II of germination under pressure. For zones (I) and (II), the results from the FCM analysis were contradictory, which may indicate that cortex degradation is not possible under these conditions, but that permeabilization of the inner spore membrane occurs. However, further research is needed to prove this assumption.

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7. Structural analysis of high pressure treated *Bacillus subtilis* spores

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Structural analysis of high pressure treated *Bacillus subtilis* spores

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ABSTRACT

The aim of this study was to investigate the internal structural changes in high pressure treated *Bacillus subtilis* spores and to correlate these changes with results from plate counts, flow cytometry and analysis of dipicolinic acid (DPA) release. For the scanning electron microscopic (EM) evaluation, a focused ion beam sectioning method was developed and compared with transmission EMs. Based on this set of methods, a mechanism was proposed in which 150 MPa at 37 °C induces a physiological-like DPA release, followed by degradation of the cortex and the DNA-binding small acid-soluble proteins (SASPs). Sectioned spores looked like hollow spheres and were inactivated prior to outgrowth. A pressure increase to 550 MPa retarded completion of germination and a porous internal network structure was visible by scanning EM, possibly indicating the lack of SASPs degradation. Higher pressures (600–900 MPa) and temperatures (60–80 °C) may inhibit the degradation of the spore cortex.

Industrial relevance: The high pressure thermal sterilization (HPTS) process is an emerging technology to produce high quality low acid food products, which are shelf-stable at ambient temperature. However, an industrial scale process has not yet been implemented. The mechanisms suggested in this paper may help to understand the mechanisms of inactivation of bacterial spores treated with various pressure and temperature combinations. In addition, a focused ion beam milling procedure was developed that enables the analysis of internal structure changes in bacterial endospores. These three-dimensional scanning electron micrographs (EMs), which have a reasonable resolution, could allow individual spore tomography, and not only for pressure treated spores.

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1. Introduction

Bacterial endospores produced by the bacteria classified in the genera *Bacillus* and *Clostridium* are a major concern with regard to the shelf-life of low-acid food. While the dormant spore itself causes no hazard in the food during storage, spore germination, outgrowth and subsequent growth can cause unwanted deterioration of food or even serious foodborne diseases. Thermal retorting is the process usually employed for the preservation of low-acid food, which involves temperatures above 115 °C being used for several minutes to inactivate all bacterial spores (Odlaug & Pflug, 1977). This high input of thermal energy, however, uses an undesirable over-processing of the food. Consequently, alternative preservation technologies are being explored to meet consumer demand for high quality foods with a high nutritional value. One of the most promising technologies in this regard is the application of high isostatic pressure for pasteurization and sterilization (Knorr et al., 2011; Lau & Turek, 2007). Pressure levels ranging from 100 to 300 MPa, in combination with ambient or higher pressures (>500 MPa) and elevated temperatures, were identified as having the capability to significantly reduce (Gould & Sale, 1970; Heinz & Knorr, 1998) or fully inactivate bacterial endospores (Ananta, Heinz, Schlüter, & Knorr 2001; Margosch et al., 2006; Mathys, Reineke, Heinz, & Knorr, 2009; Olivier et al., 2011; Rajan, Ahn, Balasubramaniam, & Yousef, 2006; Reineke et al., 2012).

Extensive research in recent decades has established a pressure-assisted thermal sterilization (PATS) process, in which thermal sterilization temperatures have to be reached during pressure dwell times. In addition, a high pressure thermal sterilization (HPTS) process was proposed, which additionally takes the impact of pressure on spore inactivation into account. A major concern hampering the industrialization of this technology, however, is that the exact inactivation mechanisms of bacterial spores by high pressure (HP) are unknown.

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Several authors have stated that the inactivation of spores under HP is either a two-step inactivation (Margosch, Gänzle, Ehrmann, & Vogel, 2004; Paidhungat, et al., 2002) or a three-step inactivation (Heinz & Knorr, 1996; Mathys, Chapman, Bull, Heinz & Knorr, 2007). Despite their difference, all authors have confirmed that one of the first events in the inactivation mechanism is a germination or at least a loss of heat resistance of the spore under HP, caused by a release of the spores' large depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA).

Wuytack, Boven, and Michiels (1998) found the highest inactivation of *Bacillus subtilis* spores to be with treatment conditions of 200 MPa and 40 °C, whereas the amount of germinated spores was nearly equal at all tested pressure levels (100–600 MPa). In addition, they reported that spores treated at 100 MPa and 40 °C showed a higher sensitivity to UV-light and hydrogen peroxide and had degraded their DNA protective α/β-type small acid-soluble proteins (SASP). These small proteins are highly concentrated in the spore core where they comprise 3–6% of total spore protein. The α/β SASP bind to and saturate spore DNA and cause a large structural change in the DNA; during spore germination these proteins are degraded (Setlow, 2007). In the dormant spore the saturation of DNA with α/β-type SASPs is the major mechanism preventing spore DNA damage by H_2O_2. An increase in the hydrogen peroxide-sensitivity of spores following HP treatment indicates that SASPs have been degraded, since H_2O_2 can permeate through the inner spore membrane and act in the core (Melly, et al., 2002).

Consequently, these spores are in stage II of the germination (Setlow, 2003) and possibly close to outgrowing. An increase of the process temperature (>50 °C) and pressure (>600 MPa) strongly accelerates the DPA release rate of spores of *B. subtilis* (Margosch et al., 2004; Reineke, Schlumbach, Baier, Mathys, & Knorr, in press) and other species (Margosch et al., 2004). Under more extreme processing conditions the release of DPA from the spore core is not initiated by HP triggering the spores' nutrient germinant receptors (nGRs), but most likely by direct opening of a Ca^{+2}–DPA channels in the spore's inner membrane (Paidhungat et al., 2002). In addition, irreversible structural changes in the inner spore membrane may occur under these conditions, which change the membrane's permeability properties (Reineke et al., in press).

As well as monitoring DPA release through high performance liquid chromatography (HPLC), a second methodology to investigate endospore germination after HP treatment is flow cytometry. Through the addition of different fluorescent dyes to the HP-treated sample, this method offers the possibility of a rapid detection of the physiological state of the spores (Black et al., 2005; Laflamme et al., 2005; Mathys et al., 2007).

The authors listed above used membrane-permeant, DNA-staining fluorescent dyes, such as SYTO16, to determine the population of germinated spores. In this regard, Black et al. (2005) stated that for a successful binding of SYTO16 to the DNA, the spore's peptidoglycan cortex has to be hydrolyzed. Using a double staining with SYTO16 and propidium iodide (PI), a membrane-impermeant fluorescent dye, Mathys et al. (2007) were able to distinguish between germinated and inactivated *Bacillus licheniformis* spore populations after a HP treatment. Furthermore, they described a third population, which they called the unknown population. Based on these data they suggested a three-step inactivation of spores under HP, involving a germination step with cortex hydrolysis, an unknown step, and finally an inactivation step with physical damage to the spore's inner membrane.

Such a three-step inactivation mechanism by HP–temperature combinations was also confirmed by flow cytometry analysis for *B. subtilis* spores after a HP treatment (Reineke et al., in press). However, for pressure dwell times above 3 min at high treatment temperatures (>60 °C), which are the relevant process conditions for a HP thermal sterilization, a diverse staining behavior or even no staining of the treated spores was possible. These findings were suggested to be due to a rapid inactivation of the spore's cortex lytic enzymes (CLE) and hence an inability of the charged fluorescent dyes (PI and SYTO16) to penetrate through the intact spore cortex, even if the inner spore membrane is partly or fully ruptured (Reineke et al., in press).

To evaluate the suggestion noted above, in the present work established methods, including cell count, flow cytometry analysis and the quantification of DPA release via HPLC, were used to investigate the physiological state of HP-treated wild-type spores of *B. subtilis* and some isogenic strains lacking components of the germination apparatus. *B. subtilis* was used for this work, since it is one of the best understood prokaryotes in terms of molecular and cell biology and up to now no official sterilization indicator microorganism for HPTS has been defined. The second aim of this study was to optimize and establish a rapid cross section cutting and microscopic technique to evaluate whether cortex degradation under certain pressure temperature conditions is possible and to correlate the micrographs with the physiological state of the spores determined by established methods.

2. Material and methods

2.1. B. subtilis strains used and spore preparation and purification

In this study, we used *B. subtilis* strain PS832 (wild-type) (Paidhungat & Setlow, 2000) and three isogenic derivatives: FB113, which lacks the two genes encoding the two redundant CLEs, CwlJ and SleB, that degrade spores' peptidoglycan cortex during germination; FB114, which lacks the genes encoding SleB as well as the major nGRs, GerA, GerB and GerK; and FB115, which lacks the genes encoding the nGRs and CwlJ (Paidhungat, Ragkousi, & Setlow, 2001). Wild-type spores (strain PS832) as well as spores of the three mutant strains were prepared at 37 °C on 2×SG agar plates without antibiotics, according to a method described elsewhere (Nicholson & Setlow, 1990; Paidhungat & Setlow, 2000). After sporulation, the spores were harvested and spores were cleaned by repeated centrifugation (3-fold at 5000 ×*g*), washed with cold distilled water (4 °C), and sonicated for 1 min. The purified spore suspensions contained ≥95% phase-bright spores and nearly no spore agglomerates, as was verified by a particle image analyses system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.). The spores were then stored in distilled water in the dark at 4 °C.

2.2. Isostatic HP treatment

Each spore suspension was diluted prior to HP treatment at a ratio of 1:10 in pressure-stable N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer solution (Mathys, Kallmeyer, Heinz, & Knorr, 2008) (pH 7, 0.05 M), to a final concentration of approximately 10^8 spores mL^{-1}. The diluted samples were placed in 1.6 mL plastic tubes with screw caps (CryoTube Vials, Nunc Brand Products, Roskilde, Denmark), or small aliquots of 300 μL were pipetted into shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany, inner diameter 3 mm, outer diameter 3.6 mm). Tubes were hermitically sealed with a soldering iron.

The pressure trials were performed in two HP systems. For all kinetics up to 550 MPa and 60 °C, a HP-System (0101-7000-S, Sitec Sieber Engineering AG, Zurich, Switzerland) with a 100 mL vessel volume was used, where water served as the pressure-transmitting medium. To achieve isothermal conditions during the pressure dwell time, the system was equipped with a double-jacket HP vessel, connected to a thermostatic bath. This system is capable of HP treating four 1.6 mL samples and an additional 1.6 mL vial for a temperature measurement in the geometrical center of the sample.

For kinetics at 900 MPa and 60 and 80 °C, a U111 unit (Unipress, Warsaw, Poland) with a 3.7 mL vessel volume was used. Due to the smaller size of the pressure vessel, the sample volume was reduced

to 4×300 μL. In this pressure equipment, Bis(2-ethylhexyl)-sebacate served as the pressure-transmitting medium and the pressure vessel was immersed in a thermostatic oil bath (cc2, Huber GmbH). To ensure isothermal conditions during pressure dwell time, the temperature was measured in the geometrical center of a dummy sample. Representative pressure–temperature profiles for both set-ups are published elsewhere (Reineke, Mathys, & Knorr, 2011).

2.3. Determination of spore germination and inactivation

To distinguish between heat-sensitive and inactivated spores after pressurization, an aliquot of each sample was mild-heat treated at 80 °C for 20 min to inactivate germinated but not dormant spores. After heating, appropriate dilutions of each sample were surface-plated in duplicate on Nutrient-agar plates (CM 003, Oxoid Ltd., Hampshire, England) and incubated at 37 °C for 2 days.

Aliquots of samples HP-treated at 150 MPa and 550 MPa were also suspended in room temperature hydrogen peroxide (0.4 M), and after 8 min diluted 1:1 in a catalase solution (C1345-1G, Sigma-Aldrich-Chemie GmbH, Steinheim, Germany), with a final concentration of 2000–5000 units/mL to rapidly break down the remaining H_2O_2 to H_2O. The aim of this treatment was to estimate whether the HP-treated spores were able to degrade their SASPs (Wuytack et al., 1998) and hence proceed through stage II of germination.

2.4. Measurement of DPA release

Spores' release of DPA following HP treatment was determined individually for each kinetic point. For quantification, the suspension was filtered through a 0.2 μm nylon filter (Rotilabo syringe filter, Carl Roth GmbH, Karlsruhe, Germany). The permeate was analyzed by HPLC (Dionex Corporation, Sunnyvale, CA, USA) as described elsewhere (Tabor, Macgee, & Holland, 1976).

The total amount of DPA in each individual batch was determined after autoclaving at 121 °C for 20 min (Janssen, Lund, & Anderson, 1958). Pressure-induced DPA release was calculated relative to the total DPA content of each individual spore batch. Data are represented as means of at least two independent experiments.

2.5. Flow cytometry analysis

For sample preparation for flow cytometry a double staining was used, according to the method of Mathys et al. (2007). The fluorescent dyes used, SYTO16 (Invitrogen, Carlsbad, CA, USA) and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA), are both able to stain DNA, but whereas the membrane-permeant SYTO16 acts as an indicator for spore germination, the membrane-impermeant PI indicates membrane damage.

The treated spore suspensions were diluted with ACES buffer solution (0.05 M, pH 7) to achieve a flow rate of about 1000 events/s as well as a constant ratio of spores for staining. The concentrations of the fluorescent dyes were 15 μM PI and 0.5 μM SYTO16 in the diluted spore suspension. Afterwards, the samples were stored in the dark at ambient temperature for 15 min.

Flow cytometry analyses were carried out using a BD FACSCalibur flow cytometer (BD Biosciences, San José, CA, USA) equipped with a 15 mW, 488 nm air-cooled argon ion laser. BD CellQuest Pro (Version 5.2, BD Biosiences) was used as operation and acquisition software. To achieve a clear determination of the different spore populations and to discriminate non-spore particles, the amplification of the forward scatter (FSC) was set to E01 (10-fold) and the FSC threshold was set to 72. The amplification of the side scatter (SSC) was set to 721 V and the SSC threshold was set to 706.

2.6. Sample preparation for transmission electron microcopy (TEM)

Spore samples were prefixed in a 2.5% glutaraldehyde (354400, Merck KGaA, Darmstadt, Germany) and 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature and afterwards centrifuged at 10,000 ×*g*. For postfixation, the spore pellets were suspended in 1% osmium tetroxide (124505, Merck KGaA, Darmstadt, Germany). Samples were again centrifuged for 10 min at 10,000 ×*g*, and pellets were suspended in a melted 1.5% agar medium (LP0011, Agar No. 1, Oxoid LTD., Basingstoke, Hampshire, UK). The solidified agar was cut into 0.5 cm pieces and dehydrated with an ethanol series (5054.2, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) ascending in decimal steps. For embedding, a four component resin was used, consisting of: Glycidether 100 (Art.-Nr. 8619.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany); DBA as a hardener (dodecenylsuccinic anhydride, Carl Roth GmbH + Co. KG, Karlsruhe, Germany); MNA as a hardener (8639.1, Nadic methyl anhydride, Carl Roth GmbH + Co KG., Karlsruhe, Germany); and BDMA as an accelerator (dimethylbenzylamine, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The resin blocks were cut with an ultramicrotome (Reichert Ultracut S, Leica, Wetzlar, Germany) into semi-thin sections of 70 μm. For negative staining a solution comprising 2% aqueous uranyl acetate (Serva Feinbiochemica, Heidelberg, Germany) and lead citrate (Ultostain2, stabilized solution of lead citrate, Laurylab, Saint-Fons, France) was used. The examination of the specimen was carried out using a "EM 10 CR" transmission electron microscope (Zeiss, Oberkochen, Germany).

2.7. Focused Ion beam sectioning of spore samples

2.7.1. Critical point drying

Since scanning EM must be done in a high vacuum, water has to be removed from the samples. Hence, to avoid creating structural damage to the spores, critical point drying was used to avoid the effects of the surface tension of water on the structure of spores occurring during drying. Prior to the critical point drying, the spore suspension was centrifuged (6 min at 10,000 ×*g*) and the pellet suspended in 2 mL full strength acetone for 24 h. The dehydrated samples were transferred into individual chambers of the specimen holder of a critical point dryer (BAL-TEC CPD 030, BAL-TEC GmbH, Witten, Germany), in which silicon wafers were laid out as an underlayer in each chamber. After repeated flooding of the specimen holder with liquid CO_2 and with 10 min stirring intervals, the system was heated above the critical temperature of CO_2 (31 °C, 7.38 MPa). A subsequent pressure reduction leads to a controlled evaporation of CO_2, which preserves the three-dimensional structure of the spores.

2.7.2. Focused ion beam (FIB) sectioning

For focused ion beam (FIB) sectioning and scanning electron microscopic (SEM) imaging, a Helios NanoLab 600 dual-beam microscope (FEI, Hillsboro, USA) was used. To improve the conductivity of the sample, a silver suspension (Leitsilber ACHESON, Plano, Marburg, Germany) was dripped onto the specimen holder in contact with the silicon wafer and it was thoroughly air-dried. The samples were transferred to the dual-beam microscope via an air lock to work in a clean vacuum. The FeiSystemControl software was used to control the ion and the electron beam as well as all other parameters simultaneously. In order to reduce thermal change and charging of the biological material, the samples were coated with an about 500 nm thick platinum (Pt) layer: a precursor gas was injected close to the sample surface via the needle of the gas injection system (GIS). The precursor gas adsorbs on the surface, and the electron or ion beam scanning over the defined area cracks the adsorbed molecules, leaving a deposit of the required material, namely Pt. It is important that first a electron beam induced Pt deposition (at 3 keV, 11 nA, 5 min) is applied, since the ion beam induced Pt deposition changes the sample's surface due to ion bombardment. After Pt deposition,

cross sections of the spores were milled at an ion beam energy of 30 keV and an ion beam current of 9 nA by scanning the beam line-by-line over the determined area. The cross sections were imaged with the SEM at low electron energies (1 kV) in the high resolution immersion mode of the electron column with detection of the inlens secondary electron (SE) signal. For SEM imaging, the imaging low-scan mode was used.

2.8. Reproducibility of results

All experiments were done at least in duplicate, and the data presented are either the means of two replicate experiments or single representative experiments. For the analysis of FIB sectioned spores at least 50 were milled from each sample.

3. Results and discussion

3.1. Optimization of FIB sectioning and comparison with TEM

The examination of bacterial spores proved difficult with SEM because of non-conductive materials such as carbon, oxygen and hydrogen, since a high conductivity of the analyzed sample is very important for high quality SEM imaging. A non-conductive sample is charging under the electron beam reducing the resolution significantly. Hence, in the first test series spore samples were sputtered with gold, but after spore milling with the ion beam hardly any differences between the inner structure of the spore and the gold coating could be identified. Fortunately a Pt-coating led to a sufficient conductivity and due to the unique structure of the Pt-layer it could be is easily distinguished from the spore. The second – and probably more important – purpose of the Pt-layer was to remove the heat generated by the bombardment of the ion beam. Otherwise the sample might have heated up to several hundreds of degrees, changing the spore unpredictably. Again Pt was found to be more suitable compared to Au.

Because Pt deposition was essential for protection of the spores, it was utilized every time. The thickness used for the Pt layer depended on several factors. Spores that passed through structural changes during HP treatment, like full core hydration, were covered with a thicker layer than untreated spores. However, more Pt on the spores led to a thin cover or Pt bumps inside the spore, due to condensation. In this regard, it was found that single spores having direct contact to the conductive underlayer needed only a thinner Pt cover.

A second method to increase the conductivity of the analyzed sample is the evaporation of carbon, depositing only a nm-thick layer on the sample (Bassi, Cappa, & Cocconcelli, 2009; Weber, et al., 2010). Especially on dormant spores (Fig. 1a), a carbon layer proved to be an advantage. Compared to Fig. 2, the core in the micrographs in Fig. 1a appears lighter and rounder. The good imaging quality may also be attributed to the low spore concentration on the silicon wafer. Images of spores that were HP treated and subsequently carbon coated did not show any improvement regarding contrast of the internal structure.

A comparison of micrographs from the FIB sectioning (Fig. 1a) with TEM micrographs (Fig. 1b) clearly illustrates the higher resolution of the TEM microscope. Images with a 63,000-fold magnification enabled a differentiation between several of the structures of a dormant spore (Santo & Doi, 1974).

The potential of FIB milling of dormant spores was published by Weber et al. (2010) for *Bacillus thuringiensis* spores. Whereas they used in situ lift-out FIB sectioning and thinning for preparation and a TEM microscope for imaging, the method presented in this study is significantly easier and faster (~20 min). The method used here showed slightly less details of the spore's internal structure, but it enabled visualization of the internal spore structures (differentiation between the spore core and the cortex) without contrasting with heavy-metals, like those needed for TEM imaging. Another advantage is that everything is done in one microscope without the necessity of any lift-out and transfer procedure.

In this study, we found the following attributes for a suitable specimen area on the silicon wafer. In the ideal case, the investigated spores should neither be in a large aggregate nor singular to have the optimum contact to the Pt-protective layer and the least changes of the spore due to the ion beam impact. Further, a drip of silver gel close to the investigated area prevents charging by excess electrons. The FIB milling area should cover several spores or a small spore assembly, to better assess the impact of the applied process to the structure of the spore. Furthermore, Pt deposition on a smaller area reduced the amount of time required for sample preparation. The spore assembly under investigation should not rise too much because contact to the silicon wafer cannot be guaranteed. This again leads to too many excess electrons and charging of the focused area, whereas a thicker Pt layer on the spores does not improve conductivity in this case.

3.2. HP-induced spore germination at 150 MPa and 37 °C

To investigate the impact of a moderate HP treatment on structural changes occurring in the spore, wild-type *B. subtilis* spores (PS832) were treated at 150 MPa and isothermal 37 °C during the pressure dwell time for various times. The results for the loss of heat and hydrogen peroxide resistance, as well as for spore inactivation and DPA release are presented in Fig. 3.

The kinetic data clearly showed that after 5 min of this HP treatment, ~90% of spore DPA was released due to HP-induced triggering of nGRs (Gould & Sale, 1970). The associated uptake of water in the spore core correlates with the 1.69 $\log_{10}$ heat-sensitive spores after this HP treatment for 5 min. However, only a small amount

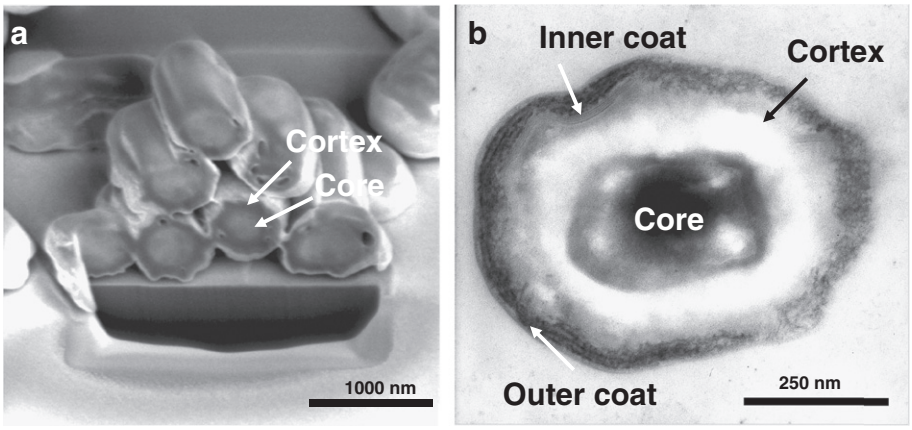

Fig. 1. a and b. Micrographs of dormant *Bacillus subtilis* (PS832, wild-type) spores a) with a carbon layer, sectioned with FIB and b) TEM with a 63,000-fold magnification.

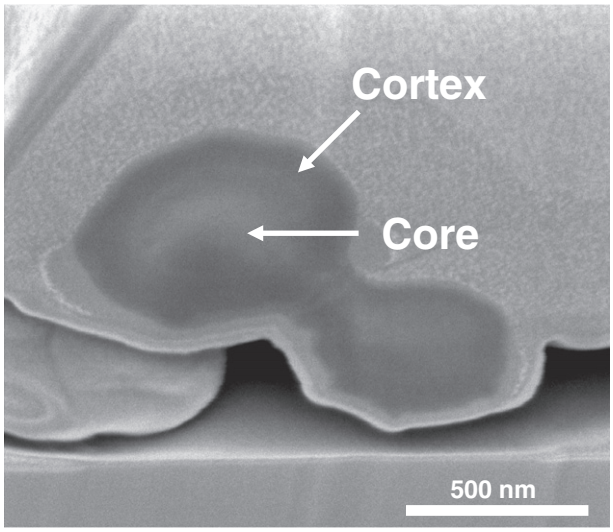

Fig. 2. Dormant *Bacillus subtilis* (PS832, wild-type) spores with Pt layer, sectioned with FIB.

of hydrogen peroxide-sensitive spores (0.29 $\log_{10}$) were detected. Longer pressure dwell times gradually increased the amount of heat-sensitive spores to 4.19 $\log_{10}$. Additionally, 3.6 $\log_{10}$ of the spore population lost its hydrogen peroxide resistance after 90 min under these HP conditions. Consequently, the spores proceeded stage I of germination, which includes a cortex degradation (Setlow, 2003) followed by SASP degradation, as expected (Wuytack et al., 1998). Whereas metabolism and SASP degradation take place at the end of stage II of germination, these events may begin partway through stage II when the core water content has risen sufficiently for enzyme action (Setlow, 2003). In addition, under these conditions a spore inactivation of 1.6 $\log_{10}$ was detected, which may indicate that vital biochemical process are inactivated.

Because of the rapid release of DPA with a pressure of 150 MPa at 37 °C, core hydration occurred during the HP treatment. Consequently, because of the high DPA concentration in the spore core (Setlow, 2003) and the low water content (de Vries, 2006; Sunde, Setlow, Hederstedt, & Halle, 2009), structural changes inside these HP-treated spores should be visible after FIB sectioning. For the FIB analysis, all HP-treated samples were covered with a thin Pt layer to dissipate the heat occurring during the milling. The pictures presented in Fig. 4 are representative

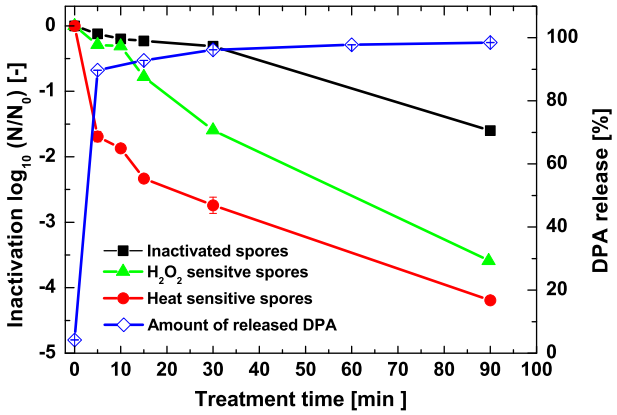

Fig. 3. Kinetic data for the formation of heat (red, ●) and hydrogen peroxide (green, ▲) sensitive spores and spore inactivation for *Bacillus subtilis* (PS832, wild-type) spores (black, ■) at isothermal (37 °C) and isobaric (150 MPa) conditions during pressure dwell time. The solid blue line (◊) denotes the amount of DPA released as measured after decompression relative to the initial DPA content of the spores.

examples from several micrographs. The critical point drying preserved the ellipsoid shape of the spores and, as expected, all spores looked like hollow spheres. Hence, the core hydration is as well visible in these micrographs. Moreover, a reduced thickness of the cortex after the pressure treatment compared to dormant spores is visible (Fig. 2), which confirms at least some cortex degradation has taken place during the HP treatment. Given that enzymatic cortex degradation during nutrient-induced spore germination takes roughly 20 min (Zhang, Thomas, Li, & Setlow, 2012), and that the disintegration of the inner and outer spore coats occurs after 40 min (Santo and Doi, 1974), a stepwise diminution of the cortex thickness should be visible in Fig. 4. However, this is not the case and all samples look alike. There was also no difference visible between HP-germinated (5–30 min) and pressure inactivated spores (90 min). This finding suggests that within the samples treated for 5 and 15 min at 150 MPa and 37 °C, the cortex degradation was triggered and had continued after decompression until the preparation for SEM analysis. The bumps present in some of the spores are preparation artifacts and are caused by condensed Pt evaporating during FIB milling.

To further confirm the possibility of cortex degradation under moderate pressure (150 MPa), a TEM micrograph for an intermediate point (15 min) was prepared, as shown in Fig. 5. The direct comparison with a dormant spore (Fig. 1b) clearly shows an expanded (dormant spore core 275.5 ± 85.5 nm vs. 434.5 ± 63.7 nm after pressure treatment) and lighter spore core, representing a reduced electron density and consequently confirming a hydration of the core. Further structures inside the core were visible, which may be mesosomes or other plasma membrane structures, like those found 20 min after initiating nutrient-induced germination by Santo and Doi (1974). A decrease in cortex thickness is also visible, similar to the FIB-sectioned spores in Fig. 4. This indicates that the CLEs of *B. subtilis* spores are active under pressure (150 MPa). It must be taken into account, however, that the enzymatic cortex degradation may also have taken place after decompression prior to sample preparation for the micrographs shown in Figs. 4 and 5. To proof this, aliquots of each sample were immediately snap frozen in liquid nitrogen after decompression and flow cytometry analysis were performed. These analyses showed that after 5 min of pressure treatment all spores were stainable with SYTO16 (data not shown), which confirmed that cortex degradation (Black et al., 2005) has taken place under a pressure of 150 MPa.

Flow cytometry analyses further showed that after a 10 min HP treatment at 150 MPa and 37 °C (Fig. 5b), the entire spore population was shifted from region R2 (germinated) into region R3 (unknown; Mathys et al. (2007)), and R4 (inactivated). After an additional mild-heat treatment at 80 °C for 20 min, the spore population was PI stainable (Fig. 5c), which confirmed that more than 2 $\log_{10}$ of the entire spore population had lost heat resistance due to core hydration.

3.3. HP-induced spore germination at 550 MPa and 37 °C

Given published data about the HP-induced DPA release (Wuytack et al., 1998; Reineke et al., in press) and the impact of HP on the triggering of nGRs (Paidhungat et al., 2002; Reineke et al., 2012), spores of strains PS832, FB113, FB114 and FB115 were pressure treated at 550 MPa and 37 °C for 60 min. These process conditions induced release of DPA, but caused only slight or no spore inactivation (Table 1). Whereas the spores with nGRs (PS832 and FB113) released nearly all their DPA, the spores lacking nGRs (FB114 and FB115) released only ~2/3 of their DPA.

For spores of strains PS832, FB114 and FB115, large amounts of heat-sensitive spores (≥2.82 $\log_{10}$) were also detected following HP treatment at 550 MPa and 37 °C for 60 min, implying that core hydration occurred during the treatment. However, in contrast to the nearly equal amounts of heat-sensitive *B. subtilis* PS832 spores at 150 MPa and 550 MPa after 60 min, there was only a marginal (−0.2 $\log_{10}$)

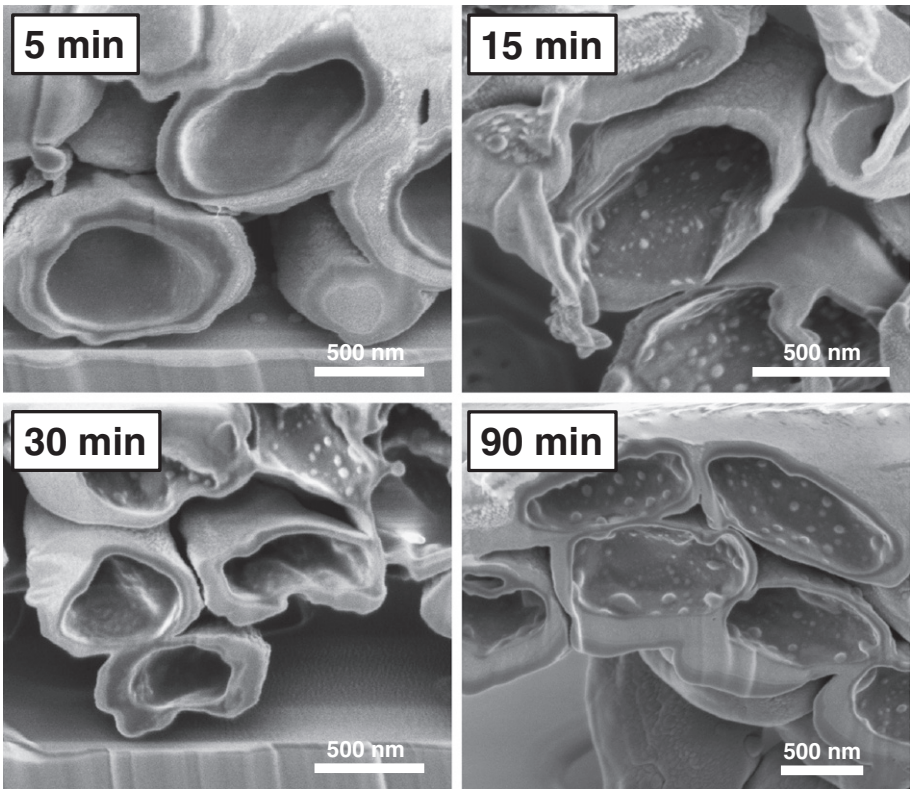

Fig. 4. a–d. FIB sectioned *Bacillus subtilis* (PS832, wild-type) spores pressure treated at 150 MPa and 37 °C after various pressure dwell times.

amount of hydrogen peroxide-sensitive spores after the 550 MPa treatment. Similar findings were reported by Wuytack et al. (1998) after a 30 min HP treatment under similar pressure and temperature conditions. These latter authors attributed this to the lack of SASPs degradation under very high pressures, and suggested that these spores were stuck in an early stage of germination. A difference in the germination behavior between 150 MPa and 550 MPa was also measured by flow cytometry (Fig. 6). Whereas after a 5 min HP treatment at 150 MPa and 37 °C all spores were detected in region R2 (germinated), this took 60 min at 550 MPa. In addition, representative FIB-sectioned and TEM micrographs showed that completion of germination is retarded for wild-type *B. subtilis* spores treated at 550 MPa 60 min (~90%). In contrast, spores treated at 150 MPa (Fig. 5a) seemed to be at the same or even a further stage of germination after 15 min. Furthermore, most (~72%) of the FIB sectioned spores treated at 550 MPa for 60 min showed a porous network in the spore core, unlike the hollow sphere appearance of the 150 MPa-treated spores (Fig. 4).

Comparison of the flow cytometry results and micrographs from the wild-type *B. subtilis* spores (PS832) with representative pictures from spores lacking nGRs (FB114 and FB115) also showed retarded germination of these spores at 550 MPa. Whereas the TEM micrographs from *B. subtilis* wild-type and FB114 spores are similar, the results from flow cytometry depicted that the lack of nGRs and the CLE SleB slows full HP germination. This effect was more pronounced for FB115 spores lacking the CLE CwlJ, which is directly activated by the release of DPA (Paidhungat et al., 2002). The FIB sectioned micrographs of FB114 and FB115 spores are also different. Neither the porous network structure nor the hollow sphere appearance was found for most of the milled spores. These two spore strains showed in most cases a solid part in the middle of the spore core, surrounded by a large cavity. The FIB–SEM pictures from the FB113 spores that retain nGRs but lack both CLEs, looked similar to micrographs from the wild-type spore. Hence this solid part may depict the residual DPA in the spore core of the strains FB114 and FB115, which represents approximately 32–33% of the total DPA amount (Table 1). The partial release of DPA may be caused by reversible structural changes in the inner spore membrane under HP (≤550 MPa), which slightly increases its permeability. Hence, after decompression the membrane properties are restored and no further DPA release was possible. This effect was not observed for PS832 and FB113 spores because of the irreversible triggering of the nGRs of these spores during compression.

Based on these different analytic, microbiological and microscopic techniques, we propose the following mechanism. Spores treated at moderate pressure (150 MPa) and temperature (37 °C) rapidly release their DPA through a pressure triggered physiological germination involving the nGRs. After core hydration, these spores are able to degrade their cortex under pressure (supported by the data from FCM, FIB–SEM and TEM) and the SASPs are also degraded (supported by the loss of resistance against hydrogen peroxide and FIB–SEM micrographs). A pressure increase to 550 MPa leads to a physically induced, unphysiological (i.e. — not involving nGRs) DPA release, even in spores without nGRs. However, the DPA release rate at 550 MPa for spores that lack nGRs is lower than for spores with nGRs, due to a superimposition of the triggering of the nGRs and the impact of pressure on the inner spore membrane and/or membrane proteins like the SpoVA proteins, which are supposed to be the Ca^{2+}–DPA channel proteins (Paidhungat et al., 2002; Li et al., 2012; Reineke et al., 2012) for spores with nGRs. Previous studies (Reineke et al., in press) distinguished three different mechanisms for the DPA release and identified a threshold pressure of 600 MPa. The pressure–temperature–time combinations investigated in this study are in the region of physiological-like germination (Reineke et al., in press), but represent the upper and lower limits of this. Hence, they should display a change of physiological-like germination to a non-physiological, pressure-induced DPA release. This difference is especially visible in the FIB–SEM micrographs and the change from the "hollow sphere" appearance (150 MPa) to the "porous network structure" (550 MPa). Hence it is assumed that the spores are unable to degrade their SASPs at 550 MPa, which is supported by these treated spores' high resistance to hydrogen peroxide and their reduced binding of SYTO16 (Kong, Zhang, Yu, Setlow,

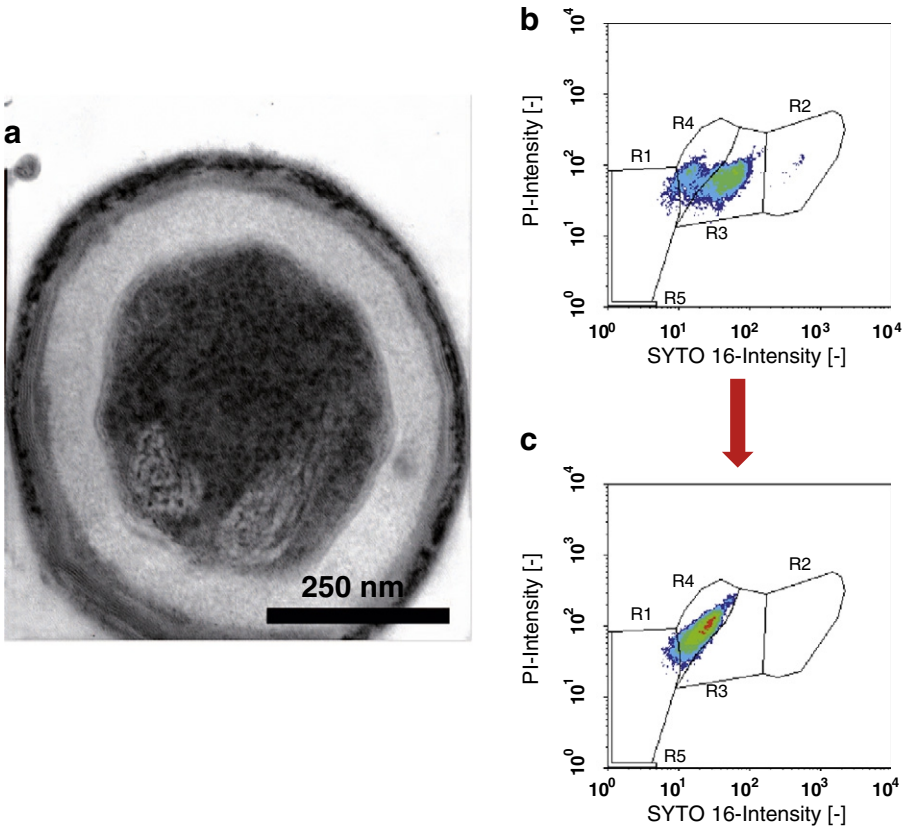

Fig. 5. a–c. a) TEM micrograph (100,000 fold magnification) of a *Bacillus subtilis* (PS832, wild-type) spore after a 10 min pressure treatment at 150 MPa and 37 °C. b, c) Corresponding results of the flow cytometric analysis (density plots) after pressure treatment (b) and after an additional mild heat treatment (c). Gatings are: R1 dormant, R2 germinated, R3 unknown, R4 inactivated and R5 background signal.

& Li, 2010; Baier, Kummer, Reineke, Mathys, & Knorr, 2012). The remaining SASPs (up to 20% of total spore core protein in a dormant *B. subtilis* spore (Setlow, 2003), which surround the DNA, may be sufficiently high (according to Wuytack et al. (1998)) that Pt could condense on it during the FIB milling. Hence, the DNA saturated with SASP could be responsible for the visible "porous network structure" in the core. That the "porous network structure" is not visible in representative FIB–SEM micrographs of the FB114 and FB115 spores could be attributed to the remaining DPA in the core. The spores that were unable to release all their DPA showed a solid inner core surrounded by a large cavity in the FIB–SEM micrographs, possibly pointing towards continuous dissolving of the DPA in the core, which is continuously replaced by water, as reported by Kong, Zhang, Yu, Setlow, and Li (2011).

3.4. HP-induced spore inactivation above 600 MPa and 60 °C

The combination of very HPs and high temperatures is known to rapidly induce DPA release (Margosch et al., 2004; Reineke et al., in press) and to lead to subsequent inactivation of spores. To evaluate the impact of these conditions on the internal structure of spores, FIB sectioned SEM as well as TEM micrographs were made. Table 2 gives an overview of the pressure, temperature and time combinations that were used, and the corresponding amounts of heat-sensitive and inactivated spores, as well as the amounts of DPA released. The increase of process temperature dramatically accelerated DPA release compared to spores treated at 550 MPa and 37 °C (Table 1), but no remarkable inactivation ($0.15 \pm 0.1.9$ $\log_{10}$) was detected. This could be due to irreversible structural damage to the inner spore membrane, leading to further diffusion of DPA out of the spore core during the more extreme heat treatment during pressure dwell time (Reineke et al., in press). More remarkable is the dramatic increase in heat-sensitive spores with short pressure dwell times. At 550 MPa and 60 °C for just 2 min, a release of 37% of DPA was associated with 2.7 ± 0.26 $\log_{10}$ heat-sensitive spores.

All TEM micrographs evaluated for these conditions (e.g. Fig. 7) show some similarities to the TEM micrograph for the wild-type spores treated at an equal HP level for 60 min at 37 °C (Fig. 6). However, based on all analyzed FIB–SEM micrographs, the internal structure differs from that of wild-type spores treated at 37 °C and 150 MPa (Fig. 4) or 550 MPa (Fig. 6). These combinations of HPs and temperatures resulted in similar internal structures to those found for spores without nGRs (FB114 and FB115; Fig. 6). The appearance of these structures was as if the DPA was partly dissolved in the spore core, which correlates with the 37% of released DPA detected by HPLC. This could be attributed to a pressure induced full DPA release in 1/3 of the entire spore population. However, due to the DPA sampling immediately after decompression and the short pressure dwell time (2 min), a second explanation could be a further DPA release after the decompression.

Table 1
Microbial counts [$\log_{10}(N/N_0)$] after 60 min dwell time at 550 MPa at 37 °C for spores of *Bacillus subtilis* strains and with the corresponding amounts of heat-sensitive spores and DPA released.

Strain	Inactivation	Heat-sensitive	DPA release [%]
PS832	0.14 ± 0.21	-3.89 ± 0.14	98
FB113	–[a]	–[a]	96
FB114	Full germination[b]	-2.97 ± 0.19	68
FB115	Full germination[b]	-2.82 ± 0.24	67

[a] Spores lack CLEs and cannot grow on nutrient agar plates.
[b] Spores lack nutrient germinant receptors and can only be germinated by pressure.

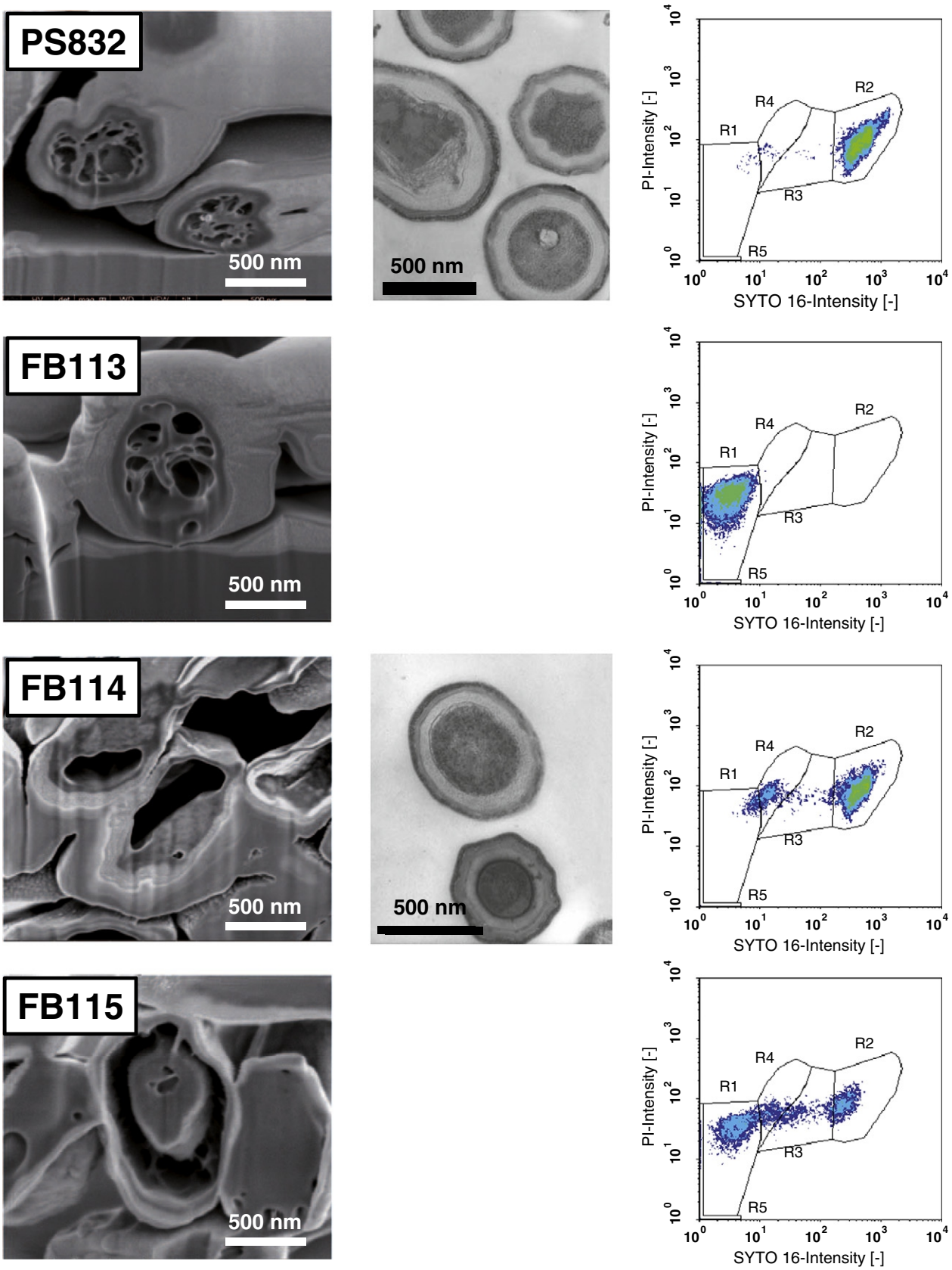

Fig. 6. FIB sectioned SEM and TEM micrographs (50,000-fold magnification) for *Bacillus subtilis* (PS832, wild-type) spores after a 60 min pressure treatment at 550 MPa and 37 °C and corresponding results of the flow cytometric analysis (density plots). Gatings are: R1 dormant, R2 germinated, R3 unknown, R4 inactivated and R5 background signal.

Table 2
Microbial inactivation [$\log_{10}(N/N_0)$] for *Bacillus subtilis* PS832 (wild-type) spores with the corresponding amount of heat-sensitive spores and the amount of released DPA. 5.3 $\log_{10}$ denotes the detection limit of the plate count method.

Pressure [MPa]	Temperature [°C]	Time [min]	Inactivation	Heat-sensitive	DPA release [%]
550	60	2	0.15 ± 0.19	2.7 ± 0.26	37
900	60	10	1.1 ± 0.14	4.39 ± 0.31	67
900	80	1	4.13 ± 0.26	> 5.3 ± 0.34	95
0.4	121	15	> 5.3 ± 0.47	–	100

A pressure increase to 900 MPa further accelerated DPA release (Table 2), and the temperature had an even more pronounced effect. At 900 MPa, inactivations of 1.1 ± 0.14 $\log_{10}$ at 60 °C and 4.13 ± 0.26 $\log_{10}$ after an additional mild heat treatment were achieved, which implies that in Fig. 8 the majority of spores are inactivated. An SEM micrograph of autoclaved *B. subtilis* wild-type spores is also presented in this figure. The FIB milled SEM micrograph for the autoclaved spores shows a fully hydrated spore core surrounded by the cortex, which is attributed to a thermal destruction of the spore's inner membrane. This in turn is associated with full DPA release (Janssen et al., 1958) and the inactivation of core enzymes (Warth, 1980; Setlow, 2000). The representative micrograph for the 10 min treatment at 60 °C and 900 MPa, is similar to the SEM micrograph in Fig. 7, showing a solid inner part in the spore core surrounded by a cavity. The results from flow cytometry showed that 59.4 ± 6.3% of spores were detected in the R3 region (unknown state) and 22.8 ± 5.9% in the R4 region (inactivated). This suggests that under these pressure–temperature conditions spores in the unknown state are not culturable, in contrast to spores in R3 detected after a 150 MPa treatment (Fig. 5). This finding may be due to more severe damage to the inner spore membrane for spores treated at higher pressures and temperatures, compared to much less and sublethal damage induced at low pressures and moderate temperatures. Further to this, Kong et al. (2010), reported an increased binding affinity of SYTO16 for DNA if the SASPs are degraded, which correlates with the increase in hydrogen peroxide-sensitive spores for longer dwell times at 150 MPa.

Surprisingly, the results noted above were countered by those from flow cytometry for the spores treated at 80 °C and 900 MPa for 1 min. Under these process conditions only 15.4 ± 3.9% of spores were detected in R3 (unknown) and 20.8 ± 1.4% in R4 (inactivated), even though a 4.13 ± 0.26 $\log_{10}$ inactivation was determined by cell count. The majority of the spore population was unstainable with SYTO 16 and PI, and was detected in R1 by flow cytometry (63.1 ± 2.6%). Such contradictory findings are also displayed in the FIB sectioned SEM micrographs. Some of the milled spores look like hollow spheres (~9%), indicating a full core hydration. However, most of the sectioned spores in all analyzed micrographs had large pores or a solid core surrounded by a large cavity.

Such a diverse staining behavior for pressure dwell times above 3 min at 60 °C, or 1 min at 80 °C, was also previously published by Reineke et al. (in press). There it was attributed to either partial cortex degradation or no cortex degradation, caused by the inactivation of the CLEs under pressure. The intact cortex will hinder the double-charged PI molecule from penetrating the spore cortex (Black et al., 2005) and binding to the DNA, even if the inner spore membrane is partly or fully ruptured. This finding is also supported by the micrographs in Fig. 8. The cortex seems to be thicker compared to the FIB–SEM micrographs in Figs. 4 and 6. Furthermore, for pressure–temperature–time combinations resulting only in a partial DPA release, again a solid inner part of the spore core was present, whereas for the spores treated at 900 MPa and 80 °C for 1 min (95% DPA release), again a "porous network structure" was found. This would support the assumption that spores are unable to degrade their SASPs at pressures above 550 MPa.

4. Conclusion

The aim of this study was to investigate the internal structural changes in HP-treated *Bacillus* spores and to correlate these changes with results from analysis of plate counts, flow cytometry and DPA release. To enable fast preparation and investigation of the internal structure of HP-treated spores, a new method of SEM imaging combined with FIB sectioning was tested, optimized and successfully used. Most important is the investigation of a suitable protection layer prior to the FIB sectioning to avoid charging and heat damages. The FIB–SEM method was compared with TEM micrographs, which were produced using established preparation methods. The FIB sectioning method proved suitable for rapid analysis of critical point dried spore samples. In addition to the advantage that imaging is possible without contrasting with heavy-metals, as is needed for TEM imaging, FIB sectioning enables a higher throughput of samples and an individual selection of spores which should be cut by the ion beam. These micrographs also display spores with a reasonable resolution and the method might therefore allow individual spore tomography. In the next step it is possible to expand the FIB-sectioning to a three-dimensional analysis if the spore is cut slice by slice with consecutive SEM-imaging.

To get insight into the mechanisms which are responsible for HP-induced DPA release or the subsequent inactivation of *B. subtilis* spores under pressure, FIB–SEM micrographs were evaluated in the context of other analytical and microbiological methods. In this study, flow cytometry and the measurement of released DPA via HPLC, as well as plate counts of inactivated, heat and hydrogen peroxide-sensitive spores, served to confirm visible changes in the SEM and

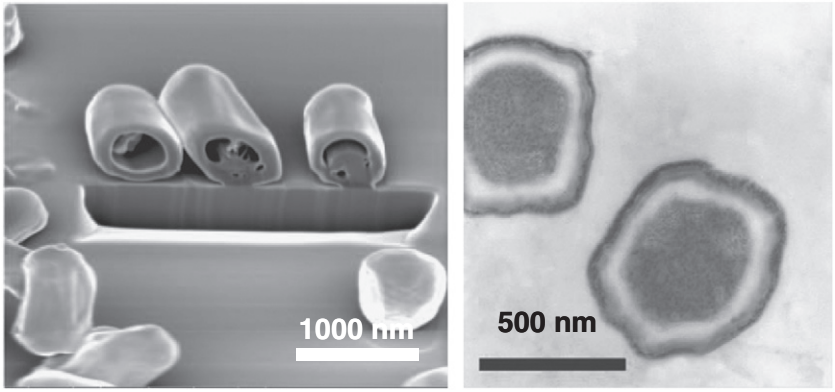

Fig. 7. FIB sectioned SEM micrograph (left) and TEM micrograph (right) each with 50,000-fold magnification of *Bacillus subtilis* (PS832, wild-type) spores treated at 550 MPa at 60 °C for 2 min. with a 50,000-fold magnification.

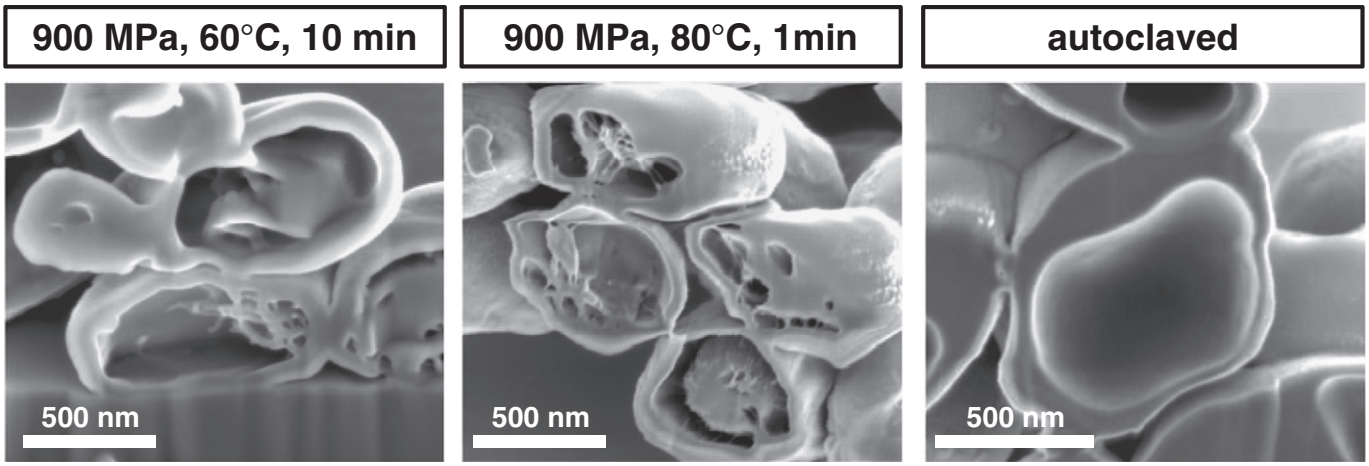

Fig. 8. FIB sectioned SEM micrograph of inactivated *Bacillus subtilis* (PS832, wild-type) spores.

TEM micrographs. Based on this set of methods, a mechanism was proposed (Table 3), which states that spores treated at 150 MPa and 37 °C release their DPA due to a HP-induced physiological-like DPA release. This rapid DPA release is followed by degradation of the cortex and SASPs. However, these spores do not begin outgrowth, presumably due to the lack of nutrients in the buffer solution, and also perhaps some inactivation of vital biochemical process(es) like ATP synthesis, (Smelt et al., 2001), since there was an ~1.6 $\log_{10}$ spore inactivation after 90 min. A pressure increase to 550 MPa led to a retarded DPA release, and spores with nGRs showed a higher DPA release rate compared to strains that lacked the nGRs. Spores that were unable to release their full amount of DPA showed a solid inner core surrounded by a large cavity in the FIB–SEM micrographs, possibly pointing towards continuous dissolving of the amorphous DPA in the core during the DPA release. Spores that had released their entire amount of DPA at 550 MPa (FB113 and PS832) showed a "porous network structure" in the spore core in the FIB–SEM micrographs, as well as a thinner cortex in the TEM micrographs. These micrographs, combined with the high resistance against hydrogen peroxide and the poor SYTO16 dye affinity, point towards an inability of the spores to degrade their SASPs. A further increase in temperature (≥60 °C) and pressure accelerated DPA release, but appeared to inhibit cortex degradation. This suggests that the structure in *B. subtilis* spores most susceptible to high pressure (≥550 MPa) and high temperature (≥60 °C) is the inner spore membrane. To further evaluate this suggested inactivation mechanism, stained inner spore membranes could be analyzed in situ in diamond anvil cells. Furthermore, cryo X-ray microscopic analysis may offer the possibility of high-resolution spore tomography without any preparation step and a rapid preservation of the physiological state of the spore immediately after decompression. The combination of this method with image analysis software to handle large quantities of micrographs, may allow an understanding of the impact of various sterilization methods on the mechanisms of inactivation of spores.

Table 3
Summary of the suggested mechanisms for DPA release from and subsequent inactivation of *Bacillus subtilis* spores.

Strain	Pressure [MPa]	Temperature [°C]	Time [min]	Suggested mechanism
PS832 (wild type)	150	37	90	1. Physiological like DPA release 2. Cortex degradation 3. SASP degradation 4. Inactivation
PS832 (wild type)	550	37	60	1. Physiological + unphysiological DPA release 2. Cortex degradation
FB113	550	37		1. Physiological + unphysiological DPA release
FB114 and FB115	550	37		1. Unphysiological DPA release (retarded) 2. Cortex degradation (retarded, due to lack of one CLE)
PS832 (wild type)	>550	60		1. Unphysiological DPA release 2. Inactivation

Strains: FB113 (Δ*cwlJ*, Δ*sleB*); FB114 (Δ*ger*, Δ*sleB*); and FB115 (Δ*ger*, Δ*cwlJ*).

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8. Conclusion and perspective

The mechanistic background for the inactivation of bacterial endospores is still a matter of discussion, not only for novel processes like high pressure (Heinz & Knorr, 2002; Setlow, 2003; Wei et al., 2010), but also for established industrial scale processes like retorting (Coleman et al., 2010; Tabit & Buys, 2010; Sanchez-Salas et al., 2011). Despite the continuous progress of analytic methods and tools, most of the published kinetic data about spore inactivation are not comparable, often due to unstable process conditions during the treatment or changes within the treatment media, which can lead to unpredictable behavior of the entire process during up-scaling. These factors are not limited to inhomogeneous temperature distributions, which can often be measured or modeled, but also include the impact of different sporulation conditions (Melly et al., 2002; Olivier et al., 2012), the heterogeneity in the spore population (Mathys et al., 2007b; Setlow et al., 2012) or even the temperature-dependent shift of the dissociation equilibrium in the treatment medium. For simple aqueous systems like buffer solutions, the equilibrium shift can be calculated, but for complex systems like real food this shift has to be measured (Reineke et al., 2011c). Even when only this single factor is not taken into account during experimental design, a biased error in the results is present, leading to incomparable results.

Within this work a pH measurement system was constructed to acquire the shift of the dissociation equilibrium of commonly used buffer solutions as well as liquid and semi-solid foods up to 130 °C. The data acquired between 90 °C and 130 °C in particular, represents an important process window for the thermal preservation of liquid foods. Furthermore, within the study (Reineke et al., 2011c; 2011b) the influence of the thermal pK_a shift on the inactivation rate of *Bacillus subtilis* spores was demonstrated for ACES and PBS buffer. Whereas the PBS buffer proved to be a proper aqueous system for pure thermal inactivation, ACES buffer showed a strong decrease of the pH-value. Hence, this study improves the comparison of spore inactivation data in literature and offers explanations for possible variations in microbial reduction levels (D-values) at similar treatment conditions. However, under high pressure, Mathys et al. (2008b) calculated a higher stability of the dissociation equilibrium for ACES buffer compared to PBS. Consequently it is essential to correctly design the experimental set up and to include all fundamental process parameters. In order to generate reliable data, which are suitable for modeling and which allow correct interpretation of the experimental results. Isothermal and isobaric conditions during the pressure dwell time are also essential for achieving comparable data sets in different high pressure systems, as was proven within this study (Reineke et al., 2012).

It is well known that spore germination and inactivation under high pressure could be achieved within a broad temperature and pressure range. The existing literature reports contradictorily results concerning the effectiveness of different pressure–temperature combinations and concerning the underlying mechanism for the inactivation. Up until now no official sterilization indicator microorganism for high pressure processing has been defined. This study therefore chose to investigate the germination and inactivation behavior of *Bacillus subtilis*, since this is one of the best explored prokaryotes in terms of molecular and cell biology. It was studied over broad pressure (0.1–1000 MPa), temperature (30–110°C) and time (1 s – 2 h, in some cases up to 24 h) domains. To identify or exclude individual microbial factors, such as nutrient germinant receptors or cortex lytic enzymes, isogenic strains of *Bacillus subtilis* lacking components of the germination apparatus were also investigated. To overcome the limited information that could be gathered from plate count, flow cytometry analysis and the quantification of DPA release via HPLC, were used to investigate the physiological state of the high pressure-treated spores. These data were compared with transmission and/or scanning electron micrographs. For a detailed scanning electron analysis, a rapid ion beam cross section cutting technique was developed. This method proved to have both a fast preparation and the capability to investigate the internal structure of pressure (Figure 8. 1) and heat-treated spores. Moreover, this technique was suitable to analyze structural changes caused by other non-thermal technologies like cold atmospheric plasma (Figure 2. 3).

Based on this set of methods, unique insights into the enzymatic and biochemical reactions occurring in *Bacillus* spores during and after a high pressure treatment were possible. This helped to understand the underlying mechanisms of spore inactivation during high pressure thermal sterilization.

It is widely accepted that, in contrast to the thermal inactivation, the inactivation of spores under high pressure is at least a two-step inactivation (Wuytack et al., 1998; Heinz & Knorr, 2002; Margosch et al., 2004; Mathys et al., 2007a; Reineke et al., 2012). The few existing mechanistic studies only focus on the first step, which is the release of DPA and accompanied by a loss of heat resistance (Gould & Sale, 1970; Wuytack et al., 1998; Wuytack et al., 2000; Paidhungat et al., 2002; Black et al., 2005), or the last step the inactivation (Ardia, 2004; Margosch et al., 2006; Mathys et al., 2009). Of these studies only some have measured and discussed the entire mechanism (Heinz & Knorr, 1996; Margosch et al., 2004; Mathys et al., 2007a). Furthermore, most of these studies investigated only single pressure–temperature combinations, short pressure dwell times, and/or either moderate or high temperatures.

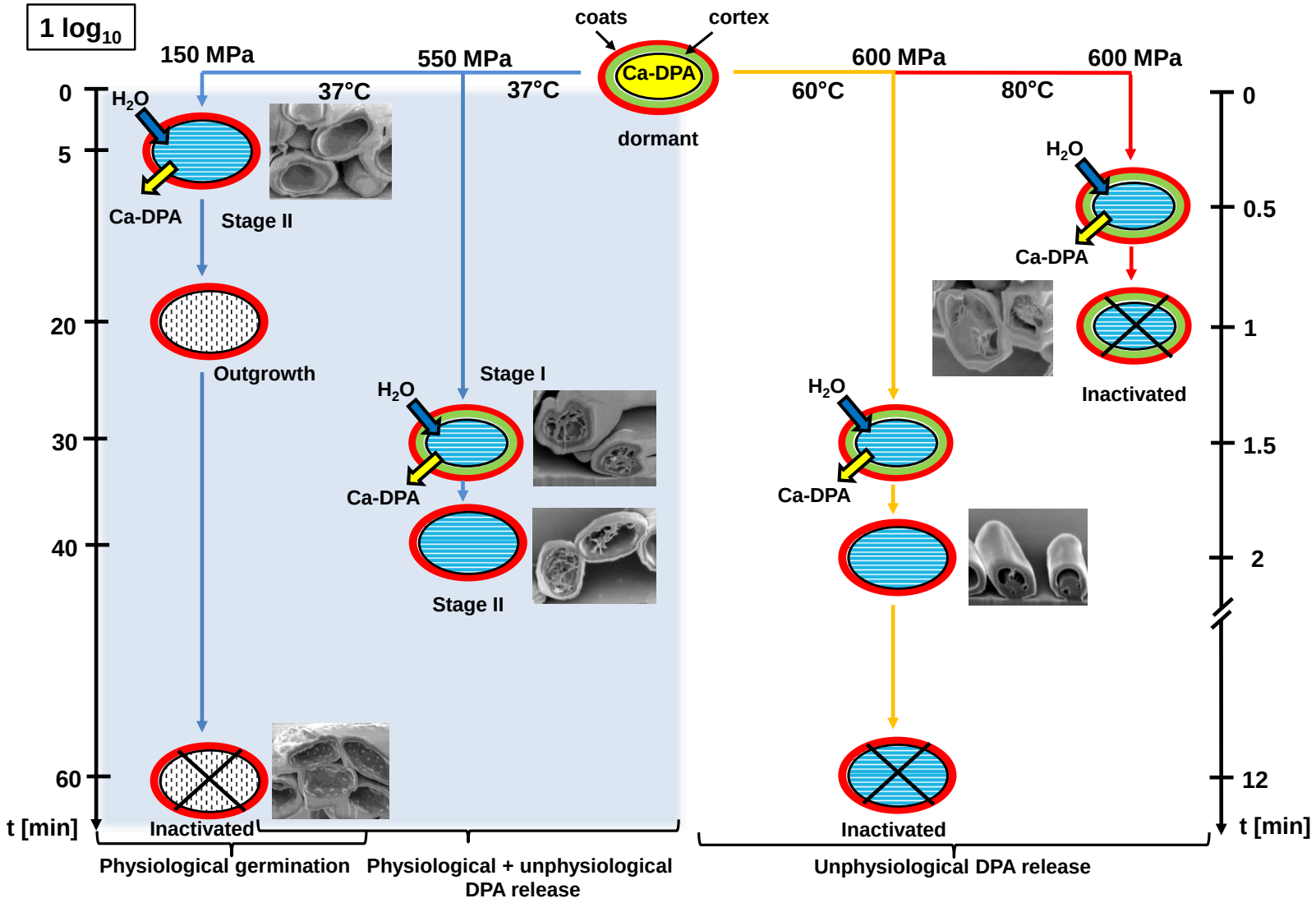

Figure 8. 1. Overview of pressure–temperature–time combinations enabling a 90 % physiological like or unphysiological DPA release and a subsequent 1 $\log_{10}$ germination [germination stages according to (Setlow, 2003)] and/ or inactivation, with corresponding focused ion beam sectioned scanning electron micrographs.

This study confirmed that spore pressure induced germination at ambient temperatures (37 °C) is possible by triggering the nutrient receptors (Figure 8. 1). Whereas this factor dominates a physiological-like germination between 100-200 MPa (Figure 8. 2), its impact continuously decreases up to pressures of 600 MPa (Reineke et al., 2012).

Contrary to data in the literature, which states that a pressure of at least 550 MPa is needed to germinate spores by an assumed direct opening of the spores Ca^{2+}-DPA-channels, we identified this germination mechanism to be also active at 200 MPa and moderate temperatures (< 50 °C) (Reineke et al., 2012). This unphysiological pressure-induced germination was still found to be dominant at higher pressures (> 400 MPa), as depicted in Figure 8. 1 and Figure 8. 2. These observations were made using spore mutant strains that lack the nutrient germinant receptors (FB114 and FB115). Conversely, under pressures > 400 MPa a retarded release of DPA was measurable compared to 150 MPa for the wild-type spore strain, accompanied by an increased amount of heat sensitive spores. Nearly no inactivation was achieved and a porous network structure was visible in the sectioned scanning electron micrographs (Figure 8. 1). It is therefore assumed that spores treated at 550 MPa and moderate temperatures are able to degrade their cortex, but not their SASPs, possibly due to an inactivation of the germination protein Gpr.

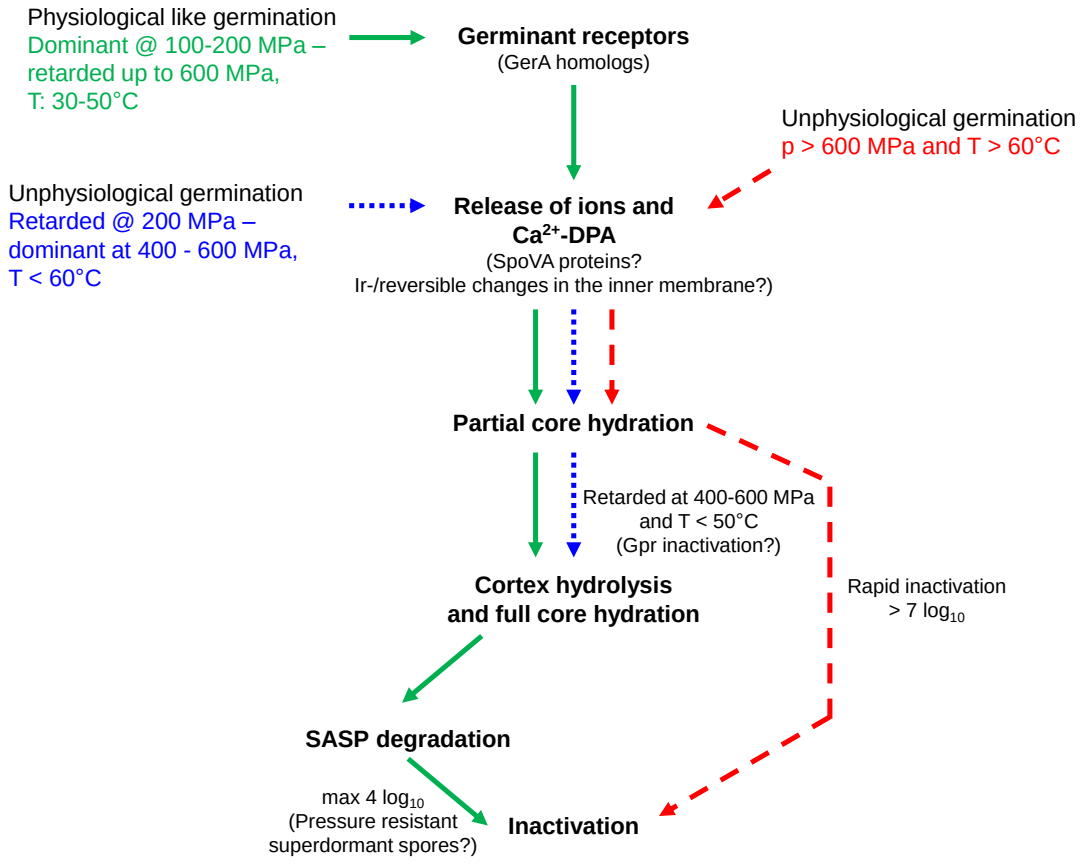

Figure 8. 2. Proposed germination and inactivation pathways of *Bacillus subtilis* in dependence of the applied pressure temperature conditions.

A SASP degradation presumably does occur, for spores treated at 150 MPa (Figure 8. 2). These spores were sensitive to a hydrogen peroxide treatment after the decompression and further showed no inner structure in the cross-section SEM micrographs (Reineke et al., 2013a) as depicted in Figure 8. 1. Moreover, a rapid DPA release was measured, pointing to a physiological-like germination (Zhang et al., 2010a), which presumably stops in the outgrowth phase (Reineke et al., 2013a). There could be many reasons for the inactivation at this stage, but is possibly due to a lack of nutrients in the buffer solution, or perhaps to some inactivation of vital biochemical process(es) such as ATP synthesis (Wuytack et al., 1998; Smelt et al., 2001). Under this moderate treatment pressure, even after 24 h pressure dwell time, a pressure-resistant spore population was found (Reineke et al., 2012), which were assumed to be superdormant spores that lack nutrient receptors.

An increase of pressure and temperature (≥ 600 MPa and 60 °C) accelerated the DPA release as well as the formation of heat sensitive and inactivated spores, but decreased the fluorescent dye binding ability of the SYTO 16 and PI, that were used for flow cytometry analysis, after pressure dwell times above 3 min. This finding may indicate an inability of the spore to degrade its cortex under pressure (Black et al., 2005) and hence a change of the inactivation mechanism (Figure 8.2 and Figure 8.3). Above this threshold pressure of 600 MPa, the treatment temperature dominates the DPA release rate and all subsequent steps in the inactivation mechanism. This suggests that the structure most susceptible to high pressures (≥ 600 MPa) and high temperatures (≥ 60 °C) is the inner spore membrane or its associated membrane proteins (Reineke et al., 2013b), which results in a rapid hydration of the spore core and hence a loss of heat resistance. Consequently, this phase denotes the rate limiting step of spore inactivation for a combined high pressure and heat treatment.

This combination of pressure and temperature may also cause an inactivation of the CLEs (Figure 8. 3), since the DPA released under pressure does not trigger the CLE CwlJ to degrade the cortex even after a sufficient time after decompression (Reineke et al., 2013b). In this regard, the CLE SleB seems to be slightly more sensitive (Reineke et al., 2011b) to pressure and heat, as opposed to its higher heat stability at atmospheric pressure compared to CwlJ. This inactivation pathway may additionally contribute to the high inactivation rates that could be achieved, but it does not seem to be the major lethal effect for at least *Bacillus subtilis* spores.

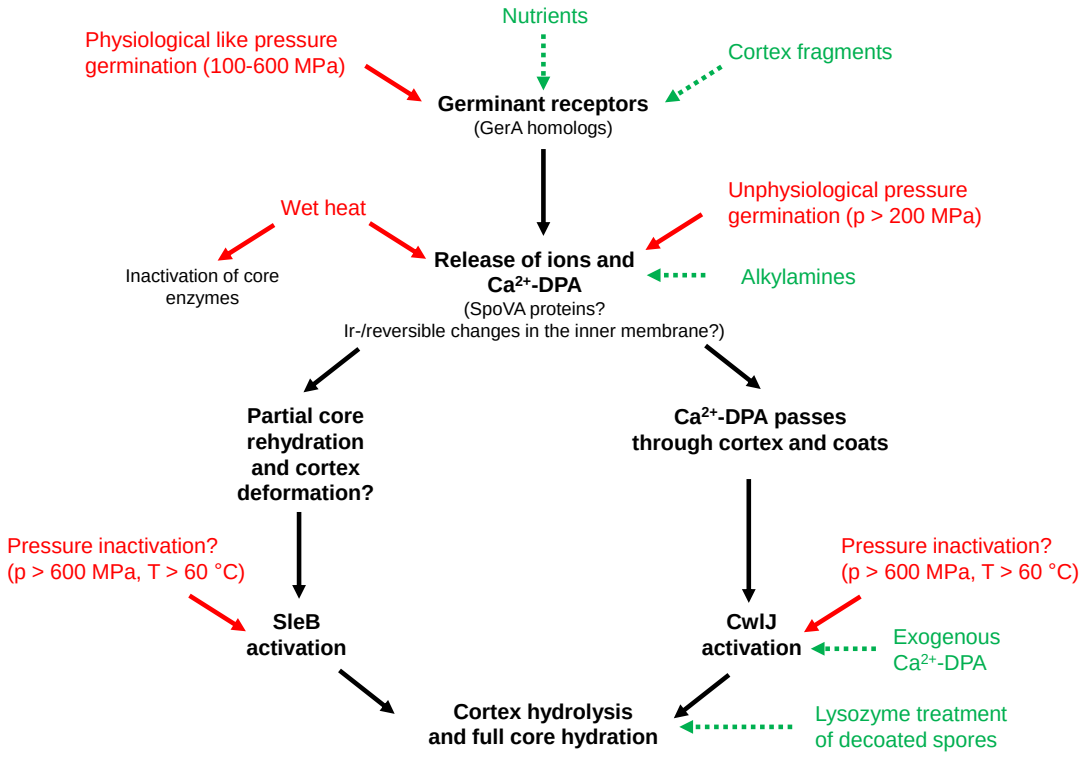

Figure 8. 3. Pathways triggered by extrinsic factors, which could lead to a loss of resistance (green dotted arrows) or to an inactivation (red arrows) of *Bacillus subtilis* spores. Adapted from (Setlow, 2003) and extended.

To adapt these data to a high pressure thermal sterilization process, detailed inactivation studies with adequate spore strains in food matrices are necessary to formulate the desired sterilization aim. It will be essential to identify and certify such a sterilization indicator strain and this study may help to find relevant process conditions. After the identification of such an indicator endospore strain, the combination of different extrinsic factors, as shown in Figure 8. 3, may open up the possibility of establishing a multi-target inactivation strategy for sterilization under high pressure to ensure the inactivation of highly pressure resistant spores. Nevertheless, food safety and consumers' health must be ensured in case-by-case studies demonstrating the applicability of the process to a larger scale.

Industrial relevance

To realize and optimize a high pressure sterilization process, it is essential to understand the black box of spore inactivation in more detail. However, to up-scale the process, kinetic data have to be comparable between different lab-scale high pressure equipment's. Within this study, data from cell counts for heat sensitive and inactivated spores were modeled with a two-step empirical multiresponse kinetic model, based on a mechanistic approach, which enabled the calculation of isorate lines in a pressure–temperature landscape (Figure 8. 4).

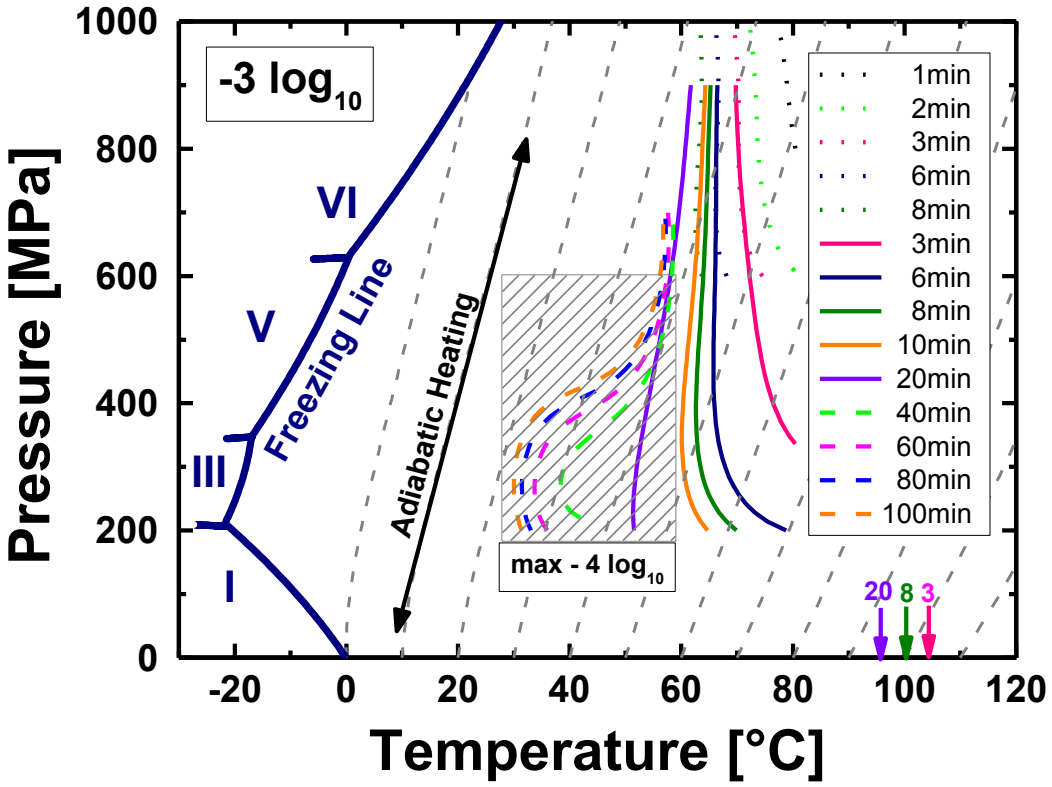

Figure 8. 4. Isorate lines for a 3 $\log_{10}$ inactivation of *Bacillus subtilis* PS832 (wild-type) for isothermal isobaric conditions derived in a SITEC-HP-Unit with a compression rate of 4 MPa/ s [dashed lines, data from (Reineke et al., 2012)] and in a Unipress-HP-Unit with a compression rate of 25 MPa/ s [dotted lines, data from (Reineke et al., 2011b) and solid lines, data from (Reineke et al., 2013b)]. Dashed gray lines represent the adiabatic heat of compression for water. Arrows represents the time needed for a pure thermal inactivation in thin glass capillaries. The solid blue line represents the freezing line between liquid water and the different pressure-dependent ice modifications.

These isorate lines were independently calculated for three separate series of experiments in two different high pressure systems. One system had a compression rate of 4 MPa s^{-1} and a vessel volume of 100 mL (dashed lines in Figure 8. 4), and the second had a compression rate of 25 MPa s^{-1} and a vessel volume of 3.75 mL (solid and dotted lines in Figure 8. 4). All isorates are calculated for the inactivation of *Bacillus subtilis* PS832 (wild-type) under isothermal and isobaric conditions. They display a nearly perfect fit in the overlapping pressure–temperature range between the experimental sets (Reineke et al., 2012). Hence, this finding depicts that kinetic data for spore inactivation, and presumably also inactivation kinetics for vegetative bacteria and enzymes, are comparable if derived under isothermal isobaric conditions, independent of the particular high pressure system used. Consequently, such data derived under defined conditions, could be useful in developing experimental protocols. They could also increase the comparability of kinetic results derived in different high pressure systems and may be useful to up-scale and validate high pressure, high temperature processes.

Figure 8. 4 further shows, that a significant spore inactivation is possible at moderate treatment pressures and temperatures for long pressure dwell times. However, for a full

spore inactivation (> 7 $\log_{10}$) in an economical treatment time, high pressures and elevated temperatures are needed. Further, a threshold pressure of 600 MPa was identified for *Bacillus subtilis* inactivation (Figure 8. 4), whereas above this pressure the process temperature dominates the inactivation rate. Given that, these process conditions presumably directly affect permeability of the inner spore membrane (Reineke et al., 2013b), such a threshold pressure may be also found for other *Bacillus* and *Clostridium* strains.

The same threshold pressure level, of 600 MPa, was identified for the formation of heat sensitive spores *Bacillus subtilis* spores (Reineke et al., 2012) and the release of DPA (Reineke et al., 2013b). Consequently, as the release of DPA was identified to be the rate limiting step of at least *Bacillus* spore inactivation, the measurement of Ca^{2+}-DPA release provides a powerful tool for a rapid estimation of the process impact. Moreover, the simplification of the HPLC-based DPA detection method (Reineke et al., 2012; Reineke et al., 2013b) to rapid DPA quantification, through the terbium-fluorescent method (Reineke et al., 2011b), may provide the possibility of screening several endospore strains and large quantities of process conditions, to find a potential indicator spore-former for the high pressure thermal sterilization.

After the establishment of a sterilization indicator strain, the ultimate goal for food preservation would be to create a predictive model that relates the heterogeneity of spores in a spore population, and their interaction with the surrounding food matrix, to their germination and/or inactivation behavior. Such a modeling approach may be realized with Monte-Carlo-Simulations, which can include resistance distributions of various spore properties and to extrinsic factors. This model would provide a mechanistic understanding and would be highly relevant for increasing food safety. Moreover, it could provide the possibility of risk assessment analysis to produce commercial sterile high pressure treated food in an industrial scale.

Annex

Annex Table 1: Bacterial spore inactivation and germination by high pressure: A literature overview [adapted from Heinz and Knorr (2002), Mathys (2008) and updated]

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Chlopin & Tammann, 1903	ant		0.1-300	0-40	l	m	Nutrient broth	Spore formers are highly resistant; lethal pressure effect increases at higher T
Hite et al., 1914	sub		< 700	Room	l	m	Different foods e.g. fruits	Pressure tolerance of spore was inferred
Larson et al., 1918	sub		300-1200	Room	l	m	Infusorial earth	Still survivors after 14 h at 1200 MPa
Basset et al., 1932	sub		< 1750	Room	l	m		Rapid inactivation of vegetative form; survival of spores up to 1750 MPa and 45 min
Johnson & Zobell, 1948	sub		0.1-60	25-94	l	m	Buffer pH 7 Water	1 lg at 60 MPa/94°C/30 min; 4 lg at 0.1 MPa/94°C/30 min
Timson & Short, 1965	sub		0.1-800	-30-100	l	m	Milk	Spore can survive phase transitions of water, survivors after 700 MPa/100°C/30 min
Clouston & Wills, 1969	pum		0.1-170	25	l/G	m/b	Water Buffer pH 6/8	2 lg at 170 MPa/25°C/270 min; 4.5 lg with simultaneous irradiation
Clouston & Wills, 1970	pum		80-100	25	l/G	m	Buffer pH 6/8	Less than 1 lg at 80 MPa/25°C/100 min
Gould & Sale, 1970	sub, cer co , pum		25-100	20-80	G	m	Water	At 50 and 100 MPa, germination optimum at 50°C
Sale et al., 1970	co, cer, sub	sp	0.1-800	25-75	l/G	m/b	Buffer pH 8	6 lg after (70°C/30 min) + (300 MPa/75°C/120 min)
Murrel & Wills, 1977	sub, pum, cer		50-70	25-44	l/G	m	Buffer pH 6/8	1 lg at 63 MPa /41°C/40 min; germination optimum at 63 MPa/ 50°C
Bender & Marquis, 1982	meg		30-100	24-60	G	m	Buffer pH 6/8	>30 MPa/45°C can induce germination
Butz et al., 1990	st		200-300	40-60	I	m	Salt solution	2.5 lg at 60°C/300 MPa and 360 min
Mallidis & Drizou, 1991	st		1-30	117-128	l	m	Water	5 lg at 10 MPa/123°C/3 min
Taki et al., 1991	li		600	60	I	m	Buffer pH 7	Inactivation at 600 MPa/ 60°C/20 min

Continues

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Seyderhelm & Knorr, 1992	st		0.1-600	20-90	l	m	Water	6 lg at 350 MPa/80°C/30 min
Hayakawa et al., 1994a	st		0.1-800	20-70	l	m	Water	6 lg at 600 MPa/70°C/ 6 x 5 min oscillatory treatment
Hayakawa et al., 1994b	st		0.1-800	20-70	l	m	Water	6 lg at 600 MPa/70°C/6 x 5 min oscillatory treatment
Nishi et al., 1994	lic, sub, pol		50-200	25-60	l/G	m	Milk nutrient broth	6 lg after (200 MPa/60°C/10 min) + (incub. 37°C/60 min) + (65°C/30 min)
Okazaki et al., 1994	sub		0.1-500	25-111	l	m	Buffer pH 7	4 lg at 400 MPa/100°C/10 min
Sojka & Ludwig, 1994	sub		20-500	40-80	l/G	m	Germination Medium	>8 lg at 50°C/6 x 30 min/cycling between 150 and 500 MPa
Fornari et al., 1995	cer, li, co, st		200-900	20-70	l	m	PBS buffer pH 7	cer: 5 lg 20°C, 200 MPa/ 1 min+900 MPa/ 1 min li:complete inactivation 800 MPa/5 min /60°C st:complete inactivation 700 MPa /5 min / 70°C co:4 lg at 900 MPa / 5 min / 70°C
Crawford et al., 1996		sp	410-820	80	l	m	Chicken	2 lg at 680 MPa/80°C/20 min and 6 lg with subsequent irradiation (3 kGy)
Gola et al., 1996	cer, li, st, co	sp	300-900		l	m	Buffer pH 7 Truffle cream	st: 5 lg at 700 MPa/70°C/5 min li: 6 lg at 700 MPa/70°C/3 min sp: 2 lg at 900 MPa/80°C/10 min
Ludwig at al., 1996	sub		0.1-500	4-70	l/G	m/b	Physio. NaCl	4.5 lg at 500 MPa/70°C/15 min; 8 lg by oscillation: 70°C/ 7x (1 min/500 MPa + 1 min/ 0.1 MPa)
Kakugawa et al., 1996	st		0.1-400	50-120	l	m	Buffer pH 7	5 lg at 150 MPa/90°C/30 min
Nakayama et al., 1996	sub, st, lic, meg, co		200-1000	5-10	l	m	Water	Less than 1 lg inactivation at 1000 MPa/10°C/40 min
Okazaki et al., 1996	sub, co	sp	0.1-400	35-110	l	m	Buffer pH 7	sub: 6 lg at 400 MPa/65°C/15 min; co: 6 lg at 400 MPa/110°C/18 min; sp: 6 lg at 400 MPa/110°C/18 min
Roberts & Hoover, 1996	co		400	25-70	l	m	Buffer pH 4-7	6 lg at 400 MPa/70°C/30 min/ pH 4/ + Nisin
Arroyo et al., 1997	cer, sub		100-400	10, 20	l	m	Tryptone soy broth	No significant reduction at 400 MPa
Heinz, 1997	sub		0.1-600	5-80	l/G	m/b	Ringer solution	6 lg at 150 MPa/70°C/30 min; depending on the p-T level germination is either initiated/ inhibited

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Hölters et al., 1997	sub/st		0.1-500	30-70	G	m/b	Physio. NaCl	Germination at 60 MPa/30°C or 30 MPa/ 50°C
Marquis, 1997	meg		100	25	l	m	Aqueous H_2O_2 solutions	0 lg at 100 MPa/25°C/3 h; 5.5 lg at 100 MPa/25°C/3 h in the presence of 1% H_2O_2
Sojka & Ludwig, 1997	sub		0.1-520	30-50	G	m/b	Physio. NaCl	> 50 MPa/ >30°C can induce germination
Wuytack et al., 1997	sub		100	40	G	m/b	Water	Rate of pressure-induced germination in mutant spores is decreased
Hayakawa et al., 1998	st		30-200	5-100	l	m	Buffer pH 7	6 lg at 200 MPa/95°C/60 min + rapid decompression (1.5 ms) 1 lg at 30 MPa/95°C/720 min + rapid decompression (1.5 ms)
Heinz & Knorr, 1998	sub		50-300	10-70	l/G	m/b	Water	6 lg at 150 MPa/70°C/30 min
Herdegen & Vogle, 1998	sub		0.1-700	40-80	l	m	Peptone solution pH 7	5 lg at (500 MPa/20°C/15 min) + (0.1 MPa/37°C/30 min) + (600 MPa/20°C/15 min)
Mills et al., 1998		sp	60-600	20-80	l	m	Water	2 lg at 0.1 MPa/80°C/10 min + 400 Ma/60°C/70 min
Raso et al., 1998a	cer		250-690	25-60	l/G	m	Buffer pH 7	8 lg at 690 MPa/60°C/1 min (sporulation at 37°C) 6 lg at 690 MPa/60°C/15 min (sporulation at 20°C)
Rovere et al., 1998	cer, li, st, co	bot, sp	700-900	50-70	l	m	Beff broth, Buffer pH 7	At 700 MPa/70°C/5 min: cer, 5 lg; li, 6 lg; st, 5 lg; co, 1 lg at 800 MPa/88°C/9 min: bot, 3 lg,
Wuytack et al., 1998	sub		100-600	40-55	l/G	m/b	Buffer pH 7	3 lg at (600 MPa/40°C/60 min) + (0.1 MPa/55°C/10 min)
Hölters et al., 1999		pas	0.1-500	30-60	l/G	m/b	Physio. NaCl	2 lg at 420 MPa/60°C/75 min 4 lg by oscillation: 60°C/6x(10 min/40 MPa + 5 min/0.1 MPa)
Reddy et al., 1999		bot	689-827	35-60	l	m	Buffer pH 7	5 lg at 827 MPa/40°C/10 min 5 lg at 227 MPa/50°C/5 min

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Rovere et al., 1999		sp	600-1200	90-110	l	m	Meat broth	D(90°C) at 400/ 600/ 800/ 1200 MPa: 76.9/ 16.7/ 5.3/ 4.38 min D(110°C) at 0.1/ 400/ 600/ 800 MPa: 13.3/ 6.1/ 1.3/ 0.7 min
Wuytack et al., 1999	sub		0.1-600	20-80	l/G	m/b	Various aqueous media	6 lg 500 MPa/ 60°C/ 30 min, different germination mechanisms at 100 MPa and 600 MPa use of fluorescent GFP-containing spores
Capellas et al., 2000	sub		60, 500	25, 40	I/G	m	Goat's milk fresh cheese	5 lg 60 MPa/40°C/210 min+ 500 MPa/40°C/15 min 2.7 lg with the same treatment at 25°C
Furukawa et al., 2000	sub		200-400	25-55	I/G	m	Water	>6 lg at 300 MPa/55°C/ 6 cycle a 5 min >4.5 lg at 400 MPa/55°C/1 cycle and 30 min
Meyer, 2000	cer	sp	2 x 690	90	I	m	macaroni cheese	Sterilization after two-pulse treatment 2 x 1 min
Meyer et al., 2000	cer, sub, st	sp	621, 690, 966	60, 90, 105	I	m	food pH > 4.5 fat<10%,a_w>0.8	2 or more cycles 621 MPa/ 105°C lead to sterility with 10 E+6/g spore load
Okazaki et al., 2000	sub, st, co	sp	0.1-400	35-120	I/G	m	PBS buffer(pH 7)	6 lg at 400 MPa/110°C/ B.co (17 min), C.sp(7 min) 4 lg at 400 MPa/113°C/ 10 min B.st with tailing
Shearer et al., 2000	sub, cer, co Acy	sp	392	45°C	I/G	m/b	milk, beef, apple tomato juice	Sucrose laurate(> 1%)+ 392 MPa 45°C ,10-15 min lead to 3- 5.5 lg reduction laurate is inhibitory not lethal on spores
Stewart et al., 2000	sub	sp	404	25-90	I	m	Citrate buffer +Nisin +Sucr. laurate	sp at 25°C/30 min/ pH 4 (2.5 lg) pH 7(<0.5 lg) sub at pH 6-7/15 min/ 70°C (5 lg) 25°C(<0.5 lg) +/- synergism of HP/ pH/ Nisin/ sucr. laurate
Wilson & Baker, 2000	sub, st	sp	51-827	75-90	I	m	Meat emulsion	Treatment: 621 MPa/98°C/5 min >5 lg (sp), > 9 lg (sub), > 10 lg (st)
Wuytack et al., 2000	sub		100-600	40	G	m	Water	Different pathway for germination at 100 and 600 MPa
Ananta et al., 2001	st		50-600	60-120	I	m	Mashed broccoli Cocoa mass	6 lg at 600 MPa/80°C/60 min in mash. broccoli 6 lg 600 MPa/90°C/45min in cocoa mass
Furukawa et al., 2001	sub, li		200	25-65	I	m	NaCl solution	4 lg(li) at 65°C/180 min; 6 lg(sub) at 65°C/90 min 1 lg more after filtration and HP
Meyer, 2001	cer	sp	345-965	70-100	I	m	Different food	Two or more cycles of ultra-high pressure were combined with temperature treatment
Moerman et al., 2001	sub, st		50- 400	20- 80	I	m	Fried minc. pork Mash.potatoes	sp 5 lg at 400 MPa/80°C/ 60 min in pork sp 5 lg at 400 MPa/80°C/ 60 min in potatoes

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Wuytack and Michiels, 2001	sub		100, 600	40	I/G	m/b	Buffer pH 3 - 8	Higher inactivation if first pressure treated at neutral pH + then exposed to low pH for 1 h sensitive to heat inactivation at low pH
De Heij et al., 2002	st		700	121	I	m	Tryptone soy broth	6 lg after 2 x 700MPa/ 121°C/ 90 s
Fujii et al., 2002	cer		500-600	20-40	I	m	Water+argon	Pressure resistance of *B. cereus* spores was affected by a strong 'water ordering' effect
Furukawa et al., 2002	sub		100	45-75	I	m	Water	>6 lg after120 min with different initial concentrations; higher No=higher resistance
Lee et al., 2002	Acy		0.1-621	22,45,71,90	I	m	Apple juice	4 lg 41°C/207 MPa/10 min, 6 lg 71°C/207 MPa and 5 min, 6 lg at 90°C/414 MPa/1 min
Krebbers et al., 2002	natural flora		700, 860	75, 85	I	m	Basil	Below detection line after 2 x 950 MPa/75°C/30 s or 2 x 700 MPa/85°C/30 s, initial 4 lg/g spores
Paidhungat et al., 2002	sub		100, 550	Room	G	m/b	KPO_4 (pH 7.4) Water	100 MPa activation of germinant receptors 550 MPa opens channels for DPA release
Balasubramiam et al., 2003	sub		827	50, 70	I	m	PBS, pH 7	Effect of pressure transmitting fluids, sodium benzoate highest dT/dp; 8 lg 827 MPa,70°C
De Heij et al., 2003	st, sub		300-800	84–122	I	m	Tryptone soy broth	6 lg 700 MPa/ 90°C/3 min in standard steel vessel 10 lg at 700 MPa/ 90°C/ 3 min in isolated vessel
Farkas et al., 2003	sub		300, 600	Room	I/G	m/b	Nutrient broth	Germination of recombinant sub was accompanied by the emergence of bioluminescence
Furukawa et al., 2003	sub		200-500	25-55	I/G	m	Water	>6 lg at 300 MPa/55°C/6 cycle a 5 min >6 lg at 500 MPa/45°C/1 cycle and 30 min
Igura et al., 2003	sub		100-300	50	I	m	Distilled water	Sporulated at 30°C highest resistance, increased after demineralization
Kalchayanand et al., 2003		sp,la,per,te	345	60	I/G		Roast beef +Biopreservat.	la alone-extended shelf-life for 84 days at 4°C; with mixture of clostridial spores for 42 days
Krebbers et al., 2003	st		300,500,700	20, 80 90 initial T	I	m	in meat balls in tomato puree	6 lg at 700 MPa/ 20°C/ 2 min 6 lg pulse 2 x 700 MPa/ 80°C/ 30 s
Lopez-Pedemonte … 2003	cer		60-400				Cheese	2.4 lg after 60 MPa/30°C/210 min and 400 MPa/30°C/15 min+Nisin
März, 2003	sub	sp	600	60-90	I	m	Salt solution	1-2 lg of sp 600 MPa/ 60°C/ 300 min 6 lg of sp at 600 MPa/ 90°C/ 180 min
Oh and Moon, 2003	cer		0.1-600	20-60	I/G	m/b	Suspension	B. cer sporulated at pH 6 showed more resistance than at pH 7 or pH 8

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Reddy et al., 2003		bot	417-827	60-75	I	m	PBS pH 7.0 crabmeat blend	2-3 lg at 827 MPa/ 75°C/ 20 min in PBS same lg reduction in crab meat -->no protection
Watanabe et al., 2003	st, co, sub, cer li		0.1-200 + CO_2	35-95	I	b	Water	HP+CO_2 resulted in 5 lg at 30 MPa/95°C/120 min
Wilson and Baker, 2003	sub, st	sp	500-900	75-130	I	m, b	Phosphate Meat	Mixed spore culture (sub, st, sp) was inactivated 6 lg at 621 MPa/85°C/1 min
Ardia, 2004	st		200-1400	90-130	I	m	PBS, ACES Buffer pH7/ 6	8 lg in p,T diagram up to 1400 MPa/170°C for 3 different holding times(10, 30, 60 s) in PBS and 20 s holding time at pH 6 and 7 in ACES
Ardia et al., 2004a	Acy		0.1-700	initial 80-95	I	m	Orange juice	6 lg in p,T diagram for 5 holding times(10-30 min)
Clery-Barraud et al., 2004	ant		280-500	20-75	I	m		D(75°C) at 0.1/500 MPa: 348/4 min; D(20°C) at 500:160 min
Kalchayanand et al., 2004		te, per, la sp	138-483	25, 50	I/G	m/b	0.1% peptone pediocin AcH nisin A	483 MPa/ 50°C/ 5 min- te: 2.5 lg reduction per, la, sp: 0.1-0.2 lg reduction antimicrobial compound after HP
Margosch et al., 2004a	sub, cer, li, am		200-800	initial 60-80	I/G	m/b	Mashed carrots	>6 lg to no reduction, large resistance variation inact.=2 stage mechanism without germination *B. amyloliquefaciens* suggested target organism
Margosch et al., 2004b	sub, cer, li, am	bot	600-800	80-116	I	m/b	Mashed carrots	5.5 lg to no reduction at 600 MPa/ 80°C/ 1 s inact.=2 stage mechanism without germination *C. botulinum* TMW 2.357 most resistant *B. amyloliquefaciens* suggested target organism
Matser et al., 2004	naturally flora		900	initial 90°C	I/G	m	Vegetables	>3 lg; all under detection limit of ~1 lg, defined as sterility;2 pressure pulses
Rodriguez et al., 2004	st	bot	400-827	60-75; 92-110	I	m	PBS pH 7.0 (bot) dest.Water (st)	st- z(T) = 34.5°C, z(p)= 370 MPa;model for steady and transient process conditions
Van Opstal et al., 2004	cer		0-600	30-60	I/G	m	Milk	6 lg-500 MPa/ 60°C/ 30 min or two-step treatment 200 MPa/ 45°C/ 30 min and then 60°C/10 min
Van Schepdael et al., 2004	st, sub		600-950	60-90	I	m	Tryptone soy	5.7 lg of B.st. at 60°C/950 MPa B. sub. at 90°C/700 MPa below detection limit
Aoyama et al., 2005a	sub, li, cer, co		60	40	I/G	m	PBS GAM broth	60 MPa/ 40°C/ 24 h sub -PBS: 1.6 lg; GAM broth: 5 lg li, cer, co- GAM broth, 1-3 lg
Aoyama et al., 2005b	sub		0.1-300	20-70	I/G	m	PBS glucose broth	Induction germination started at 10 MPa/ 40°C/1 h 2 lg at 20 MPa/ 60°C/ 1 h in broth 5 lg (PBS and broth) at 300 MPa/ 60°C/ 30 min

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Black et al., 2005	sub		150	initial 37°C	G	m/b	50 mM Tris-HCl pH 7.5	Detection of germinated spores with Syto16 and Flow Cytomerty;effects on pressure germination
De Heij et al., 2005	st, sub	bot, sp	400-800	70–110	I	m	Broth	st D(105°C) at 800 MPa = 20 s
Koutchma et al., 2005	st	sp	600-800;688 in 35 L vessel	91-121	I	m	PBS, Scrambled Egg patties	4.5 lg of C.sp at 800 MPa/108°C/3 min, z(p)~21°C in PBS, >6 lg at 688 MPa/121°C/3 min in patties >6 lg of B.st at 688 MPa/108°C/5 min in patties
Moerman, 2005	sub, st	sp, ty, sa	400	20, 50	I	m	Pork Marengo	20°C/30 min sub 1 lg, st 0.2 lg, sp ty sa 0.7 lg 50°C/30 min sub 4 lg, st 1.5 lg, sp ty sa ~3 lg
Farid, 2006	st		0.1 - 87	90 - 125	I	m	Water Milk	Heating a liquid food product to a temperature of 95°C will generate pressure of 700 bar 90 min leads to an inactivation of 4 lg
Gao et al., 2006a	st		432-768	63-97	I	m	Milk buffer	Optima at 86°C/625 MPa/ 14 min-->6 lg Response surface methodology (RSM)
Gao et al., 2006b	sub		323-668	63-97	I	m	Milk buffer	Optima at 87°C/576 MPa/ 13 min-->6 lg Response surface methodology
Islam et al., 2006	sub, coa, st		100	65-85	I	m	Potage, ketchup pH 4/ 7	4-8 lg dependent on pH (4 higher) higher inactivation in potage than ketchup
Lee et al., 2006	Acy		207,414,621	22,45,71,90	I/G	m	Apple juice 17, 35, 70°Brix	17.5° Brix 2 lg at 45°C, 5 lg at 71 and 90°C 30° Brix no effect at 45°C, 2 and 4lg at 71, 90°C 70° Brix, no inactivation
Margosch et al., 2006	am	bot	0.1-1400	70-120 const.	I	m	Tris-His buffer pH 4, 5.15, 6	p,T-diagram for 5 lg reduction of C.bot (pH 5.15) Isothermal conditions, tailing under pressure 4 lg reduction of B.am at 800 MPa/100°C
Patazca et al., 2006	st		500-700	92-111	I	m	Water	D(T,p) values 29.4 to 108.8 s at 92°C, 17 - 76 s at 100°C, 6.1 to 51 s at 111°C within 500-700 MPa
Rajan et al., 2006a	am		500-700	95-121	I	m	Egg patty mince	6.8 lg at 600 MPa/121°C/~1.7 min and 700 MPa at 110°C/ 3 min; z (700 MPa) = 28.6°C; z (12 1°C) = 332 MPa
Rajan et al., 2006b	st		400-700	105	I	m	Egg patty mince Water	4 lg at 700 MPa/ 105°C/ 5 min no inactivation difference in patties and water
Reddy et al., 2006		bot	551-827	40-100	I	m	Crabmeat blend PBS (pH 7)	>6 lg at 827 MPa/ 75°C/ 20 min in both media Crabmeat blend provided no protection
Sasagawa et al., 2006	sub		700	55°C	I/G	m	Orange juice PBS, Acetate	PEF+ HP, 7.1 lg in acetate buffer with pH 3.3 HP main lethal effect, HP 6 lg in orange juice

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Scurrah et al., 2006	cer, co, li, pum		600	25, 75-95 initial T	I	m	Skim milk 9.5 % (w,w)	No inactivation to 6 lg at 600MPa/initial 72°C/1min high variability between species
Subramanian et al., 2006	am, sh	ty	700	121	I	m/b	Distilled water	Inactivation determined by FT-IR spectroscopy, adaquate comparison to plate count between
Black et al., 2006	sub		150, 500	50-76	G	m/b	Tris/HCl buffer pH 7.5	Germination detected by flow cytometry (Syto 16) 500 MPa no nutrient-germinantion, but DPA 0-8 lg inactivation after different dwell times
Ahn et al., 2007	am, sh	sp, ty	700	105, 121	I	m	Deionized water	sp, ty 6 lg at 700 MPa/ 105, 121°C(2, 0.2 min) am 6 lg at 700 MPa/ 105, 121°C(3, 0.2 min)
Mathys et al., 2007a	li		150, 600	10, 37, 77	I/G	m/b	Citrate buffer Nutrient broth	3 step mechanisms of inactivation detected by flow cytometry(PI,S16) with unknown population
Shigeta et al., 2007	sub, cer, pol		20-100	40	I/G	m	PBS glucose broth	60 MPa/ 40°C/ 30-60 min 5 lg germination with nutrients; 2-3 lg without nutrients
Subramanian et al., 2007	sh, am	ty	700	121	I	m	TSB, clostridial medium	Biochemical changes were studied by FT-IR 121°C no changes in the DPA content pressure leads to release of DPA
Vepachedu et al., 2007	sub		150, 500	Room	G	m/b	Buffer	SpoVAproteins involved in DPA release,perhaps SpoVA proteins component of DPA channel
Mathys et al., 2008	st		500,600,900	80	I	m/b	PBS, ACES buffer (pH 5-8)	Different dissociation equilibrium shifts in buffer with effect on inactivation, up to 1.5 lg different
Gao et al. 2008		bot	300-700	30-70	I	m	Milk	6 lg optimum (modelled) at 545 MPa/ 51 °C/ 13.3 min and 129 U/ml nisin
Suklim et al. 2008	cer		100,550	40	I/G	m	Water, Blue crab meat	Max. germination and inactivation in water at 550 MPa, in crab meat higher germination but retarded inactivation Spores survived 4 °C storage
Robertson et al. 2008	6 species		600	75 start	I	m	Skimmed milk	p-transmitting media: silicon oil (T_{max} 90°C after cycling max 105 °C) higher inactivation compared to water (T_{max} 88°C after cycling 103 °C)
Black et al. 2008	sub, cer		100, 500	40	I/G	m/b	Buffer, milk	B. sub germination 4 lg, 500 MPa and 2.5 lg inactivation + 500U/ml nisin >5.7 lg / B. cer more resistant
Zhu et al. 2008		sp	700-900	80-100	I	m	Ground beef	D-value: 15.8-7.0 min at 80°C and 1.5-0.63 at 100°C under p; z-value: 19.1-19.7 °C under pressure, 12.4 °C, 0.1 MPa
Ju et al. 2008	cer		400-600	60-80	I	m	Milk buffer	RSM-model: optimum 6lg at 540 MPa, 71 °C. 16.8 min
Bull et al. 2009		sp, bot	600	105	I	m	30% Bolognese, 50% cream, Rice water	$F_{105°C}$-value calculation, for some strains synergism of pressure and temp., no protective effect of tested media, C. sp. no suitable surrogate for C. bot under pressure

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Wang et al. 2009	co		400-600	70-80	I	m	PBS buffer, Whole milk	Higher resistance in milk, tailing occurred which could be best fitted with Weibull-model
Wei et al. 2009	cer		150	40-50	I/G	m	Buffer	Treatment of wild-type and mutant strains that lack some nrGR, wild-type spore germinated slightly faster
Ratphitagsanti et al. 2010	am		700	35, 105	I/G	m	Organic acid solution, Carrot puree	Full inactivation even without org. acids (3min, 700 MPa, 105°C), after 2 min stronger inactivation in citric and acetic acid, only 105 °C or HP at 35°C caused no inactivation, 5min, After 28d storage at 32 °C no recovery of spores in carrot puree with added organic acids
Ramaswamy et al. 2010a		sp	700-900	80-100	I	m	Milk	Isothermal, isobaric treatment; no inactivation after 1s; at 900 MPa D-values of 9.1-0.73 min, synergism between p and T, z-value under pressure higher (16.5-18.2 min) compared to 0.1 MPa (9.6 °C)
Ramaswamy et al. 2010b		sp	700-900	80-100	I	m	Salmon slurry	Synergism between p and T, z-value under pressure higher (14.5-15.5 min) compared to 0.1 MPa (8.8 °C)
Gao et al. 2010a	st		625	86	I	m	Ringer solution + /Sucrose /Soybean protein / Soybean oil	pH value variation and RSM-model, 6 lg inactivation,14 min; no effect of soybean oil; pH, soybean protein and sucrose significantly effected spore inactivation
Wei et al. 2010	sub		150, 500	room	G	m	TRIS buffer	Superdormant spores germinate well after p- treatment
Reddy et al. 2010		bot	620	95, 105	I	m	TPGY-broth	Addition of lysozyme, L-alanine, L-aspartic acid, DPA, sodium bicarbonate and lactate; None of the additives improved spore recovery despite lysozyme (less than 1lg)
Balasubramanian et al. 2010	sub		690, 827	60-75	I	m	Citrate buffer (pH 3, 5,7) Tomato puree Minced crabmeat	Highest inactivation in citrate buffer (pH 3) 7lg, 825 MPa, 75 °C 5min
Gao et al. spore 2010b	cer		540	71	I	m	Ringer solution + /Sucrose /Soybean protein / Soybean oil	Soybean protein, pH and sucrose significantly effected inactivation; verification of quadratic predictive model to describe impact of food ingredients
De Lamo-Castellvi 2010	am		700	35, 105	I	m	Deionized water Mashed carrots	Addition of sucrose laurate (0.1-1%), 1% SL showed best synergistic effect under pressure, no effect at 105°C and 0.1 MPa or 35 °C and 700 MPa

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Minh et al. 2010	sub		90-550	40	I/G	m	Distilled water	Spore germination more efficient at low pressures; estimation of germination by 10min at 80 °C or 350 MPa, 40°C for 10 min, pressure resistant subpopulation
Gao et al. 2011a	co		300-700	15-75	I	m	Milk buffer + nisin (0-200 U/mL)	Optimum for 6 lg inactivation: 550 MPa, 41 °C, 12.2 min 120 U/mL nisin det. by RSM model; synergism with nisin
Baier et al. 2011	sub		150-600	37-60	I/G	m/b	ACES buffer	Correlation of germination data detected by FCM (SYTO 16) and released amount of DPA, fluorescence Tb-DPA quantification suitable for screening p-T conditions for germination
Reineke et al. 2011b	sub		550-1000	37-80	I/G	m/b	ACES buffer	No inactivation at 550 MPa, 37 °C, 2h; above 600 MPa, 60 °C treatment temperature dominates inactivation mutant strains lacking one CLE showed only slight higher Inactivation (sleB) than wild-type spore strain
Shao et al. 2011		sp	700-900	80-100	I	m	Milk (2% fat)	Pressure pulse inactivated 0.5 lg; D-value at 700 MPa from 13.6-2.4 min, at 900 MPa from 7.0-1.3 min; z-value under pressure (16.5-20.3 °C) higher than at 0.1 MPa (9 °C)
Minh et al. 2011	sub		350	40	I/G	m/b	Distilled water	Effect of sporulation conditions on pressure resistance tested; spores produced at pH were most resistant
Marco et al. 2011	cer		200-500	20 start	I	m	BHI broth + 0-2.5% olive powder	Max 1.5 lg inactivation; 2.5% olive powder slightly decreased inactivation, highest inactivation at 200 MPa
Gao et al. 2011b		per	400-800	35-95	I	m	Milk + nisin	RSM-model calculated optimum parameter for 6lg: 654 MPa, 74 °C, 13.6 min and 328 U/mL nisin
Reineke et al. 2012	sub		100-700	30-80	I/G	m/b	ACES buffer	Spore germination without nutrient receptors possible at 200 MPa, first step of inactivation is germination, multi-response kinetic modeling – isorate p-T plane for G and I
Sokolowska et al. 2012	Acy		100-500	50	I	m	Apple juice	Highest inactivation at 200 MPa, stabilization at 500 MPa, 200 MPa + 60 min at 0.1 MPa + 500 MPa resulted in 6.15 lg ,addition of 250 U/mL nisin enhanced inactivation, no effect of lysozyme
Vercammen et al. 2012	co, Acy		100-800	25-60	I/G	m	PBS buffer Tomato sauce	2lg germination of Acy at p< 300 MPa and B. co 600-800 MPa, pH 4 suppressed Acy germination but stimulated B. co germination, tomato sauce, 60 °C, pH 4.2. 3.5 lg Acy and 2.0 lg B. co inactivation
Syed et al. 2012	sub		600	60-70	I/G	m/b	TRIS buffer Skimmed milk Orange juice	Compression and decompression rate effect spore inactivation, highest inactivation for slow compression and decompression

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Aouadhi et al. 2012 (in press)	spot		300-500	30-50	I	m	Distilled water Skimmed milk	Higher inactivation in distilled water, RSM model – 5 lg optimum at: 477 MPa, 48 °C, 26 min (water), 495 MPa, 49 °C, 30 min (milk)
Olivier et al, 2012 (in press)	am, co, spot		600	110	I	m	molten Nutrient agar	Impact of sporulation conditions on HP resistance was tested; high sporulation T decreased pressure resistance Increasing mineral content at various effects B. am spores sporulated at 37 or 30 °C: D-value 0.58 – 4.0 min under pressure
Reineke et al. 2013a	sub		100-700	30-80	I/G	m/b	ACES buffer	Structural analysis of internal structure of pressure treated spores (FIB-SEM), Mechanism proposed: p < 550 MPa physiological like germination – SASP degradation possible; p > 600 MPa, T > 60°C inner spore membrane most sensitive structure under pressure
Reineke et al. 2013b	sub		100-700	30-80	I/G	m/b	ACES buffer	Isorate-pressure-temperature-diagram for DPA release, differentiation between 3 different release mechanisms in dependence of p and T, DPA release rate limiting step of *Bacillus* spore inactivation

Organism: sub, *B. subtilis*; st, *G. stearothermophilus*; cer, *B. cereus*; li, *B. licheniformis*; meg, *B. megaterium*; pum, *B. pumilis*; co, *B. coagulans*; pol, *B. polymyxa*; ant, *B. anthracis;* am, *B. amyloliquefaciens*; sh, *B. sphaericus*; *spot, Bacillus sporothermodurans*; Acy, *Alicylobacillus acidoterrestris*; sp, *C. sporogenes*; pas, *C. pasteurianum*; bot, *C. botulinum*; ty, *C. tyrobutyricum*; sa, *C. saccharolyticum*; la, *C. laramie*; per, *C. perfringens*; te, *C. tertium* .

Type (of experiment): I, inactivation; G, germination.

Methodology used: m, microbiological; b, biochemical.

Comments: lg = $\log_{10}$.

Kai Reineke – Curriculum Vitae

MAIN RESEARCH FIELD:

- Inactivation of bacterial endospores and vegetative microorganisms during high pressure and pulsed electric field treatment
- Development and optimization of methods to monitor the physiological state of endospores after pressurization
- Pressure induced gel formation in simple model systems
- pH shift in buffer solutions and food systems during thermal and non-thermal treatments
- development of fluorescent based optical methods for the detection of microbial contaminations

EDUCATION:

Since 10/ 2008:	Technische Universität Berlin, Department of Food Biotechnology and Food Process Engineering Research associate, PhD candidate Thesis under the supervision of Prof. Dr. D. Knorr: Mechanisms of *Bacillus* spore germination and inactivation during high pressure processing
Since 09/ 2008	M. Eng. (Dipl.-Ing.) Food Technology Thesis: Temperature- controlled high pressure inactivation of *Geobacillus stearothermophilus* spores and modeling in the pressure and temperature landscape
03/2007 – **05/2008**	Technische Universität Berlin Student assistant in the High pressure research and application group
10/2003	Technische Universität Berlin
09/2008	Studies of Food Technology, emphasis on processing of fruit based foods

RESEARCH PROJECTS:

- PROMETHEUS: Process contaminants: Mitigation and elimination techniques for high food quality and their evaluation using sensors and simulation. *Subproject leader FP 7 EU Project.*
- Application of high pressure processing in combination with an innovative packing, to produce high quality fresh food with extended shelf life. Supported by Federal Agency for Agriculture and Food (BLE-2816302307)
- Optical methods for cleaning and contamination validation. Support by Federal Ministry of Education and Research (BMBF, FKZ 01 RI 0645A-F)

AWARDS:

03/2013 Winner of the "Charles R. Stumbo Student Paper Competition" of the Institute For Thermal Processing Specialists (IFTPS) at Annual Meeting 2013, San Antonio, USA

04/2012 Supervisor of the team "Cruemel", winner of the German Student Award for Food Innovation (TROPHELIA 2011). FEI-meeting 2012, Bonn Germany

06/2011 Winner of the IFT Non-thermal Processing Division (NPD) Graduate Paper

06/2012 competition at IFT11 „IFT Annual Meeting & Food Expo", New Orleans, USA, and co-author of the third place in 2012 at IFT12 „IFT Annual Meeting & Food Expo", Las Vegas, USA,

03/2011 3rd Place of the "Charles R. Stumbo Student Paper Competition" of the Institute For Thermal Processing Specialists (IFTPS) at Annual Meeting 2011, San Antonio, USA

06/2010 Winner of the "George F. Stewart IFT International Research Paper Competition" Award 2010 at IFT10 „FT Annual Meeting & Food Expo", Chicago, USA

11/2008 Winner of the EFFoST (European Federation of Food Science and Technology) "Student of the Year" Award 2008 at the First European Food congress in Ljubljana, Slovenia

RESEARCH GRANTS:

03/2011 Travel grant of Comvita New Zealand Ltd for participation at the Comvita Science symposium

TEACHING ACTIVITY

- Emerging technologies in food processing (lecture)
- Innovative Technologies (integrated seminar)
- Recovery of food constituents (practical course)
- Thermal technology for food processing (practical course)
- Co-supervisor of 7 master/diploma thesis and 8 bachelor/ scientific student thesis

REVIEWER ACTIVITIES

- Innovative Food Science and Emerging Technologies
- High Pressure Research
- Journal of Food Engineering
- Food Microbiology
- Critical Reviews in Food Science and Nutrition

MEMBERSHIP IN SCIENTIFIC ORGANIZATIONS

- ·German Association of Food Technologists (GDL e.V.)
- Institute of Food Technologists (IFT)

INTERNSHIP EXPERIENCE

05/2009 – 07/2009	German Institute of Food Technologies, Quakenbrück, German Food science and technology institute, intern student in the R&D group for non-thermal technologies, intern student in the R&D group for senor development
02/2006 – 03/ 2006	Fleischerei Staske GmbH, Berlin, Germany Slaughter and butcher, intern student
07/ 2005 - 09/2005	Berliner Wasserbetriebe, Berlin, Germany Berlin waterworks, microbiological analysis of drinking and waste water, intern student

LANGUAGES

German Native

English Fluent

French basic skills

SCHOOL EDUCATION

1995-2002	Alexander von Humboldt-Gymnasium; Berlin, Germany (High school)
1989-1995	Elementary school

DATE AND PLACE OF BIRTH

16/04/1983	Berlin, Germany
Nationality	German

CONTACT DETAILS

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ANNEX

List of Publications

Berlin, 10.02.2013

PUBLICATIONS

Peer-reviewed primary publications:

1. **Reineke, K., Ellinger, N., Berger, D., Baier, D., Mathys, A., Setlow, P. & Knorr, D. (2013)** Structural analysis of high pressure treated *Bacillus subtilis* spores, Innovative Food Science and Emerging Technologies, 17, 43-53

2. **Georget, E., Reineke, K., Heinz, V., Knorr, D. Ananta, E. & Mathys, A. (accepted)** Spore Inactivation Mechanisms during Industrial Food and Equipment Sterilization, Encyclopedia of Biotechnology in Agriculture and Food

3. **Reineke, K., Schlumbach, K., Baier, D., Mathys, A. & Knorr, D. (2013)** The release of dipicolinic acid – the rate limiting step of endospore inactivation during the high pressure thermal sterilization process, International Journal of Food Microbiology, 162, 55-63

4. **Reineke, K., Doehner, I., Schlumbach, K., Baier, D., Mathys, A. & Knorr, D. (2012)** The different pathways of spore germination and inactivation in dependence of pressure and temperature, Innovative Food Science and Emerging Technologies, 13, 31-41

5. **Schmitt, F.J., Südmeyer, H., Börner, J., Löber, J., Olliges, K., Reineke, K., Kahlen, I., Schöngen M., Hätti, P., Eichler, H.J. & Cappius, H.J. (2011)** Handheld measurement device for fast and non-contact optical measurement of protein films on surfaces, Optics and Lasers in Engineering, 49, 1294-1300

6. **Abbas, Q., Reineke, K., Saldo, J., Buffa, M., Guamis B. & Knorr, D. (2012).** Effect of compression and decompression rates during High Hydrostatic Pressure processing on inactivation kinetics of bacterial spores at different temperatures, Food Control, 25 (1), 361-367

7. **Cai, Z., Riedel, H., Thaw Saw, N. M. M., Mewis, I., Reineke, K., Knorr, D. & Smetanska, I. (2011)** Effects of elicitors and high hydrostatic pressure on secondary metabolism of *Vitis vinifera* suspension culture, Process Biochemistry, 46 (7), 1411-1416

8. **Reineke, K., Mathys, A. & Knorr, D. (2011)** The Impact of High Pressure and Temperature on Bacterial Spores: Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. Journal of Food Science, 76 (3), 189-197

9. **Knorr, D., Froehling, A., Jaeger, H., Reineke, K., Schlueter, O. & Schoessler, K. (2011).** Emerging Technologies in Food Processing. Annual Reviews in Food Science and Technology, 2, 203-235

10. **Baier, D., Reineke, K., Doehner, I., Mathys, A. & Knorr D. (2011).** Fluorescence based methods for the detection of pressure induced spore germination and inactivation. High Pressure Research, 31 (1), 110-115

11. **Reineke, K., Mathys, A. & Knorr D. (2011).** Shift of pH-value during thermal treatments in buffer solutions and selected foods. Journal of Food Properties, 14 (4), 870-881

12. **Mathys, A., Reineke, K., Heinz, V. & Knorr, D. (2009).** High pressure thermal sterilization- Development and application of temperature controlled spore inactivation studies. High Pressure Research, 29 (1), 3-7.

13. **Reineke, K., Mathys, A., Heinz V. & Knorr, D. (2008).** Temperature control for high pressure processes up to 1400 MPa. Journal of Physics: Conference Series. **121** 142012-142016

14. **Mathys, A., Reineke, K., Heinz, V. & Knorr, D. (2008).** Gentle sterilization of food by high pressure treatment (in German). Lebensmitteltechnik 4/08, 68-69.

Other publications:

15. **Reineke, K., Doehner, I., Baier, D., Mathys, A. & Knorr, D. (2011)** The different pathways of spore germination and inactivation of *Bacillus subtilis* under high pressure and elevated temperatures. Procedia Food Science, 1, 792-799

16. **Reineke, K., Weich, H. & Knorr, D. (2011)** The influence of sugars on pressure induced starch gelatinization. Procedia Food Science, 1, 2040-2046

17. **Meneses, N., Jaeger, H., Reineke, K., Schloesser, K., Surowsky, B. & Knorr, D. (2010).** "Simulation of Nonthermal Technologies". Proceedings of: FoodInnova 2010 International Conference on Food Innovation. Pedro Fito and Fidel Toldrá Eds.

18. **Reineke K., Mathys A., Heinz V. & Knorr, D. (2007).** Temperature control for high pressure processes up to 1400 MPa for gentile food sterilization. In: Proceedings (in German) of GDL Congress “Food Technology 2007”, VDI Verlag

Chapter in Edited Books

1. **Knorr, D., Froehling, A., Jaeger, H., Reineke, K., Schlueter, O. & Schoessler, K., (submitted)** Emerging technologies for targeted food processing. In: Advances in Food Process Engineering Research and Applications, Springer Science & Business Media. New York, Heidelberg

2. **Jaeger, H., Reineke, K., Schoessler, K. & Knorr, D. (in press).** Effects of emerging processing technologies on food material properties. In: Food Materials Science and Engineering, Bhandari, Bhesh; Roos, Yrjo (Eds.), Wiley-Blackwell: Indianapolis

3. **Knorr D., Reineke K., Mathys A., Buckow, R. & Heinz, V. (2010).** High pressure assisted effects on bacterial spores, vegetative micro-organisms and enzymes. In: J. M. Aguilera, G. V. Barbosa-Cánovas, R. Simpson & J. Welti-Chanes. Food Engineering at Interfaces. Springer Science & Business Media. New York, Heidelberg.

PRESENTATIONS

Invited oral presentations at scientific meetings:

1. **Reineke, K. Schmitt, F.J., Cappius, H. & Knorr, D. (2012).** Use of optical sensing techniques for cleaning inspection of metallic surfaces in the food industry, Oral presentation at the Symposia on Senor Technology at the Anuga Foodtech Expo, Cologne, Germany

2. **Reineke, K., Schmitt, F.J., Südmeyer, H., Eichler, H.J., Cappius, H.J. & Knorr, D. (2011)** Optical cleaning validation on metallic surfaces in the food industry. Oral presentation at the 2. Symposium of fresh convenience/ fresh cut products (in German), Potsdam, Germany

3. **Reineke, K. (2011).** High pressure sterilization and effects on macro molecules. Oral presentation at the Comvita Science Symposium 2011, Auckland, New Zealand.

4. **Reineke, K., Mathys, A. & Knorr, D. (2010).** Temperature dependent shift of pH-value in buffer solutions and model foods – a tool to gain deeper insight into thermal food treatments. Oral presentation at IFT10 „FT Annual Meeting & Food Expo“, Chicago, USA

Oral presentations at scientific meetings:

5. **Reineke, K., Schlumbach, K. Baier, D., Mathys, A. & Knorr, D. (2011).** The different pathways of spore germination and inactivation in dependence of pressure and temperature. Oral presentation at EFFoST Annual Meeting, Berlin, Germany

6. **Reineke, K., Schlumbach, K. Baier, D., Mathys, A. & Knorr, D. (2011).** High pressure thermal sterilization: Modeling Approaches for the different pathways of spore germination and inactivation. Oral presentation at iFood 2011 – Innovation Food Conference, Osnabrueck, Germany

7. **Reineke, K., Doehner, I., Baier, D., Mathys, A. & Knorr, D. (2011).** The different pathways of spore germination and inactivation of *Bacillus subtilis* under high pressure and elevated temperatures. Oral presentation at iCEF11, Athens, Greece

8. **Reineke, K., Baier, D., Doehner, I., Mathys, A. & Knorr, D. (2011).** The different germination and inactivation mechanisms of bacterial spores under pressure. Oral presentation (in German) at ProcessNet FA Sitzung “Lebensmittelverfahrenstechnik 2011”, Vlaardingen, Netherlands.

9. **Reineke, K., Doehner, I., Mathys, A. & Knorr, D. (2010).** Pressure induced germination of *Bacillus subtilis* spores – A mechanistic study. Oral presentation at the "6th International Conference on High Pressure Bioscience and Biotechnology (HPBB2010)", Freising, Germany.

10. **Baier, D., Reineke, K., Doehner, I., Mathys, A. & Knorr, D. (2010).** Comparison of fluorescence based methods for rapid detection of the pressure induced germination of bacterial spores. Oral presentation at the "6th International Conference on High Pressure Bioscience and Biotechnology (HPBB2010)", Freising, Germany.

11. **Reineke, K., Doehner, I., Baier, D., Mathys, A. & Knorr, D. (2010).** New insight into the pressure induced spore germination. Oral presentation at 48th European High Pressure Research Group (EHPRG) International Conference, Uppsala, Sweden

12. **Baier D., Reineke, K., Doehner I., Mathys, A. & Knorr, D. (2010).** Fluorescence based methods for the detection of pressure induced spore germination and. Oral presentation at 48th European High Pressure Research Group (EHPRG) International Conference, Uppsala, Sweden

13. **Schmitt, F.J., Südmeyer, H., Reineke, K., Kahlen, I., Börner, J., Schöngen M., Hätti, P., Eichler, H.J. & Cappius, H.J. (2010).** Fast and non-invasive optical study of protein films on surfaces. Oral presentation at DPG Spring Meeting, Regensburg, Germany

14. **Schmitt, F.J., Südmeyer, H., Reineke, K., Kahlen, I., Börner, J., Schöngen M., Hätti, P., Eichler, H.J. & Cappius, H.J. (2010).** Pigment fluorescence in protein environment. Oral presentation at DPG Spring Meeting, Regensburg, Germany

15. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2009).** Influence of high pressure and elevated temperature on bacterial spores - inactivation mechanisms above 500 MPa. Oral presentation at 47th European High Pressure Research Group (EHPRG) meeting, Paris, France.

16. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2009).** Influence of high pressure and elevated temperatures on the inactivation mechanism of bacterial spores. Oral presentation (in German) at GDL Congress "Food Technology 2009", Lemgo, Germany.

17. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2009).** Combination of high pressure and temperature as an alternative sterilization process. Oral presentation at "5th International Technical Symposium on Food Processing, Monitoring Technology in Bioprocesses and Food Quality Management", Potsdam, Germany

18. **Mathys, A., Reineke, K., Heinz, V. & Knorr, D. (2008).** High pressure sterilization. Oral presentation at EFFoST **„**First European Food Congress", Ljubljana, Slovenia

19. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2008).** Temperature- controlled high pressure inactivation of *Geobacillus stearothermophilus* spores and modeling in the pressure and temperature landscape. Oral presentation at 46th European High Pressure Research Group (EHPRG) meeting, Valencia, Spain.

20. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2008).** Sterilization under high pressure- New mechanistic inactivation studies and adiabatic process concepts. Oral presentation (in German) at ProcessNet FA Sitzung "Lebensmittelverfahrenstechnik 2008", Weihenstephan, Germany.

21. **Reineke, K., Mathys, A., Heinz, V. & Knorr, D. (2007).** Temperature control for high pressure processes up to 1400 MPa for gentile food sterilization. Oral presentation (in German) at GDL Congress "Food Technology 2007", Hamburg, Germany.

Poster presentations at scientific meetings:

1. **Reineke, K., Schlumbach, K., Baier, D., Mathys, A. & Knorr, D. (2012).** The release of dipicolinic acid – the rate limiting step of endospores inactivation during high pressure thermal sterilization. Poster presentation at IFT12 „IFT Annual Meeting & Food Expo", Las Vegas, USA

2. **Meneses, N., Reineke, K. & Knorr, D.** Bacterial endospore inactivation by pulsed electric fields: a proof of principle. Poster presentation at IFT12 „IFT Annual Meeting & Food Expo", Las Vegas, USA

3. **Reineke, K., Doehner, I., Baier, D., Mathys, A. & Knorr, D. (2011).** Impact of high pressure on endospore germination and inactivation – A mechanistic study. Poster presentation at 8th European Congress of Chemical Engineering, Berlin, Germany

4. **Reineke, K., Doehner, I., Baier, D., Mathys, A. & Knorr, D. (2011).** Influence of high pressure and elevated temperatures on the different pathways of spore germination and inactivation of *Bacillus subtilis.* Poster presentation at IFT11 „IFT Annual Meeting & Food Expo", New Orleans, USA

5. **Reineke, K., Weich, H. & Knorr, D. (2011).** The addition of sugars as tool for a controlled pressure induced starch gelatinization. Poster presentation at IFT11 „IFT Annual Meeting & Food Expo", New Orleans, USA

6. **Reineke, K., Weich, H. & Knorr, D. (2011).** The Influence of sugars on pressure induced starch gelatinization. Poster presentation at iCEF11, Athens, Greece

7. **Reineke, K., Kuhn, A., Doehner, I., Mathys, A. & Knorr, D. (2010).** Role of the cortex lytic enzymes during the high pressure inactivation of bacterial spores. Poster presentation at EFFoST “Food and Health”, Dublin, Ireland

8. **Reineke, K., Kuhn, A., Doehner, I., Mathys, A. & Knorr, D. (2010).** Role of the cortex lytic enzymes during spore inactivation under high pressure. European PhD Conference in Food Science and Technology. BerlinFood. 8 - 10 September 2010. Berlin, Germany.

9. **Reineke, K., Mathys, A. & Knorr, D. (2010).** Influence of high pressure and temperature on bacterial spores – Inactivation mechanisms above 500 MPa. Poster presentation at IFT10 „IFT Annual Meeting & Food Expo“, Chicago, USA

10. **Weich, H., Reineke, K. & Knorr, D. (2010).** Controlled pressure induced starch gelatinization by the addition of sugars. Poster presentation at 48th European High Pressure Research Group (EHPRG) International Conference, Uppsala, Sweden

11. **Reineke, K., Mathys, A. & Knorr, D. (2010).** Mechanisms of bacterial endospores inactivation during high pressure thermal sterilization. (in German) Poster presentation at ProcessNet FA Sitzung “Lebensmittelverfahrenstechnik 2010”, Frankfurt, Germany.

12. **Baier, D., Reineke, K., Mathys, A. & Knorr, D. (2010).** Fluoreszenzbasierte Detektion der Keimung bakterieller Endosporen während der Hochdruckbehandlung. Poster presentation at the ProcessNet technical committee meeting "Lebensmittelverfahrenstechnik 2010" in Frankfurt / Main, Germany.

13. **Reineke K., Mathys, A., Heinz, V. & Knorr, D. (2009).** Inactivation mechanisms of bacterial spores during ultra high pressure treatments. Poster presentation at EFFoST “New Challenges in Food Preservation”, Budapest, Hungary

14. **Doehner, I., Reineke, K., Mathys, A. & Knorr, D. (2009).** Temperature dependent pH-shift and its influence on the inactivation of bacterial spores. Poster presentation at EFFoST “New Challenges in Food Preservation”, Budapest, Hungary

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig verfasst habe. Alle benutzten Hilfsmittel und Quellen sind aufgeführt.

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir, wie in der vorstehenden Publikationsliste aufgeführt, vorgenommen worden.

Berlin, 10.09.2012

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