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Comprehensive study of the heat resistance of dried *Bacillus subtilis* spores

Julia Hauck Tiburski

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UNIVERSITE DE BOURGOGNE

AGROSUP DIJON

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Par **Julia HAUCK TIBURSKI**

Comprehensive study of the heat resistance of dried *Bacillus subtilis* spores

Soutenue le 17 décembre 2013 devant la Commission d'Examen :

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Résumé

En réponse à un stress nutritif, les espèces du genre *Bacillus* sont susceptibles de former des spores métaboliquement dormantes résistantes à d'autres formes de stress. Ces spores peuvent se retrouver à forte concentration dans beaucoup d'aliments secs, ce qui peut provoquer des intoxications alimentaires ou dégrader les aliments lorsqu'ils sont réhydratés. Comme leur destruction est très difficile, la plupart des méthodes couramment utilisées pour décontaminer les aliments secs sont peu efficaces. L'objectif de ce travail est de comprendre l'influence de l'hydratation de la spore sur l'inactivation des spores sèches de *B. subtilis*. Une étude fondamentale a été menée en soumettant les spores placées dans les capsules d'Analyse Enthalpique Différentielle à différents traitements thermiques et en associant simultanément les thermogrammes obtenus à la viabilité des spores traitées. Les résultats montrent la persistance d'une teneur en eau relativement élevée dans le protoplaste des spores équilibrées à faible a_w (0,13). De plus, une relation forte a été mise en évidence entre la teneur en eau du protoplaste de la spore et sa sensibilité thermique. La spectroscopie IR à transformée de Fourier a montré que cette sensibilité est fortement liée à la dénaturation/agrégation des protéines et à la libération de l'acide dipicolinique. Ces résultats ont finalement permis de développer un procédé d'inactivation thermique sous pression (entre 2 et 7 bar) des spores sèches. Le maintien d'une pression d'azote dans le réacteur chauffé permet d'empêcher l'évaporation de l'eau du protoplaste des spores et donc de favoriser leur inactivation. A terme et après développement, ce procédé peut être un moyen original de décontamination d'aliments secs.

Mot clés : spores; *Bacillus subtilis*; AED; IRTF; décontamination; hydratation; aliments secs.

Abstract

In response to starvation, species from the genre *Bacillus* are able to form metabolically dormant spores which are very resistant to multiple forms of stress. They are found in quite high concentrations in some dried foods which, upon rehydration, may lead to food deterioration or food-borne diseases. Moreover, their destruction is rather difficult and most of the techniques commonly used to treat dry foods result in a very low spore inactivation. The aim of this work is to better understand the role spore hydration in the inactivation of dried *Bacillus subtilis* spores. A fundamental study was conducted using Differential Scanning Calorimetry pans as reactors to perform a heat treatment in dried spores and simultaneously relate the thermograms to spore viability. Results show the persistence of a relatively high water concentration in the core of extremely dry spores. Besides, a strong relation between this core water concentration and spore thermal sensitivity was demonstrated. This destruction was found to be highly related to protein denaturation/aggregation and dipicolinic acid release through Fourier Transform Infrared Spectroscopy analysis. From this fundamental study, a procedure for the inactivation of dried spores using low pressures (2-7 bar) and high temperature was developed. The system consisted of a heated reactor in which gaseous nitrogen was compressed to prevent the evaporation of water from the spores and so favor spore inactivation ($> 5 \log_{10}$). This method of inactivation could be an interesting new way to optimize the decontamination of dried foods.

Key-words: spores; *Bacillus subtilis*; DSC; FTIR; decontamination; water content; dried foods.

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LIST OF SYMBOLS AND ABBREVIATIONS

64 PP	6-4 photoproduct
$\alpha\beta^-$ spores	Mutant spores lacking SASP
AGFK	Asparagine, Glucose, Fructose and KCl
AP	Apurinic/apyrimidinic sites
a_w	Water activity
BCP	Bromocresol Purple
BER	Base excision repair
Ca^{2+}	Calcium cation
$CaCl_2 \cdot 2H_2O$	Calcium Chloride Dihydrate
CCFH	Codex Committee on Food Hygiene
CFU	Colony forming units
CH_3CO_2K	Potassium acetate
CLE	Cortex lytic enzyme
CO_2	Carbon dioxide
$CuCl_2$	Copper(II) chloride
$CuSO_4 \cdot 5H_2O$	Copper (II) sulfate pentahydrate
D	Decimal reduction time
D_2O	Deuterium oxide
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid or pyridine-2,6-dicarboxylic acid
DSB	Double strand break
DSC	Differential Scanning Calorimetry
EU	European Union
FAO	Food and Agriculture Organization of the United nations
FDA	Food and Drug Administration
$FeSO_4 \cdot 7H_2O$	Iron(II) Sulfate Heptahydrate
FTIR	Fourier Transform Infrared Spectroscopy
GR	Germinant receptors
H^+	Hydrogen cation
H_2O_2	Hydrogen peroxide
HP	High Pressure

HTST	High Temperature Short Time
IR	Infrared
J	Joule
K ⁺	Potassium cation
KCl	Potassium chloride
KH ₂ PO ₄	Potassium phosphate monobasic
K ₂ HPO ₄	Dipotassium phosphate
LiBr	Lithium Bromide
LiCl	Lithium Chloride
log	Logarithm 10 of the number of spores
LTLT	Low-temperature long-time
Mg ²⁺	Magnesium cation
MgSO ₄ .7H ₂ O	Magnesium Sulfate Heptahydrate
Mn ²⁺	Manganese cation
MnCl ₂ .4H ₂ O	Manganese(II) Chloride Tetrahydrate
n	Number of mols
N ₀	Number of spore before treatment
N _t	Number of spores after treatment
Na ⁺	Sodium cation
NaBr	Sodium Bromide
NaCl	Sodium Chloride
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
Patm	Atmospheric pressure
Pf	Final pressure
Pi	Initial pressure
PG	Peptidoglycan
pH	Hydrogen potential
PMB	Procédès Microbiologiques et Biotechnologiques
py	Pyrimidine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SASP	Small acid-soluble proteins

SP	Spore photoproduct
Spl	Spore photoproduct lyase
SSB	Single strand break
U.S.	United States of America
UV	Ultraviolet
WHO	World health organization
W	Watt
w/w	Weight by weight
x	Molar fraction
z	Thermal death time
ZnSO ₄	Zinc Sulfate
σ	Sigma factor

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INTRODUCTION

1. INTRODUCTION

Bacterial spores are produced in nature as a mean to survive extreme environmental conditions enabling long-term survival in conditions that could otherwise kill vegetative bacteria (Nicholson et al. 2000). Some bacterial species forms spores as a survival mechanism, namely the Gram-positive *Bacillus*, *Alicyclobacillus*, *Clostridium*, *Sporolactobacillus*, and *Sporosarcina* and the Gram-negative *Desulfotomaculum* species. Among these, species of *Bacillus* (*B. cereus*, *B. anthracis*) and *Clostridium* (*C. perfringens*, *C. botulinum*, *C. difficile*) cause foodborne diseases in humans and are considered agents for bioterrorism (Nicholson 2004).

Bacterial spores are resistant to multiple forms of stress such as heat (wet and dry), pressure, UV radiation, chemicals and desiccation that would normally kill vegetative bacteria (Nicholson et al. 2000). Consequently, spores can survive food processing and conservation methods, which can lead to food spoilage and to food poisoning.

However, spore-forming bacteria may also present beneficial aspects. Indeed, their use as indicators of sterilization (*Geobacillus stearothermophilus*), natural insecticide (*Bacillus thuringiensis*) or for the production of enzymes and antibiotics (*B. subtilis*) and probiotic (*B. subtilis*) begins to be recognized and developed in the industrial world (Höfte and Whiteley 1989; Albert et al. 1998; Schallmey et al. 2004; Cutting 2011). New spore applications, such as the use as vaccination vehicles are being currently developed (Oggioni et al. 2003).

Low moisture foods have historically been considered to present a minimal risk of causing illness, because they do not support the growth of pathogens. However, over the last few decades, a number of outbreaks in a number of different countries have been associated with the consumption of low moisture food products. The most common bacterial pathogens linked to these outbreaks were *Salmonella* and *Bacillus* spp. These outbreaks underscore the need to ensure appropriate hygienic practices and adequate inactivation treatment in the production of low moisture foods. In addition, they caused the food industry to change its practices to better combat the potential for low-moisture food contamination (FAO/WHO 2012; Van Doren et al. 2013). Between 2007 and 2010, three large-scale salmonellosis outbreaks, only in the United States, were attributed to consumption of *Salmonella*-contaminated spices/seasonings (Sotir et al., 2009; CDC, 2010; Higa, 2011). Indeed, the infectious dose of *Salmonella* can be

relatively small; 100 to 1000 organisms are enough to cause the infection in some people. FDA reported that *Salmonella* contamination of spices was the cause of 95% of the U.S. food recalls associated with spices over the period 1980-2000 (Vij et al., 2006).

Spores of the *Bacillus* genus are found in quite high concentrations in dried foods (exceeding 10^2 - 10^5 spores/g) in ingredients such as spices, cocoa powder, milk powders and cereals (Chen et al. 2004; Lima et al. 2011; Witkowska et al. 2011). However, the decontamination of dried foods is difficult since the low water content causes changes in the protein conformation making them more resistant to treatments. Although spores are dormant in dried products, their survival represents a problem when this products are added to high-moisture foods and a suitable environment is established, which may cause them to germinate and multiply.

The factors involved in spore resistance have been partially explained by its structure and the presence of specific internal components. They present a multilayered structure very different from that of growing cells (Nicholson et al. 2000) and different layers play a role in spore resistance. The spore's coat protects it from chemicals and lytic enzymes and also acts as a permeability barrier (Driks 1999). The cortex and the underlying germ cell wall are essential in maintaining the integrity of the spore inner membrane. The inner membrane exhibits low permeability to small molecules, perhaps even water, which is one factor involved in the resistance of spores to some chemicals, in particular DNA damaging chemicals. The final layer is the core that contains the spore DNA, RNA and most enzymes. The high level of Ca-DPA (dipicolinic acid) (25% of core dry weight), the saturation of DNA with a/b-type small, acid-soluble spore proteins and the low water content in the core (25–50% of wet weight depending on the species) all contribute to spore resistance properties (Setlow et al. 2006). It is believed that the remarkable resilience of spores to severe stress is a direct consequence of the low water content within the spore core which in aqueous medium contains only a fraction of the water found in the respective vegetative cell (Black and Gerhardt 1962b).

To meet safety requirements and consumers' expectations the food industry is devoting considerable resources and expertise to the development of processing methods efficiently eliminate unwanted micro-organisms without negatively affecting food quality. Production of safe food requires the control of the raw products entering the food chain, the suppression of microbial growth and the reduction or elimination of the microbial load by adequate treatments. Therefore, the use of a decontamination treatment is essential to limit microbial

contamination to levels below 4 log₁₀ CFU/g and assure the absence of pathogens such as *Salmonella* in the final product.

Commonly used treatments for these dried products are currently steam, ohmic heating, irradiation, chemical agents, or Joule-effect treatments, as the formerly standard ethylene fumigation has been restricted in parts of the world, such as the EU (Schweiggert et al. 2007). Furthermore, dried foods suppliers prefer to avoid the use of irradiation technology due to the psychological barrier and labeling obligations (Laroche et al. 2005). In general, regardless of the chosen decontamination treatment, a compromise must be found between the maximum efficiency of the process and the maximum product quality.

Besides, a number of studies done in the PMB laboratory have dealt with the problem of powder decontamination. A process based on very short heat stresses (from 0.1 up to 30 s) at very high temperatures (in the range of 200 to 600 °C) followed by an instantaneous cooling due to a cold gas (-80 °C) for heat treatment of seeds and food powders was developed and patented (Gervais et al. 2002; Fine and Gervais 2005). Emerging technologies as pulsed UV light (Fine and Gervais 2004) and high gas pressure (Espinasse et al. 2008; Colas de la Noue et al. 2012) were used on the inactivation of dried microorganisms (yeasts and spores) offering prospects for new processes for the decontamination of dehydrated products in which microorganisms present higher resistance to conventional treatments.

Irradiation is the only method highly effective in spore inactivation and although it has proven to be an efficient, environmentally clean, energy effective and even officially approved method, it is scarcely used because of its poor consumer acceptance. Unfortunately, all the other current methods present a limited ability for the decontamination of dried spores (usually around 2 log).

In this context, the objective of this study was to investigate the role of water, its distribution and its implication in the resistance of dried spores. This understanding will allow the optimization of dried food decontamination.

This thesis manuscript begins with an introduction of the subject followed by a literature review where the major points of spore formation, structure and resistance are presented as well as the current methods used for the decontamination of dried food products. Next, the material and methods chapter presents the strains, the productions of spores, and the

treatments performed. The Results and Discussion chapter is divided in two parts: a fundamental, using DSC with an original approach to heat treat spores and at the same time recover data from thermal transitions, water content and inactivation and the second part presents an application of the findings for the development of a system to optimize the destruction of dried spores.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 The bacterial spore

Historically, spores were best known as agents of human and animal disease or food poisoning. Some important events related to spore discovery and the history of spore research are presented in Table 1. The first observation of bacterial spore dates from 1938 when Ehrenberg described refractile bodies inside bacterial cells. However the first studies were conducted independently by Cohn and in *B. subtilis* and later by Koch in the pathogen, *B. anthracis*. Cohn demonstrated the heat resistance of spores of *B. subtilis* and Koch first described in *B. anthracis* the developmental cycle of sporeformers, vegetative cell to spore and spore to vegetative cell (Gould 2006).

Since then, spores have been thoroughly investigated by the community of the microbiologists and many aspects of its formation, structure, genetics and resistance properties were elucidated. From the 1970s, the increasing availability of new genetic techniques provided new methods to accelerate spore research. From that moment, the attentions have been drawn to the model organism *B. subtilis* 168. *B. subtilis* is an aerobic, endospore-forming, rod shaped bacterium commonly found in soil, water sources and in association with plants.

It was the first sequenced Gram-positive bacterium (Kunst et al. 1997) and is to date the most-studied and best understood *Bacillus* species (Nicholson 2004). Therefore, *B. subtilis* has served as a model for bacterial cell biology, especially cell differentiation. Its status as a "generally regarded as safe" (GRAS) organism and the fact that it is easily transformed and completely sequenced makes *B. subtilis* a great laboratory model for the study of the exceptional resistance properties of bacterial spores (Sietske and Diderichsen 1991; Nicholson et al. 2000). However this impressive resistance is a major issue when hygienic and sterile conditions are a prerequisite, such as in the food industry and in medical environments. Consequently, spores are responsible for many cases of food spoilage involving products like herbs, spices and others dry ingredients as well as the cause food-borne illnesses (Brul et al. 2011).

Table 1. Important events in the development of bacterial spore research

Year	Event
1613	Black Bane (<i>B. anthracis</i>): first European pandemic. It caused over 60,000 deaths in humans and cattle.
1735	First case of botulism was recorded and it was associated to consumption of german sausages.
1765	Lazzaro Spallanzani proved microorganisms could be killed by boiling and was one of the first to disprove spontaneous generation.
1820	Justinus Kamer gives a complete clinical description of what physicians now recognize as botulism.
1836	Ehrenberg describes the presence of refractile bodies inside bacterial cells.
1850	Pierre Rayer and Casimir-Joseph Davaine discovered small filiform bodies “about twice the length of a blood corpuscle” in the circulation of sheep with anthrax.
1861	Pasteur refutes the theory of spontaneous generation
1872	Ferdinand Cohn recognized and named the bacterium <i>Bacillus subtilis</i> , capable of growth in the presence of oxygen and forms a unique type of resting cell called endospores.
1876	Robert Koch demonstrates that anthrax is caused by <i>Bacillus anthracis</i> . Anthrax served as the prototype for Koch’s famous postulates regarding the transmission of infectious diseases.
1877	John Tyndall develops a sterilization method to inactivate spore called Tyndalization.
1881	Louis Pasteur develops a live attenuated vaccine against anthrax for cattle.
1896	Van Ermengem discovers <i>Clostridium botulinum</i> , the agent of botulism.
1949	Hills show the role of l-alanine and other amino acids in spore germination.
1953	Development of phase-contrast microscopy.
1953	Identification of DPA by Powell and Strange
1958	Discovery of transformation in <i>B. subtilis</i> by Spizizen.
1960	Description of asymmetric septation, engulfment and the sequential formation of the major structural features of the spore by electron microscopy by Fitz-James.
1966	Subdivision of the sporulation sequence into seven distinct stages by Ryter et al.
1969	Recognition of ‘commitment’ to sporulation by Sterlini and Mandelstam.
1990	Identification of germination enzymes, first by Foster and Johnstone
1993	Description of sporulation genetics by Errington.
1996	The controlling sigma cascade is uncovered by Stragier and Losick.
1997	Complete sequencing of <i>B. subtilis</i> 168 by Kunst et al.
2001	Anthrax attack in the US postal service.
2004	The Genus <i>Bacillus</i> is re-classified and comprises of 88 species and 2 subspecies (Fritze 2004).

References: (Spizizen 1958; Errington 1993; Stragier and Losick 1996; Kunst et al. 1997; Fritze 2004; Nicholson 2004; Gould 2006; Abel-Santos 2012).

2.2 Structure of a *Bacillus* spore: how can it explain spore extreme resistance?

The structure (Figure 1) and chemical composition of the spore differ considerably from those of the vegetative cell. These differences largely account for the unique spore resistance to environmental stresses. Spores are extremely small (1-1.8 μm of length and 0.48-0.98 μm of diameter) and consist of multiple concentric shells encasing dehydrated genetic material at the core (Carrera et al. 2007). Both spore structure and chemical composition are discussed in further detail below as well as their function in spore resistance.

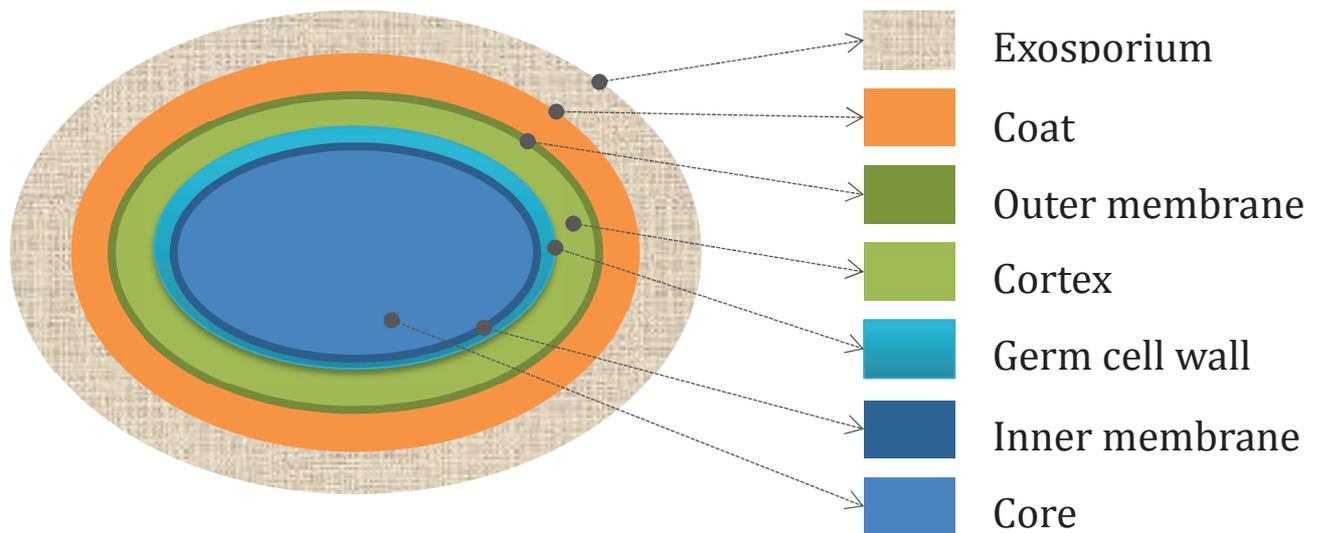


Figure 1. Spore structure. The various layers are not drawn to scale, and there can be many sublayers in the coat and exosporium. The exosporium layer is not present in spores of all species. Adapted from Setlow (2003).

2.2.1 Spore core

At the centre of the spore lies the core, which contains the spores' DNA, RNA and most of its enzymes (Setlow 2006; Setlow 2007). The core is relatively dehydrated (Beaman et al. 1982), contains high levels (c. 5–15% of core dry weight) of the small molecule DPA (Paidhungat et al. 2000), DNA is saturated with SASP (Setlow 1988) and it has a low permeability (Cowan et al. 2004). All these conditions are strongly linked to the resistance properties of the spore,

many of which are in some way involved in protecting spore DNA from damage, and they will be discussed below.

2.2.1.2 Core water content

Bacterial spores contain a highly reduced level of water as compared with its vegetative cell counterparts. In protoplast of growing cells the water content is about 75-80% of wet weight whereas in spores the core water represents only about 27-55% of wet weight (Lindsay et al. 1985; Beaman and Gerhardt 1986). Although the mechanisms for the reduction of core water during sporulation are still not known, the maintenance of the dehydrated core is achieved by the low permeability of the inner membrane, the DPA content and the pressure exerted by the cortex (Pedraza-Reyes et al. 2012).

The low water content of the core is believed to be the major factor contributing to the spore dormancy and heat resistance. Generally, the lower the core water content, the higher the wet heat resistance (Nicholson et al. 2000; Setlow 2006). Melly et al. (Melly et al. 2002b) suggested that core proteins in a more highly dehydrated spore core have greater resistance to wet heat, presumably as a result of reduced molecular motion. Killing of spores by hydrogen peroxide is also affected by core water content, with higher core water levels being associated with greater sensitivity to peroxide, although the reason for such a relationship is unclear (Popham et al. 1995).

Spore core water content can be varied in several ways, including by the variation of the sporulation temperature, higher temperatures generally leading to a lower core water content (Melly et al. 2002b), or using strains that lack the ability to produce DPA (Paidhungat et al. 2000).

The molecular mobility of spore core components is strongly dependent on hydration, the more hydrated the spores are, more mobile are its components. Furthermore, there is a reversible water migration between inner spore compartments and the environment, whereas DPA seems to be immobilized in a water-insoluble network in the core (Leuschner and Lillford 2000).

It has been proposed that the spore core was in a glassy state and so spore dormancy could be explained by the low diffusive molecular motions in the highly viscous glass state (Sapru and Labuza 1993; Ablett et al. 1999; Stecchini et al. 2006). This hypothesis came from an endothermic transition observed by differential scanning calorimetry on *Bacillus subtilis*

spores but later this interpretation was questioned (Leuschner and Lillford 2003) and more recently dismissed (Sunde et al. 2009). In fact, core water has high mobility through the spore, as shown by recent spin relaxation rates in H₂O/D₂O exchange and therefore spore's dormancy is probably linked to the dehydration-induced conformational changes of enzymes. Moreover, it was established that core water molecules are bound to the hydration layers of the proteins but still remain mobile (Sunde et al. 2009).

2.2.1.3 Spore mineral content

Bacterial spores accumulate minerals presenting very high levels of divalent ions, which contribute to spore stability and heat resistance. The most abundant minerals in spores are calcium, manganese, magnesium, potassium and sodium, and among them manganese is essential to sporulation and calcium is necessary for an optimum sporulation and production of stable spores (Slepecky and Foster 1959; Bender and Marquis 1985; Marquis and Bender 1985).

The mineral content of spores can be modulated by the modification of the sporulation medium, sporulation temperature or by removal of metal ions by ion exchange (Alderton et al. 1964; Bender and Marquis 1985; Atrih and Foster 2001). With these techniques it is possible to obtain spores with extremely low levels of monovalent cations (H⁺, Na⁺ or K⁺) or, conversely, spores with high levels of divalent cations (Ca²⁺, Mg²⁺, Mn²⁺) (Nicholson et al. 2000).

Several studies have shown that core mineralization is crucial to spore resistance to different types of stresses. Increased core mineralization is related to a decrease in core water content which is known to contribute to wet heat resistance (Marquis and Bender 1985; Beaman and Gerhardt 1986; Cazemier et al. 2001). The accumulation of intracellular Mn⁺² complexes may protect spore's proteins against treatments such as ionizing radiation that generate reactive oxygen species which may be due in part to the spores' high levels of DPA conjugated to divalent metal ions, predominantly Ca²⁺ (Granger et al. 2011). It has also been observed that increasing the Mn levels on diploid spores, such as *B. megaterium*, elevated their resistance to both wet heat and dry heat, H₂O₂ and UV radiation (Ghosh et al. 2011).

The exact mechanism of protection provided by spore mineralization is not completely elucidated, and since that wet heat and H₂O₂ kill spores by protein damage and dry heat and radiation by DNA damage, there is clearly more than one effect. It has been suggested that

Mn²⁺ increases DNA repair capacity by induction of specific DNA repair enzymes during sporulation and also by increasing the ability to detoxify ROS (Ghosh et al. 2011). Finally, another important mechanism is the reduction and maintenance of spore low core water that protect spores from wet heat (Bender and Marquis 1985).

2.2.1.4 α/β -Type Small Acid-Soluble Proteins

The DNA in dormant spores of *Bacillus* is complexed with a group of unique proteins, the Small Acid Soluble Proteins (SASPs), which are synthesized only within the developing spore late in sporulation, slightly prior to DPA. They are present exclusively in the spore core in amounts sufficient to saturate the DNA (Setlow 1988) (Figure 2). These small proteins (60–75 aa) are extremely abundant in spores, comprising 3–6% of total spore protein (Setlow 2006).

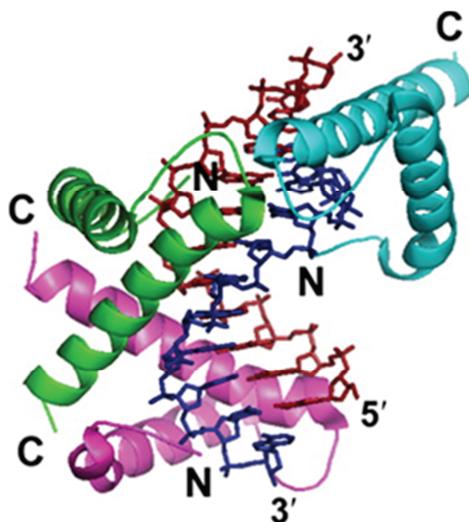


Figure 2. Crystal structure of the α/β -type SASP–DNA complex. Stereo diagram of the complex. The positions of the N and C termini of each protomer are labeled. Green, SASP1; magenta, SASP2; cyan, SASP3; red, dG DNA strands; deep blue, dC DNA strands (Lee et al. 2008).

A major function of SASPs is to supply amino acids for protein synthesis during spore germination. SASPs are degraded early in spore germination and contribute to more than 90% of the total protein degraded during germination (Setlow 1988).

There are two major SASP in *Bacillus* spores (termed α and β), as well as a number of minor ones, for example the γ -type SASP. In general, the two major α/β -type SASP comprise 40-50% of the total SASP pool with the γ -type SASP comprising 30-40% (Johnson and Tipper 1981). Only the α/β -type SASP is known to have a different function besides being an amino acid reservoir. Studies have been conducted to elucidate the role of the γ -type SASP but the results were not conclusive (Vyas et al. 2011). However, Ruzal et al. (2013) recently showed that mutants without γ -type SASP germinated less efficiently with alanine and Ca-DPA and had different coat properties, suggesting that the γ -type SASP is involved in the coat assembly during sporulation.

The binding of these proteins alters DNA's structure and properties dramatically, and these proteins are significant factors in spore resistance to heat and many chemicals, and a major factor in spore resistance to UV radiation (Setlow 2006). The binding of α/β -type SASPs results in the energetic stabilization and protection of DNA, presumably this is why this complex is formed in spores and the reason for the increased rigidity of its components due to intermolecular interactions (Lee et al. 2008).

Each α/β -type SASP interacts with the DNA backbone on both edges of the DNA helix which causes DNA to assume a A-like (Figure 3) conformation that is more UV resistant than its other forms (Mohr et al. 1991; Setlow 1992a; Lee et al. 2008). This binding protects the DNA backbone from chemical and enzymatic cleavage and reduces significantly the rate of depurination (Setlow et al. 1992; Fairhead et al. 1993). It is important to mention that the protein-DNA interactions do not involve water molecules, so consequently these proteins remain tightly bound to DNA even in dry spores, providing protection against desiccation damage to DNA.



Figure 3. DNA conformations

The spore photochemistry is also modified by the presence of α/β -type SASPs. Indeed, when exposed to UV radiation instead of forming cyclobutane-type pyrimidine dimers (Py \leftrightarrow Py) and (6–4)-photoproducts (64PP) like growing cells, spores form thymine-thymine adduct (spore photoproduct). Although SP is a potentially lethal photoproduct, it is repaired early in spore outgrowth by one of the spore's repair systems (Fairhead et al. 1993; Setlow 2007). On the contrary, spores without α/β -type SASP produce a large amount of of Py \leftrightarrow Py and 64PP (Setlow and Setlow 1987; Nicholson et al. 1991).

Mutants in genes encoding α/β -type SASPs lacks about 80% of the wild-type SASP protein pool (Setlow et al. 2002) and are significantly more sensitive to wet and dry heat, UV radiation, desiccation, ionizing radiation and genotoxic chemicals including nitrous acid, hydrogen peroxide and formaldehyde (Fairhead et al. 1993; Setlow and Setlow 1993a; Fairhead et al. 1994; Tennen et al. 2000; Huesca-Espitia et al. 2002; Douki et al. 2005; Moeller et al. 2008), although $\alpha\beta^-$ spores and wild-type spores exhibit similar resistance to other chemicals like peroxyxynitrite, CuCl_2 , hypochlorite and chlorine dioxide (Genest et al. 2002; Young and Setlow 2003; Shapiro et al. 2004).

Recent studies have described both the structure of the α/β -type SASP-DNA complex by X-ray diffraction (Lee et al. 2008) and protein-DNA binding conformations and interactions (Ge et al. 2011).

2.2.1.5 Dipicolinic Acid

Another special feature of the spore core, firstly identified by Powell (1953), is the presence of a huge amount of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)], (Figure 4) which makes up 5-15% of the dry weight of bacterial spores. DPA is not present in growing cells and is synthesized in the mother cell very late in sporulation, at about the time of acquisition of spore heat resistance (Daniel and Errington 1993).

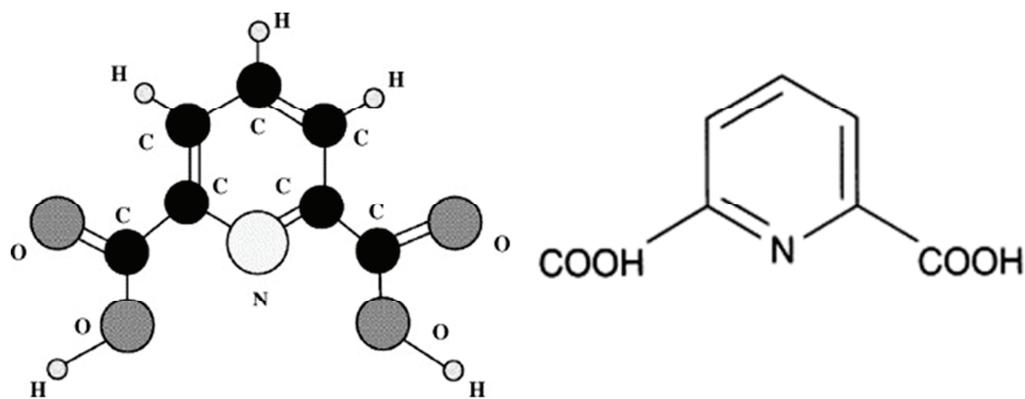


Figure 4. Dipicolinic acid structure

DPA exists in spores as a 1:1 chelate with divalent cations, predominantly Ca^{2+} , and does not leak out significantly from spores stored in water (Setlow 1994). In the first minutes of spore germination the DPA is excreted, along with the associated divalent cations (Setlow 2003).

The Ca-DPA levels differ significantly in a spore population but its concentration in the spore core is well above the solubility of the complex (800 mM to 1M) which indicates that the majority of Ca-DPA is in an insoluble form (Huang et al. 2007).

One role of DPA is to lower the core water content, since DPA loss early in germination is paralleled by its replacement with water. Additionally, genetically DPA-less dormant spores have more core water than wild-type dormant spores, are extremely unstable and germinate and lyse during purification (Paidhungat et al. 2000). DPA has also been suggested to play a role in spore dormancy which is supported by the fact that the low core water content is likely the major factor in the spore's dormancy and DPA is essential to achieve a low core water content (Setlow 2006)

Another function of DPA in spores is to contribute to the protection of spore DNA against many types of damage like wet heat and desiccation which is especially important in $-\alpha-\beta$ spores (Setlow and Setlow 1995). However, the mechanism whereby DPA protects DNA against dry heat and desiccation is not known.

Considering their higher core water content, DPA-less spores are less resistant to wet heat. However, it is not clear if this effect is due only to a change in spore core hydration or also to the reduction in core mineralization which accompanies the loss of DPA from spores or yet to specific effects of DPA on spore resistance (Paidhungat et al. 2000).

DPA complexed with Mn^{2+} or Ca^{2+} has also been shown to protect proteins from ionizing radiation in vitro (Granger et al. 2011), which again may serve the same protective role in vivo. Conversely, DPA actually increases the sensitivity of spores to UV radiation, as demonstrated in spores that lack DPA (Setlow and Setlow 1993b; Paidhungat et al. 2000).

2.2.2 Inner membrane

There are two membranes in the dormant spore, the inner membrane which is derived from the forespore compartment, and the outer membrane which is derived from the mother cell (Errington 1993). The inner membrane serves as a selective permeability barrier, it surrounds the spore core preventing harmful molecules from passing through (Cortezzo and Setlow 2005).

The inner membrane also plays a role in spore germination since essential parts of the germination apparatus, like the germination receptors and gene products of the *SpoVA* operon (associated with the opening of channels for DPA release) (Hudson et al. 2001; Paidhungat and Setlow 2001; Vepachedu and Setlow 2005) are located there.

In addition, the dormant spore's inner membrane can change its volume without new membrane synthesis, decreasing as much as 2-fold late in sporulation and increasing up to 2-fold in the first minute of spore germination, when the spore's large peptidoglycan cortex is degraded and the germ cell wall expands (Errington 1993; Cowan et al. 2004)

2.2.2.1 Core permeability

The inner spore membrane is a strong permeability barrier to the penetration of small molecules into the spore core, and the rate of transit of a molecule as small as methylamine across the inner membrane is extremely slow (Black and Gerhardt 1962a; Swerdlow et al. 1981; Cowan et al. 2004; Sunde et al. 2009).

Consequently, the inner membrane plays a major role in spore resistance to many chemicals, in particular those that cross this membrane to damage spore DNA and is also a determinant factor in maintenance of the low water content in the spore core (Genest et al. 2002; Young

and Setlow 2003; Cowan et al. 2004; Cortezzo and Setlow 2005). In other words, the low permeability directly protects spores against DNA damaging chemicals and indirectly it is important in the wet heat resistance as it helps to maintain the low core water in the spore.

The reason for the relative impermeability of the spore's inner membrane to small molecules is not completely clear, although the great majority of the lipids in this membrane are largely immobile. Another reason could be that the membrane appears very compressed in the dormant spore, as its volume doubles in the first minutes of spore germination (Cowan et al. 2004; Sunde et al. 2009).

2.2.3 The cortex

The spore cortex is formed by a thick layer of peptidoglycan (PG) and it resides beneath the spore coat. The PG from the cortex is different from the PG of vegetative cells, notably regarding the complete absence of teichoic acids from the N-acetylmuramic acid residues in spore PG (Atrih et al. 1996). It has been proposed that the spore cortex is involved in the maintenance of the heat-resistant, dormant state and this is likely to be mediated by the ability of the cortex to prevent rehydration of the spore core, by creating a mechanical constraint. The PG in spore cortex is loosely cross-linked and this low level of cross-linking has been suggested as a possible mechanism responsible for attaining and maintaining maximum core dehydration, a hypothesis referred to as the contractile cortex concept. However, more recent studies have demonstrated that the level of cross-linking in spore PG does not alter spore dehydration but can, however, interfere in spore germination (Popham 2002).

The cortex also contains enzymes (Sleb and Cwlj) that allow its hydrolysis during late stages of germination. Cwlj is synthesized in the mother cell and localized specifically between cortex and coat. Sleb is also present at this location but also at the inner membrane (Chirakkal et al. 2002). The cortex degradation results in rapid rehydration of the spore core with a consequent resumption of metabolic activity and loss of resistance properties.

2.2.4 The outer membrane

Around the cortex lies the outer spore membrane that is formed in sporulation, during engulfment, and is actually an inverted inner membrane from the mother cell. Although essential for spore formation (Piggot and Hilbert 2004), its exact function in the dormant spore remains unclear. Indeed, removal of the outer membrane along with much spore coat

protein has no notable effect on spore resistance to heat, radiation and some chemicals (Nicholson et al. 2000).

Furthermore, the outer membrane does not prevent the uptake of the small uncharged, lipophilic molecule, methylamine, and presumably does not obstruct the passage of germinants, which must penetrate as far as their receptors in the inner membrane (Setlow and Setlow 1980; Swerdlow et al. 1981).

There has been some doubt as to whether the outer membrane actually serves as an intact membrane in the mature spore since there are no reports on the its isolation and purification and also due to the difficulty in observing it (Leggett et al. 2012). However, there are reports asserting that the outer membrane in dormant *B. megaterium* spores is intact (Crafts-Lighty and Ellar 1980) and also that it acts as a functional barrier to the diffusion of large ions (Vries 2006). Other studies have also indicated the presence of proteins in the outer membrane that may play a role in the coat assembly (Ramamurthi et al. 2006) and also the presence cortex lytic enzymes (Chirakkal et al. 2002).

2.2.5 The spore coat

The spore coat is a multilayered shell that protects the bacterial genome during stress conditions (Setlow 2012). The coat layers are mainly composed of protein, around 30% of the total spore protein, and more than 70 proteins unique to spores were already identified (Kim et al. 2006) (Driks 1999). The synthesis of the coat protein occurs exclusively in the mother cell compartment of the sporulating cell and this transcription is ordered by the sequential activation of two alternative σ s factors for RNA polymerase, σ E followed by σ K (McKenney et al. 2013).

Recently, fluorescence microscopy coupled with high-resolution image analysis has been applied to the dynamic process of coat assembly and has shown that the coat is organized into at least four distinct layers as represented in Figure 5 (Henriques and Moran 2007; McKenney et al. 2010; McKenney et al. 2013).

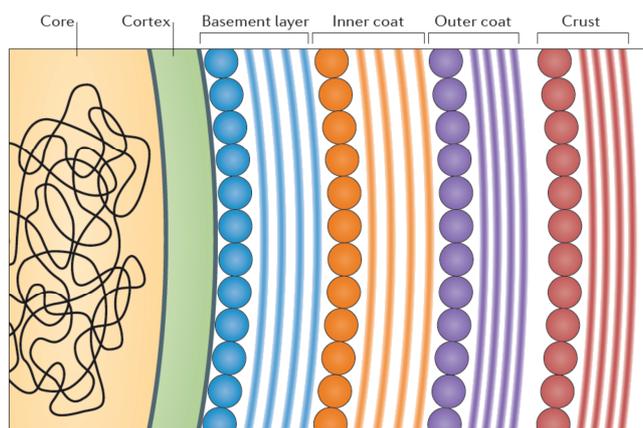


Figure 5. Structure of spore coat (McKenney et al. 2013)

The major known function of the coat is spore protection and, indirectly, preservation of the spore genome. The coat can be conceptualized as a molecular sieve that excludes large molecules, such as lysozyme, while allowing the passage of small-molecule germinants (Driks 1999). The coat also appears to house enzymes with direct roles in germination, like cortex lytic enzymes.

2.2.5.1 Spore coats - resistance

The spore coat is important in spore resistance to some chemicals (Young and Setlow 2003), to exogenous lytic enzymes that can degrade the spore cortex (Driks 1999), to predation by protozoa (Klobutcher et al. 2006; Laaberki and Dworkin 2008) and recently it has been shown that the coat also plays a minor role in spore wet heat resistance (Ghosh et al. 2008; Sanchez-Salas et al. 2011).

The precise mechanism whereby the coat protects against reactive chemicals is not known, but coat proteins may react with and detoxify such chemicals before they can gain access to and damage more sensitive targets further in the spore's interior. The spore coat also appears to contain enzymes such as superoxide dismutase and perhaps catalase that may assist in detoxification of some reactive toxic chemicals, as well as the CotA laccase, which can contribute to spore hydrogen peroxide resistance (Henriques et al. 1998; Riesenman and Nicholson 2000; Driks 2002; Henriques and Moran 2007).

2.2.6 The exosporium

The exosporium is the most external layer and is present in some species including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus megaterium*, *Clostridium sporogenes* and *Clostridium difficile* but not in others such as *Bacillus atrophaeus* and *Bacillus subtilis* (Henriques and Moran 2007). Its discovery is usually attributed to deBary (deBary 1885) and is described in *B. anthracis* spores by Koch as ‘a round transparent mass which appeared like a small light ring surrounding the spore (Carrera et al. 2007).

Typically, the exosporium is a loose fitting, balloon-like multilayered shell that surrounds the entire spore, including the coat, but is not connected to the spore or the coat (Henriques and Moran 2007 {Driks, 1999 #1066). Rather, a gap separates the two structures and the nature of the material in this gap, if any, is unknown (Driks 2003). The exosporium is made up of proteins and lipids, including some glycoproteins found only in spores that contain an exosporium (Redmond et al. 2004).

The exosporium is the least understood part of the spore structure, but its presence on pathogenic *Bacilli*, as *B. anthracis* and *B. cereus*, suggests a possible role in interactions with host organisms. The exosporium also accounts for the remarkably strong adhesion of *B. cereus* spores to various materials such as stainless steels or polymers. This adhesion strength has been ascribed to its exceptional hydrophobicity (Faille et al. 2010) and also to the size of its exosporium (Tauveron et al. 2006).

Because it is absent in species, such as *Bacillus subtilis*, which tolerate a wide range of environmental stresses, the exosporium may have more to do with accommodation to specific niches or lifestyles than with general protection or germination (Driks 2003).

2.2.7 Another factors influencing spore resistance

2.2.7.1 Sporulation conditions

Studies have shown that sporulation conditions can affect the spore resistance to different stresses, probably by altering spore structure and composition (Melly et al. 2002b). Among the sporulation factors that have already been investigated are the temperature of sporulation, water activity, pH and mineral content of the medium and the state of the medium.

Spores produced at higher temperatures have lower core water content and present modifications in their coats and cortex. The decrease in the core water content is believed to be the reason of the higher wet heat resistance and the modifications on the coat properties

make the spores more resistant to chemicals (Warth 1978; Condon et al. 1992; Melly et al. 2002b; Cortezzo and Setlow 2005). However, spores produced at higher temperatures are more sensitive to high pressure treatments (Raso et al. 1998).

The decrease of the a_w of the sporulation medium produced smaller spores with a considerable decrease in spore thermotolerance (Nguyen Thi Minh et al. 2008). This decrease in heat resistance could be related to the changes in the lipid composition of spore membrane and the coat properties that are observed when spores are produced in a hyperosmotic medium (López et al. 1998).

Spores produced at higher pH are more resistant to wet heat and high pressure according to Mihn et al. (2011) and Mazas et al. (1997), however the mechanisms by which sporulation pH could affect spore resistance are still unknown. It has been suggested that it could be due to changes in spore mineralization levels (Alderton and Snell 1963) or in membrane fluidity (Nguyen Thi Minh et al. 2011).

The increase in spore mineralization has major effects on the augmentation of spore resistance to UV radiation and wet and dry heat (Ghosh et al. 2011). The mechanisms by which mineralization protects the spore are diverse since the target of spore inactivation is different depending on the treatment. Regarding wet heat, the resistance could be related to the decrease in core water with the addition of minerals (Cazemier et al. 2001). On the other hand, for UV and dry heat the protection may come from some modifications in the DNA repair system and proteins protection from ROS (Ghosh et al. 2011).

The method (liquid or solid media) used for spore production also affects their properties even if an identical medium and temperatures are used. Spores prepared in liquid media are more sensitive to wet heat and some chemicals and germinate more rapidly. These changes are probably related to modifications observed in the lipid composition of the inner membrane and minor differences in spore coat (Rose et al. 2007).

2.2.7.2 Repair systems

There are three distinct strategies for dealing with potential types of DNA damage. They are the low core water content and SASP and they act in DNA protection. The third mechanism associated to DNA protections is spore's DNA repair system. During dormancy, DNA damage generated due to environmental stresses will continually accumulate in spores and will only be repaired during germination as enzyme action and energy metabolism are

reinitiated (Setlow 1992b). However, at this time the amount of DNA damage accumulated could overwhelm DNA repair capacities.

There are a number of different mechanisms currently known to repair the DNA damage, among them the most important are: Spore photoproduct lyase (SPL), recombination and nucleotide excision repair (NER) and base excision repair (BER).

Base excision repair (BER) was described for the first time by Salas-Pacheco et al. (2005). Since then, several studies have shown that BER is a major pathway involved in repair of a wide variety of DNA base damage resulting from exposure to various chemical (e.g., hydrogen peroxide) and physical (e.g., dry and wet heat, UV and ionizing radiation) insults (Moeller et al. 2007; Moeller et al. 2008; Barraza-Salas et al. 2010; Moeller et al. 2011). ExoA and Nfo are the major *B. subtilis* AP endonucleases involved in this pathway and they can repair strand breaks and nick DNA at apurinic/aprimidinic (AP) sites, leading to gap filling. If not repaired these lesions can inhibit DNA replication and be mutagenic preventing spores from correct outgrowth.

Since spore DNA is bound to SASP proteins and consequently in an A-like conformation, different photoproducts are formed when spore are exposed to UV-radiation. This spore-specific lesion is a special thymine dimer 5-thymine-5,6-dihydrothymine, which is commonly called spore photoproduct (SP). When spores leave the dormancy phase and start germinating, these SPs must be repaired as they have proved lethal to the germinated bacteria (Setlow 1995). The unique spore system to repair this lesion is the spore photoproduct lyase (SPL).

SPL is activated only during early germination to monomerize the SP dimer back to the two original thymine residues in an adenosyl-radical dependent ("Radical SAM") reaction (Li 2012) (Slieman 2000). SP is the exclusive DNA photo-damage product in bacterial endospores; its generation and swift repair by SPL are responsible for the spores' extremely high UV resistance (Setlow 2001).

Nucleotide excision repair (NER) is one of the major cellular pathways that removes bulky DNA adducts and helix-distorting lesions, like UV-induced photoproducts (alteration of the nucleotide structure), that produce a block to DNA replication. This repair system in *B. subtilis* closely resembles the analogous system in *E. coli*, which has been extremely well characterized (Lin and Sancar 1991; Friedberg 1996).

The bacterial nucleotide excision repair system is mediated by the *uvr*-system (UvrA, UvrB and UvrC). These repair proteins work in concert to identify and remove a large array of DNA lesions that vary in structure and chemistry. Essentially, following UV irradiation (or treatments with other DNA-damaging treatments) lesions are enzymatically removed from the DNA. Expression of the *uvr*-system is inducible by DNA damage both during vegetative growth and during the outgrowth phase of germination of UV-C irradiated spores (Setlow and Setlow 1996).

2.3 Spore life cycle

2.3.1 Sporulation

Sporulation of *Bacillus* species is induced by starvation for carbon and / or nitrogen and is also important that the population density is high, also, no specific nutrient act as a trigger (Higgins and Dworkin 2012). The sporulation process is initiated by phosphorylation of the master transcription regulator, Spo0A (Stragier and Losick 1996).

The secret of the success of spore formation lies in the altruistic behavior of the mother cell, which uses all of its resources to endow the prespore with resources, particularly protective layers, thereby maximizing the chances of survival for the mature spore (Errington 2003).

During the endospore development, five RNA polymerases (RNAP) sigma factors appear, displacing one another and conferring on RNAP different specificities for the recognition of different classes of promoters. The sigma factors appear in order σ H (pre-septum formation), σ F (early forespore, active septation) , σ E (mother cell specific), σ G (late forespore, replaces σ E), and σ K (late mother cell, replaces σ E). This process of altering transcriptional specificity is the fundamental mechanism that regulates sporulation gene expression. The sequential appearance of each sigma factor determines the temporal pattern of gene transcription.

In summary, sporulation is regulated by two cascades of sigma factors, one in the forespore and the other in the mother cell. Each cascade influences the other through a series of signals so that the whole complex developmental process is properly coordinated.

The sporulation process is usually divided in seven stages (0-VII), as shown in Figure 6 (Stragier and Losick 1996). Stage 0 is defined as vegetative growth and upon starvation an axial chromatin filament is formed (Stage I). This filament results from the condensation and stretching of two copies of the chromosome across the cell in order to reach and attach itself

to the cell poles. Stage II initiates with an asymmetric division near one pole of the cell resulting in the formation of a smaller cell, the forespore, and a larger cell, the mother cell. The forespore will contain only one third of a chromosome, however SpoIIIE, a DNA translocase, will transfer the rest of the chromosome through the septum into the developing spore. The two compartments engage in differential activation of genes because of compartment specific σ -factors (Robledo et al. 2012).

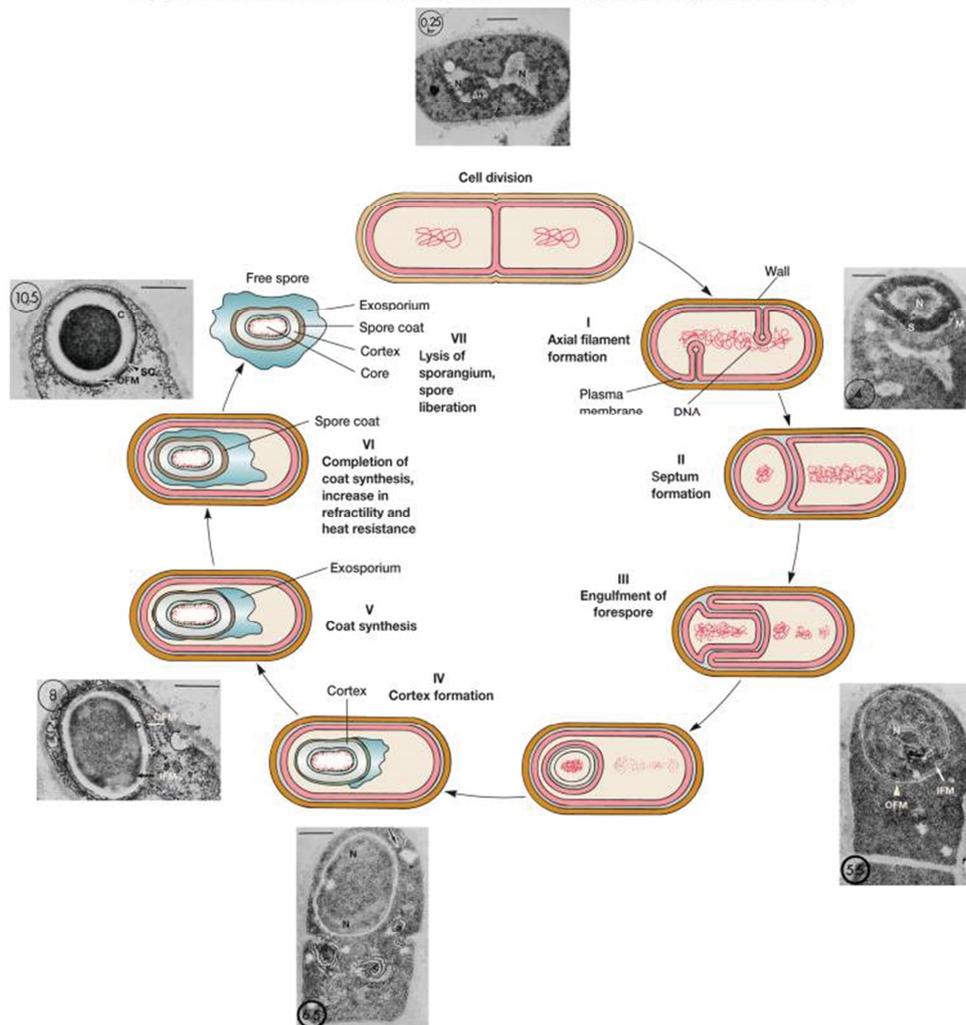


Figure 6. Sporulation stages of *Bacillus* sp. (Prescott, 2002)

The next step is the engulfment of the forespore by the mother cell membrane which results in the forespore being surrounded by two membranes (Stage III). Stage IV is characterized by the synthesis of a thick peptidoglycan cortex between the outer and inner forespore membranes and also of the germ cell wall, which separates the inner membrane from the core. The formation of the cortex is accompanied by a large decrease in the volume and water content of the forespore protoplast, and there is also a decrease in forespore pH by 1 unit. The

forespore later takes up tremendous amounts of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] that has been synthesized in the mother cell, which decreases forespore protoplast water content even further. In this stage occurs also the synthesis of SASPs in the forespore which makes the forespore nucleoid rather condensed and in a ringlike structure. Next, the mother cell produces a proteinaceous coat that assembles on the outside surface of the mother-cell membrane around the forespore. The coat consists of a lamellar inner layer and an electron-dense outer layer and provides a thick, protective barrier that encases the mature spore.

In this stage (V) the protoplast develops an increasing heat resistance and a bright appearance of the spore in phase contrast illumination can be observed. From now the spore is extremely resistant to physical stress like heat, pressure, or radiation, and to the attack of chemical agents or enzymes. The last stages are the maturation of the forespore (Stage VI) and finally the lyses of the mother cell releasing the spore in to environment (Stage VII) (Higgins and Dworkin 2012).

2.3.2 Germination and outgrowth

Spores of *Bacillus* species can remain dormant for long periods and are extremely resistant to a variety of environmental stresses. However, under appropriate conditions, spores can come back to active growth through a process called germination followed by outgrowth (Paidhungat and Setlow 2000; Paidhungat and Setlow 2001; Setlow 2003). Germination is a critical step for spore destruction since it loses its remarkable resistance during this process.

Germination can be divided into nutrient and non-nutrient mechanisms. The first pathway can be triggered by L-alanine or L-valine or a combination of L-asparagine, D-glucose, D-fructose, and K⁺ (AGFK) (Setlow 2003). These nutrients set off the germination process by binding to and interacting with the germinant receptors (GR) located in the spore's inner membrane (Paidhungat and Setlow 2001; Korza and Setlow 2013). Rather, non-nutrient germination can be triggered by lysozyme (Popham et al. 1996), Ca-DPA (Paidhungat and Setlow 2000; Perez-Valdespino et al. 2013), cationic surfactants (Perez-Valdespino et al. 2013) and high pressures (Wuytack et al. 1998; Reineke et al. 2013). Recently, another germination pathway independent of known GRs has been identified (Shah et al. 2008). In this pathway, spores are germinated by muropeptides derived from breakdown of PG from growing cells of the same or closely related species. These various non-nutrients can bypass individual components of the nutrient germination pathway as shown in Figure 7.

The process of germination has been studied and reviewed recently by several authors (Moir 2006; Cronin and Wilkinson 2007; Setlow 2008; Ghosh and Setlow 2010; Yi and Setlow 2010; Paredes-Sabja et al. 2011; Bassi et al. 2012; Christie 2012; Giebel et al. 2012; Popham et al. 2012), therefore only a brief overview is given here.

Normally, before germination is initiated there is an activation phase that allows the synchronization of germination, usually a sub lethal heat shock is used; however the mechanisms of spore activation are still not known (Setlow 2003; Mathys et al. 2007). A number of events occur in a defined sequence during spore germination. The first one is the “commitment to germination” that occurs upon spore exposure to nutrient germinants causing a chain reaction ultimately leading to a fully germinated spore, even if the germinant is removed (Stewart et al. 1981; Yi and Setlow 2010; Yi et al. 2011).

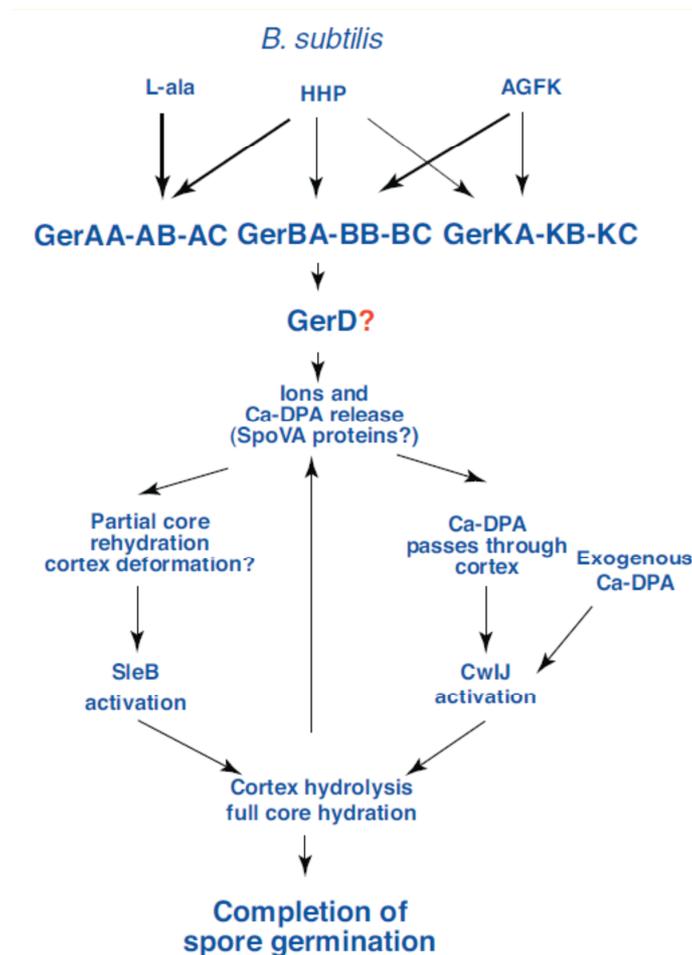


Figure 7. Models for information flow during the germination of spores of *B. subtilis* proposed by Paredes-Sabja et al (2011). Dark arrows denote the germinant receptor (GR) preference of the germinants, and thin arrows denote weak effects of the germinant on the GR. Upward arrows indicate that cortex hydrolysis increases rates of DPA release.

The germination process is usually divided in two stages as shown in Figure 8. The first events take place in Stage I which comprises receptor-mediated changes in the permeability of the spore's inner membrane resulting in the release of monovalent cations from the spore core. The release of cations causes an increase in the internal pH from ~ 6.5 to 7.7 , a change essential for spore metabolism once core hydration levels are high enough for enzyme action (Swerdlow et al. 1981). Next, the vast pool of dipicolinic acid is released from the core in parallel with other small molecules like amino acids (Setlow et al. 2008). Then, water moves in the opposite direction to partly rehydrate the spore core and although the moisture content is not sufficient for enzyme activity, the heat resistance of these partly germinated spores is reduced (Cowan et al. 2003).

Stage II is characterized by the hydrolysis of the spore's cortex by germination specific peptidoglycan hydrolases, SleB and CwlJ, which are activated by the DPA release (Figure 7). CwlJ is located at the cortex-coat junction whereas SleB is present at the inner spore membrane and in outer layers (Moir and Smith 1990; Moir et al. 2002; Moir 2006). The degradation of the cortex removes the physical constraint on the spore core, allowing further core hydration and consequently, core and germ cell wall expansion and loss of dormancy. At this stage protein mobility in the core returns and enzyme activity is possible meaning that the spore has lost all its heat resistance (Christie 2012).

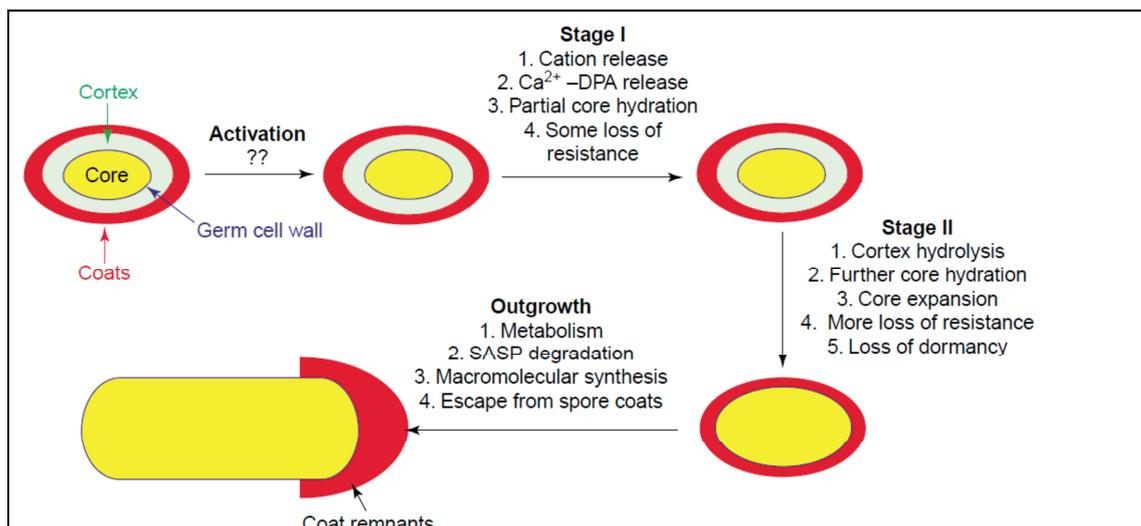


Figure 8: Stages of spore germination (Setlow 2003).

The germinated spore then initiates the outgrowth stage, which comprises the resume of metabolism, SASP degradation by proteases (Gpr), macromolecular synthesis and escape from the spore coats (Popham et al. 2012).

Spore germination is essential for dormant spores to return to vegetative growth and although much has been learned about it, a number of key questions still remain unanswered. According to Paredes-Sabja et al. (2011) the mechanisms of action of germinant receptors and of DPA release, the identity of all cortex degrading pathways and the mechanism of activation of SleB are not yet well understood.

2.4 Spore Resistance to different environmental stresses

2.4.1 Desiccation

Bacterial spores are extremely resistant to desiccation and can survive repeated freeze-drying/rehydration cycles, in contrast to vegetative cells, which are killed by such processes (Ashwood-Smith and Grant 1976; Fairhead et al. 1994). Not only they can survive desiccation but they can also maintain its viability over many years of dried storage as shown by Marshal et al. (1963). Freeze-dried spores of *Bacillus* and *Clostridium* were kept for 6 years at different water activities and showed no loss in viability when the water activity was between 0.2 and 0.8.

Bacterial spores can also be dried by atomization with little or no loss in viability. Xuenyong et al. (2008) showed that when the inlet air temperature of 155–165°C and the outlet air temperature of 66–70°C are employed, the spores count approaches that of the freeze dried sample.

Exposure to vacuum predominantly causes the removal of water and consequently hydrophobic bonds of membranes and proteins are disrupted and metabolism practically comes to a complete stop. This extreme removal of water also causes substantial changes regarding the structure of DNA leading to changes in conformation and strand breaks (Dose 1986; Dose et al. 1992). Spores submitted to vacuum desiccation presented an increase in mutation frequency even if they were not inactivated since most of these lesions are repaired during germination (Northrop and Slepecky 1967).

To date the only factors that have been shown to contribute to spore resistance to desiccation is the protection of spore DNA by α/β -type SASP and DNA repair systems, since the mechanisms of killing by dry heat are mutagenesis and DNA damage (Pedraza-Reyes et al. 2012). As a result, whereas spores of wild-type *B. subtilis* are resistant to up to six cycles of freeze-drying and rehydration, α/β spores undergo a 30-70% loss in viability during each cycle (Fairhead et al. 1994).

Munakata et al. (1997) used spores with mutations on the DNA repair system, one defective in the nucleotide excision repair and spore-specific repair of UV-induced spore photoproducts (-uvr -spl) and another also defective in RecA (-uvr -spl -recA). They showed that forced dehydration of DNA in the microenvironment of the spore core causes unique damage leading to an increase in the mutation frequency in the *GyrA12* allele and the accumulation of DNA double strand breaks and DNA-protein crosslinks. The study also showed that these lesions seem to be preferentially repaired by *rec* dependent process of DNA repair.

2.4.2 UV radiation

Spores of *Bacillus* species are generally 7-50 times more resistant to UV radiation (260 nm) than are growing cells of the same species (Setlow 1995; Setlow 2007). As DNA is the target for UV radiation, UV damage to spore DNA must either be prevented in some fashion or be rapidly repaired in the first minutes of spore germination. The major mechanisms of DNA protection and reparation include a difference in the UV photochemistry of DNA in spores and the efficient and relatively error-free repair of the novel photoproduct (5-thymineyl-5,6-dihydrothymine) formed by UV light in spore DNA (Douki et al. 2005).

There appear to be three factors that contribute to the spore DNA photochemistry: the saturation of spore DNA with α/β -type SASP; the low water content in the spore core; and the high level of DPA in the spore core (Setlow 2006).

Studies in vivo and in vitro have shown that α/β -type SASP have profound effects on DNA properties. Indeed they are the major determinant of spore resistance to UV radiation (Popham et al. 1995; Setlow et al. 2000). Spores of *Bacillus subtilis* lacking the majority of their α/β -type SASP are more sensitive to UV than growing cells (Douki et al. 2005). The change in the UV photochemistry of spore DNA because of α/β -type SASP appears due to a change in DNA conformation induced by the binding of these proteins from B-type helix in SASP-free DNA to an A-like helix in DNA saturated with SASP (Setlow 2001).

Studies have shown that a reduction in DNA hydration increases SP production and suppresses formation of other photoproducts on UV irradiation (Rahn and Hosszu 1969; Nicholson et al. 1991) suggesting that the low core water content may also play a role in spore DNA photochemistry. However, there has been no systematic study of spore DNA photochemistry as a function of core hydration (Setlow 2006).

DPA in the core also influences spore DNA photochemistry markedly, as DPA acts as a strong photosensitizer (Paidhungat et al. 2000). Spores with decreased DPA levels are more resistant to UV radiation and DPA increases the yield of SP. This effect of DPA may explain the higher resistance of vegetative cells (which lack DPA) to UV radiation compared to that of $\alpha\beta^-$ spores (Setlow and Setlow 1993b).

However the protection mechanism cited above cannot explain spore UV resistance alone; since SP can be a lethal lesion, the other factor essential for spore UV resistance is DNA damage repair. The two major spore DNA repair pathways are nucleotide excision repair (NER) and spore photoproduct lyase (Spl) that operate in the early minutes of spore germination. The first excises SP and fills in the resulting single-stranded gap and the latter process uses an enzyme termed spore photoproduct lyase (Spl) that monomerizes SP to two thymidine residues (Pedraza-Reyes et al. 2012).

The NER repair is dependent on RecA so spores lacking either Spl or the RecA-dependent repair pathways are two- to sixfold more UV sensitive than are wild-type spores, and RecA Spl spores are approximately 50-fold more UV sensitive (Setlow 2001).

2.4.3 Dry heat

Bacterial spores are also more resistant to dry heat than their corresponding vegetative cells (Nicholson et al. 2000). A dry heat treatment at 90°C killed 90% of vegetative cells in 5 minutes whereas only 10% of spores were killed after 60 minutes of the same treatment (Setlow and Setlow 1995). The killing of spores by dry heat appears to be due to DNA damage accumulated during treatment since both DNA damage (strand breaks and AP sites) and mutations were observed in spores exposed to dry heat (Northrop and Slepecky 1967) (Setlow and Setlow 1995; Huesca-Espitia et al. 2002).

Most factors potentially responsible for spore resistance to dry heat have not been investigated thoroughly. Although there have been studies with spores of only a few species, in at least one species (*B. stearothermophilus*), variations in spore core mineralization affected spore

resistance to dry heat, with demineralized spores having significantly lower resistance (Alderton and Snell 1969). However, the mechanism of this effect is not understood, and it has not been seen with spores of all species examined.

Consequently, the major known factor of protection against dry heat are the α/β -type SASPs as they protect spore DNA which is, in this case, the target of spore inactivation. Furthermore, $\alpha^- \beta^-$ spores are as sensitive to dry heat as dry growing cells (Setlow and Setlow 1995) (Setlow 2006).

Accordingly, DNA repair systems are also important in spore dry heat resistance. There are different systems involved in the repair of strand breaks and AP sites due to dry heat treatment. RecA deficient *B. subtilis* spores are much more sensitive to dry heat than are wild-type spores, suggesting that RecA-dependent DNA repair systems are involved in the repair of dry heat damage to spore DNA (Setlow and Setlow 1996). The BER system (base excision repair) includes two endonucleases, ExoA and Nfo, that can remove AP sites and they are also related to DNA repair after dry heat treatment (Urtiz-Estrada et al. 2003; Salas-Pacheco et al. 2005; Barraza-Salas et al. 2010). Mutants without either ExoA or Nfo were reported to be more sensitive to a dry heat treatment, but the double mutant were as sensitive as the single mutants (Salas-Pacheco et al. 2005). Recently it was also shown that a large increase (50 fold) in the spores' Nfo level was able to make $\alpha^- \beta^-$ spores even more resistant than wild-type spores dry heat (Barraza-Salas et al. 2010).

Spores of *B. subtilis* contain a single chromosome therefore making impossible for the homologous recombination pathway to operate (since it requires at least two homologous chromosomes) on the repair of double strand breaks during spore germination. Spores can, however, use an alternative repair pathway called non-homologous-end joining (NHEJ) (Weller et al. 2002; Bowater and Doherty 2006). This system involves the genes *ykoU* and *ykoV* and mutants without these genes were found to be significantly more sensitive to dry heat than were wild-type spores (Wang et al. 2006).

In conclusion, α/β -type SASPs and the repair systems BER (Nfo/ExoA), NHEJ (*ykoU/ykoV*) and RecA mediated appear play an essential role in the preservation and repair of fry heat induced DNA damage.

2.4.4 Moist heat

The most common method for spore inactivation is wet heat treatment, although this requires quite high temperatures, because spores are resistant to temperatures that are around 45°C higher than growing cells (Warth 1978). This method is routinely used for the inactivation of spores in food products.

A number of factors are responsible for spore moist-heat resistance, including low core water, the optimum growth temperature of the bacterial strain and the sporulation temperature, the spore core's high level of DPA, core mineralization and α/β -type SASPs. Since both core mineralization and a higher sporulation temperature result in more dehydrated spore core, it is assumed that core dehydration is the primary component determining spore heat resistance (Bender and Marquis 1985; Marquis and Bender 1985; Beaman and Gerhardt 1986) (Nakashio and Gerhardt 1985; Cazemier et al. 2001).

Thermophiles invariably have more wet heat resistant spores than do mesophiles. Some of the higher wet heat resistance of thermophiles spores is likely because of their relatively low core water content. However, the higher intrinsic thermostability of macromolecules, in particular proteins, from thermophiles also likely contributes to the increased wet heat resistance of their spores (Setlow 2006).

Several studies have showed that spores prepared at higher temperatures are generally more heat resistant than spores prepared at lower temperatures (Condon et al. 1992). However, the impact of the temperature of sporulation on heat resistance appears more or less important depending on the bacterial species. *B. subtilis*, *B. cereus* and *B. coagulans*, for example, showed a 10 fold increase in D with an increase in the order of 25 to 30°C in the sporulation temperature however *B. stearothermophilus* presented an increase in D from 15 to 1000 folds (Beaman and Gerhardt 1986).

The increase in the sporulation temperature can alter spores' properties that have a role in the wet heat resistance. The spore density increases (Beaman and Gerhardt 1986), the core water content decreases and there are changes in the protein composition of the coat and the cortex (Melly et al. 2002b) and modifications in the fatty acids profile (Planchon et al. 2011). Spores also present a higher degree of mineralization that contribute both for the lower water content as for the higher density (Palop et al. 1999).

However regarding the modification of spore's properties there are differences between species. Baweja et al (2008) observed that the amount of DPA in *B. anthracis* spores formed

at an elevated temperature (45°C) was two-fold higher as compared to the control spores but Melly et al (2002b) observed no such modification in spores of *B. subtilis*.

In addition to high levels of DPA, the spore core also contains high levels of divalent cations, most chelated with DPA. This significant mineralization of the core is also important in spore wet heat resistance, with higher mineralization generally associated with higher spore wet heat resistance (Beaman and Gerhardt 1986). One function of DPA in spore resistance is to lower the core water content, probably by replacing some core water. This process can elevate spore resistance to wet heat by protecting core proteins from inactivation or denaturation (Baweja et al. 2008).

Spores of different *Bacillus* strains prepared on nutrient agar with a mix of metal ions (Ca^{2+} , Mg^{2+} , Fe^{2+} , K^+ and Mn^{2+}) all appeared to be more resistant to a wet heat at 114°C for 4 min treatment than the spores prepared with only Mn^{2+} . The protoplast wet densities of all the tested spore types prepared on nutrient agar with the mix of metal ions were higher than those of the spores prepared on nutrient agar with only Mn^{2+} (Cazemier et al. 2001).

Other than affecting levels of core water, it is not clear why spore core mineralization and the specific nature of the mineral ions would affect spore wet heat resistance. Perhaps this is due to interaction of spore macromolecules with some DPA-metal ion lattice that permeates the spore core (Leuschner and Lillford 2000). Tied in with this latter uncertainty is whether DPA plays any direct role in spore wet heat resistance other than by reducing core water content (Setlow 2006).

Spore killing by wet heat does not occur through DNA damage that might be expected at elevated temperatures. Indeed, spore DNA is well protected by the saturation of the spore DNA with a/b-type SASP (Setlow 1988). However, mutant *B. subtilis* strains lacking the majority of a/b-type SASP are significantly more sensitive to wet heat and are killed in large part (if not completely) by DNA damage, including abasic sites presumably generated as a result of depurination (Setlow et al. 2000).

There have been also studies showing that the coat can also be important in spore wet heat resistance since some coat-defective spores, including *cotE gerE* spores have decreased wet heat resistance (Ghosh et al. 2008). A recent study (Sanchez-Salas et al. 2011) suggested that modifications in spore structure following spores' release from the sporangium, including

increased cross-linking coat proteins by transglutaminase (Tgl) result in increased spore wet heat resistance.

Even though the mechanisms of spore resistance to moist heat are fairly well understood, the mechanisms whereby spores are killed by this treatment are not completely elucidated, although it is known that spores are not killed by DNA damage.

The work by Coleman et al (2007) lead to significant conclusions about the events and the mechanism of *B. subtilis* spores inactivation by moist heat and later they extended these conclusions to *B. cereus* and *B. megaterium* spores (Coleman et al. 2010). They proposed a model for the inactivation of spores due to wet heat treatment that is presented in Figure 9.

During moist-heat treatment, spores suffer increasing amounts of damage to a number of proteins at different rates. When loss of some crucial protein becomes too great, the spores die; however, these dead spores retain DPA and the capacity to initiate germination but cannot progress in outgrowth. The specific defect leading to the inability of these spores to outgrow is not known, but is may be due to the inactivation of essential proteins (Coleman et al. 2007; Coleman et al. 2010).

As moist-heat treatment continues, there is eventually sufficient damage to one or more proteins in the spore's inner membrane such that there is a change in the membrane permeability, leading to the rapid release of the spore's DPA. It was shown that DPA release is an all-or-nothing phenomenon and does not take place in discrete steps which, rather the DPA level remains constant for 5–50 min and then suddenly declines to zero in 1–2 min; presumably the large variation in the lag times prior to rapid DPA release is a reflection of heterogeneity in spore populations (Zhang et al. 2010). Although the mechanism for this rapid CaDPA release is not clear, it seems likely to be due to a breakdown in the impermeability of the spore's inner membrane during wet-heat treatment that allows CaDPA and other small molecules to escape, although not allowing the loss of large molecules such as nucleic acids (Zhang et al. 2011).

DPA release is accompanied by a large amount of protein damage that occurs slightly before and after the full release of DPA which suggests that protein denaturation may be an early event during wet-heat inactivation of spores and the major mechanism of spore killing by wet heat. With the loss of spore DPA and its likely replacement by water as in spore germination, the spore's core water content almost immediately rises significantly and this will

undoubtedly result in much more rapid heat inactivation of spore core proteins (Coleman et al. 2007; Coleman and Setlow 2009; Coleman et al. 2010).

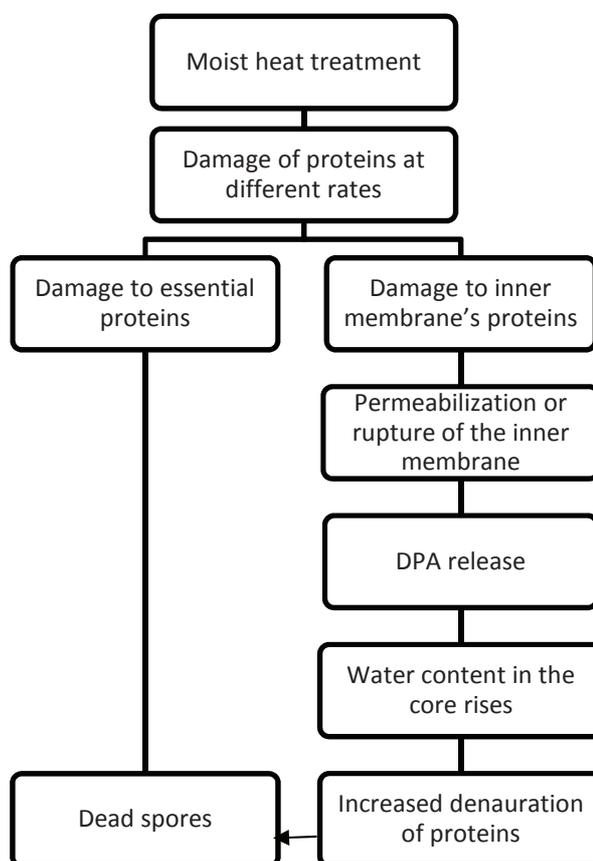


Figure 9. Model for the wet heat inactivation for spores of *Bacillus*. (Coleman et al. 2007; Coleman et al. 2010)

However, despite all the mechanisms that were elucidated, there are still some remaining questions about the inactivation of spores by wet heat including the mechanism of DPA release during heat treatment, how protein inactivation can trigger subsequent events during wet-heat treatment and finally the identity of the protein responsible for spore death and the exact moment of spore death.

2.4.5 Chemicals

Spores are extremely resistant to a variety of chemicals, including acids, bases, oxidizing agents, alkylating agents, aldehydes and organic solvents. However the mechanisms of inactivation by chemicals vary enormously and will be only briefly discussed here.

Some toxic chemical like formaldehyde, nitrous acid, ethylene oxide and ethylmethanesulphonate kill spores via DNA damage, as the survivors accumulate mutations and a *recA* mutation sensitizes spores to these agents (Setlow and Setlow 1996; Setlow et al. 1998; Loshon et al. 1999) (Tennen et al. 2000). In addition, hydrogen peroxide kill spores lacking the majority of their DNA protective α/β -type SASP by damaging these spore's DNA (Setlow et al. 1997; Cortezzo and Setlow 2005).

Most oxidizing agents including Sterilox®, peroxydinitrite, ozone, chlorine dioxide and hypochlorite, likely kill spores by damaging the inner membrane, however the nature of the damage is not known. Presumably the rate of permeation of sufficient oxidizing agent to the sensitive site in the spore's outer layers is a major factor determining the rate of spore killing by such agents. (Loshon et al. 2001; Genest et al. 2002; Young and Setlow 2003; Cortezzo et al. 2004). In addition, Mildly lethal treatment of spores with a variety of oxidizing agents also sensitizes the survivors to a subsequent treatment (e.g. wet heat) to which an undamaged inner membrane may be required for full spore resistance (Cortezzo et al. 2004).

For some chemical agents including larger aldehydes such as glutaraldehyde and ortho-phthalaldehyde, the mechanism of spore killing remains unclear, although these latter two chemicals do not kill spores by DNA damage (Tennen et al. 2000; Cabrera-Martinez et al. 2002). Spore killing by ethanol and strong acid involve the disruption of a spore permeability barrier, while spore killing by strong alkali is due to the inactivation of spore cortex lytic enzymes (Setlow et al. 2002).

In addition to different mechanisms of spore killing by different chemicals, there are also a number of mechanisms involved in spore resistance to different chemicals. The spore coat is of major importance in resistance to a large number of chemicals, in particular most oxidizing agents (Genest et al. 2002; Melly et al. 2002a) (Young and Setlow 2003; Young 2004), although the coat has only a minor role in spore resistance to hydrogen peroxide (Riesenman and Nicholson 2000). A second important factor in spore chemical resistance is the spore's inner membrane, which exhibits extremely low permeability to small hydrophilic and hydrophobic molecules (Black and Gerhardt 1962b). Finally, the third factor important in spore resistance to some chemicals is the saturation of spore DNA with α/β -type SASP (Setlow et al. 2000) (Nicholson et al. 2000).

2.4.6 Ionization

Ionizing radiation can be used to inactivate bacterial spores and the mechanisms of inactivation include “direct” and “indirect” processes. Direct processes involve absorption of photon energy by a target molecule, resulting in damage to the target. Indirect processes occur when photon energy is absorbed by a nearby molecule, like water, resulting in the formation of highly reactive species which in turn react with target molecules in the microorganism (Blatchley et al. 2005). The biological effects of ionizing radiation are thought to arise from the formation of double-strand break (DSB) and clustered DNA damage (two or more lesions including base lesion, single-strand break (SSB), abasic site or DSB) (Nicastro et al. 2002).

Spores are significantly more resistant than are growing cells to ionizing radiation. The reasons for this elevated spore resistance are not all clear, but there are factors that known to be implicated in this resistance as the spore’s low core water (Nicholson et al. 2000) and its DNA repair system (Barraza-Salas et al. 2010; Moeller et al. 2011).

The low core water content of the spore presumably reduces the ability of γ -radiation to generate damaging hydroxyl radicals. However, this has not been proved, as there has been no systematic study of the relationship between core water content and the level of radiation resistance. Nevertheless, Blatchley et al. (2005) showed that spores of *B. cereus* submitted to irradiation in aqueous or dry media did not differ regarding inactivation, i.e., a dose of 4 kGy produced a inactivation of around 2 logs of spores in both treatments.

There is also no information on the possible role of DPA in spore resistance to irradiation, although DPA is involved in spore resistance to UV radiation. However, the a/b-type SASP are not important in spore irradiation resistance (Nicholson et al. 2000).

The efficiency of radiation decreases with the increase in the number of spores. It was calculated that an additional dose of 1.7 kGy is required for each increment of 1 log in the initial spore count. This implies that there is no limit to the dose required to decontaminate a sample since the decontaminating dose depends on the microbial load (Salih 2001).

2.4.7 High Pressure

The application of 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 (Matser et al. 2004).

The favored mechanism for explaining bacterial spore inactivation by HP is pressure/temperature-induced spore germination in which the spores lose their inherent resistance and are inactivated by subsequent treatment conditions due to acquired sensitivity. The mechanism of spore inactivation proposed by Reineke (2013) is presented in Figure 10.

When the studies about the spore resistance to high pressure were less developed a question of whether the spore heat resistance was correlated to their pressure resistance was raised. However Nakayama et al. (Nakayama et al. 1996) tested six different strains of spores and showed that there is no correlation between the spore resistance to wet heat and pressure, i.e., strains sensible to wet heat can be highly resistant to pressure and vice-versa.

The sporulation conditions have an effect on spores properties like the cortex and coat composition, the core water content (Melly et al. 2002b), the inner membrane permeability and fatty acid composition (Cortezzo and Setlow 2005), the mineralization of the core (Cazemier et al. 2001) and the levels germinant receptors (Black et al. 2005; Nguyen Thi Minh et al. 2011). Consequently, these changes in spore composition can influence their resistance to stresses like high pressure.

Raso et al. (1998) were the first to study the effect of sporulation temperature in the spore resistance to high pressures. They produced spores of *B. cereus* at three different temperatures, 20, 30 and 37°C, and showed that those spores produced at 20°C were more resistant to pressure than the ones produced at 30 and 37°C. This result was attributed to the dormancy of the spore population sporulated at 20°C. Black et al. (2005) confirmed those results on *B. subtilis* spores showing that the rate of pressure germination increase with the temperature of sporulation and spores with only a single germinant receptor also exhibited large increases in pressure germination as the sporulation temperature increased. Since the triggering of germination by pressure is likely the rate limiting step in pressure killing of spores the low ability to pressure germinate reduces the spore inactivation by high pressure. The same conclusions were found by Igura et al. (2003) and Margosh et al. (2004).

Previous work in our laboratory have also identified that the pH of the sporulation medium affects spore resistance to high pressure since spores of *B. subtilis* produced at pH 10 were more resistant to pressure treatment than those produced at pH 6 and 7.5 (usual pH of the sporulation medium) (Nguyen Thi Minh et al. 2011). On the contrary, changes in the water

activity of the sporulation medium had no effect on the pressure resistance of spores (Nguyen Thi Minh et al. 2008).

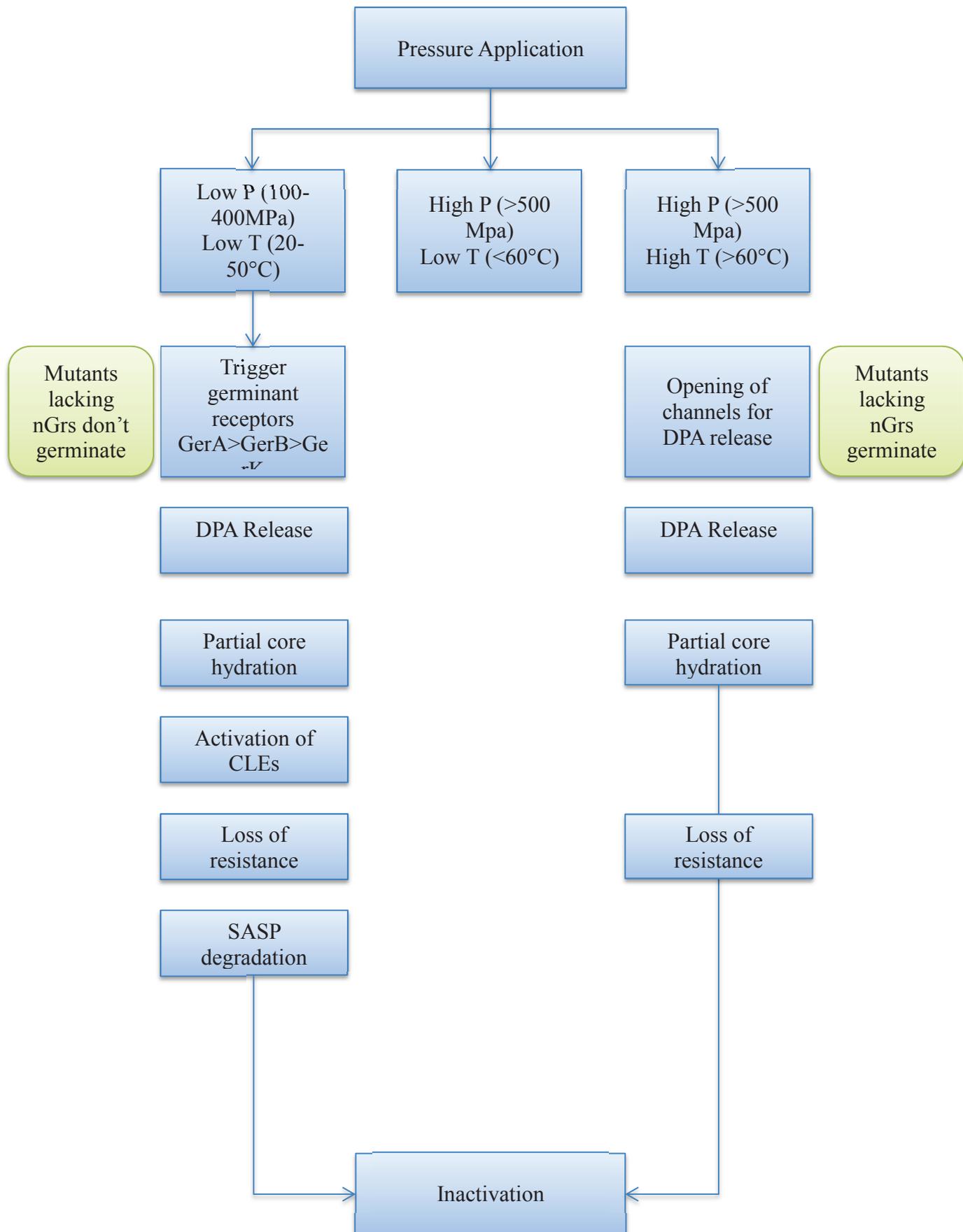


Figure 10. Mechanisms of spore inactivation by high pressure application (Adapted from Reineke (2013))

The mineralization of the spore core also changes the spore resistance to high pressure. Igura et al. (2003) investigated the effect of minerals on resistance to pressure of *B. subtilis* spores and conclude that the resistance to pressure increased after demineralization of spores and decreased after remineralization 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 (CLEs) during germination under HP. The same reduced pressure resistance was observed when minerals were added to the sporulation medium (Margosch et al. 2004).

2.5 Decontamination of dry foods

There is a general rule that most bacteria prefer a water activity higher than 0.90, whereas yeast and molds will persist in $a_w > 0.70$. A food item is considered high moisture if its a_w is >0.95 , medium moisture with $a_w >0.85$, and low moisture is generally $a_w <0.84$.

Given recent wide-spread contamination events, including pistachios (a_w 0.72), milk chocolate (a_w 0.65) and hydrolyzed vegetable protein (a_w 0.60), the industry is now realigning its recommendations to better combat the potential for low-moisture food contamination. The concerns of these outbreaks are led primarily by *Salmonella* species, as well as *E. coli*, but are not limited in scope to these two organisms.

In November 2012, the 44th Session of the Codex Committee on Food Hygiene (CCFH) agreed to develop a Draft Code of Hygienic practice for Low moisture Foods (FAO/WHO 2012). This code will apply to the control of microbiological hazards in foods having a water activity of 0.85 or below and that:

- are exposed to the processing environment following a microbial inactivation step;
- products that are not subjected to an inactivation step,
- or products in which low moisture ingredients that may be contaminated with pathogens are added after an inactivation step.

The methods currently used for the decontamination of low moisture food products are discussed below.

2.5.1 Gamma irradiation

Irradiation is a physical treatment that consists of exposing foods to the direct action of electronic, electromagnetic rays to decrease the population or prevent the growth of undesirable biological organisms in food. Food irradiation has generally come to describe the use of ionizing radiation (energetic charged particles such as electrons and alpha particles, or energetic photons such as gamma rays and X-rays) (Lacroix 2005).

The irradiation sources that are permitted for use in food processing are gamma rays produced from the radioisotopes cobalt-60 (1.17 and 1.33 MeV) and caesium- 137 (0.662 MeV). The biological effects of irradiation create damage in the genetic material of the cell causing a lesion of the DNA or breaking both strands of DNA (Blatchley et al. 2005).

Decades of research have conclusively shown that food irradiation can have numerous beneficial applications, including for example, the disinfestations of insects in fruits and grains the inhibition of sprouting in potatoes and onions, the delayed ripening of fresh fruits and vegetables, and the enhanced safety and sterilization of fresh and frozen meat products, seafood, and eggs (Farkas and Mohácsi-Farkas 2011; Hallman 2013).

The largest use of food irradiation worldwide today is for spices, as shown in Figure 11, with more than 185.600 tons irradiated each year (Kume et al. 2009). The primary reason for irradiation of spices is to control disease-causing microorganisms (Hallman 2013).

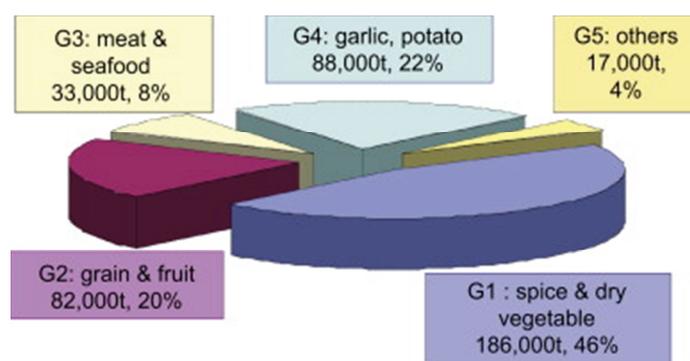


Figure 11. Food irradiation items in the world in 2005. (G5) other food items including health foods, mushroom, honey, etc (Kume et al. 2009).

Commercial food irradiation in EU was active, especially in Belgium, France and the Netherlands. However, it decreased rapidly after the enforcement of an EU regulation in 1999

requiring the strict checking of labels on irradiated foods (Commission of the European Communities, 1999). For example, commercial food irradiation in France reached 20,000 ton in 1998, but dropped to 3000 ton in 2005 (Kume et al. 2009). Further data is presented in Table 2 where we can see that the amount of products submitted to ionization in France continues to drop, especially dry foods like herbs and spices.

Spores are more resistant to γ -rays than vegetative cells are (Nicholson et al. 2000). Since 1999, according to WHO/FAO recommendations, there is no upper limit for gamma radiation dose and it was attested that “food irradiated to any dose appropriate to achieve the intended technological objective is both safe to consume and nutritionally adequate” (WHO 1999) . However, irradiation at high doses cannot be applied since ionizing radiation causes oxidation and degradation of some aromatic components of the spices (Hayashi 1998; Variyar et al. 1998).

Table 2: Categories and quantities of products irradiated in France.

Products	Dose (kGy)	Treated quantity (t)				
		2003	2004	2005	2006	2007
Poultry	5	2290	635	1849.2	1780	1239
Frozen frog legs	5	1006	813	939.8	965	687
Gum arabic	3	141	28	133.7	149	131
Herbs, spices, dried vegetables and fruits	10	919	206	134.3	110	60
Casein	6	15	54	43.5	-	22
Shrimps	5	-	20	10.5	-	-
Total		4371	1756	3111	3004	2139

On the other hand, rates of 5 and 10 kGy have been found to be sufficient to decrease the population of spore-forming flora in spices, like black pepper (Emam et al. 1995) but smaller doses can be used in poultry, gum arabic and shrimp (Table 2).

The greatest problem with irradiation is the consumer acceptance of the technology (Cardello 2003; Cardello et al. 2007). Any irradiated foodstuff and foodstuff or compound food

containing one or more irradiated food ingredient must be labeled with the words “irradiated” or “treated with ionizing radiation” or contain the “radura” symbol. Consequently, due to consumer suspicion, unawareness and resistance, industry is decreasing the use of irradiation on larger scale for spices.

2.5.2 Fumigation and chemical sanitizing

Fumigation with a suitable toxic gas or vapor is a recommended treatment, particularly for contaminants in the form of insects or other small animals. For many years, the most widely used method to destroy microorganisms in dry food ingredients was fumigation with ethylene oxide ((CH₂)₂O or Etox). However Etox is now prohibited in the EU, but is still allowed in the US and Australia. The drawback of Etox is that it is highly inflammable and that it leaves a residue after treatment, which rapidly decreases, however, after two weeks of storage. In addition, it has been proved that the ethylene oxide molecule is carcinogenic to humans, and it has been classified as a category 1 carcinogen by the International Agency for Research on Cancer (IARC) (Tateo and Bononi 2006).

The 2001 incident involving envelopes contaminated with *Bacillus anthracis* in the US mail spores were placed into the U.S. mail, resulted in the contamination of mail processing and distribution facilities and office buildings. These spore-contaminated facilities were subsequently decontaminated primarily by fumigation with hydrogen peroxide, chlorine dioxide, and formaldehyde (Rogers et al. 2008).

The use of hydrogen peroxide vapor may offer the possibility of eliminating persistent pathogens from both food processing equipment and environments, Malik et al. (2013) have demonstrated that an inactivation of up to 6 log of spores can be achieved depending on the hydrogen peroxide concentration. However it will add moisture to the treated specimens.

Ozone, in both the gaseous and aqueous states, is an effective fumigant for insect killing, mycotoxin destruction and microbial inactivation which has a minimal or no effect on food. Ozone is generated on-site and decomposes quickly, leaving no residues, after application to food (Tiwari et al. 2010). Some studies show that up to 3 log reductions of micro-organisms in cereal grains can be achieved depending on ozone concentration, but this level of reduction is only achieved at high relative humidity conditions (> 50%). *Bacillus cereus* spores were reduced by 1.5 log in red pepper flakes at ozone concentrations of 7.0 ppm and 70% RH (Akbas and Ozdemir 2008). A ozonation treatment with 0.4 ppm of ozone at 50% RH reduced

in up to 4 log the population of *B. cereus* spores in rice grains (Shah et al. 2011). However it is possible to inactivate spores in extremely dry environments using very high ozone concentrations (4000 ppm) but to a degree that is largely dependent on the spore type and substrate material (Mahfoudh et al. 2010).

2.5.3 UV light

Irradiation with ultraviolet light (UV) can be used to inactivate many types of organisms, including viruses and reduce the microbial load in food (Guerrero-Beltran and Barbosa-Canovas 2004). The most effective wavelengths are located between 200 and 280 nm (the so-called UVC), especially at 254 nm, while at 320 nm its efficiency is almost null (Bintsis et al. 2000).

The application of UV light with germicidal effects has been used in three areas: air disinfection, liquid sterilization and inhibition of microorganisms in surface, in other words, the germicidal effect is obtained only by applying UV light *directly* to the target (Bintsis et al. 2000). Therefore, in order to decontaminate powders or dry foods there are two possibilities: suspending the food in an aerosol like form and treat it as air disinfection, or keeping the food agitated in order to uniformly expose its surface to UV light.

One of the advantages of UV light is that it does not leave any residue in the treated products and could then be a proper method decontaminating spices without any detrimental defect to the other quality parameters since the temperature increase will be limited (Erdoğdu and Ekiz 2011).

There are two modes of UV-light treatment: continuous (i.e. a constant light cycle) or pulsed (i.e. alternating light and dark cycles). Pulsed UV light is more effective and rapid at micro-organism inactivation than continuous UV light, because the energy released is multiplied many times. The power dissipation from a continuous UV-light system ranges from 100 to 1000 W, whereas a pulsed UV-light system can produce a peak power output as high as 35 MW. In pulsed UV-light treatment, the energy is stored in a high-power capacitor and released intermittently, producing several high-energy bursts in a short period of time (Krishnamurthy et al. 2004).

However, there are no reports of a successful use, i.e., high microbial inactivation rates, using UV light in dry food products. Hidaka and Kubota (Hidaka and Kubota 2006) used UV light in wheat grains and the required sterilization time to obtain a 90% reduction rate was 6.3 h for

bacteria and 5.6 h for molds, which was is efficient for an industrial process. Fine and Gervais (Fine and Gervais 2004) evaluated the efficiency of pulsed UV light for microbial decontamination of black pepper and wheat flour powder, and an extremely low microbial destruction was reported. Erdođdu and Ekiz (Erdođdu and Ekiz 2011) also reported an inactivation of only 0.6 log of mesophilic bacteria after a 60 minutes UV treatment in cumim seeds.

2.5.5 Microwave irradiation

Microwaves are a portion of the electromagnetic spectrum with wavelengths ranging from 1 mm to 1 m, characterized by frequencies of 300 MHz to 300 GHz. Domestic microwave appliances operate generally at a frequency of 2.45 GHz, while industrial microwave systems operate at frequencies of 915 MHz and 2.45 GHz. In the food industry, microwave energy is used cooking, thawing, tempering, drying, freeze-drying, pasteurization, sterilization, baking, heating and re-heating (Chandrasekaran et al. 2013). The heat generated by microwaves can significantly reduce the time required for commercial pasteurization and sterilization. However, bacterial destruction by microwave sterilization present some problems such as an unpredictable and non-uniform energy distribution and the difficulty in monitoring and predicting the microwave heating pattern during processing (Datta 2001).

The dielectric properties of a food determine whether a material can be successfully heated by a microwave field and they are altered by its moisture content, density and temperature. Few studies have examined the use of microwaves to decontaminate spices or food powders, especially because microwave heating is seriously hindered at the low moisture content of dry commodities (Vajdi and Pereira 1973).

Usually, microwave treatments result in low reductions of the bacterial population, especially spores, in dried food. Emam et al. (1995) treated black pepper in a microwave oven and observed a significant reduction in mould and coliforms but not in *Bacillus* and *Clostridium*. Some studies have shown that very long treatments times (> 40 minutes) to destroy dry spores with a microwave treatment (Luck and Daniels 1981; Jeng et al. 1987). A microbial load reduction of only 2 log can be realistically achieved, which is insufficient for highly contaminated spices (Vajdi and Pereira 1973).

2.5.6 Heat treatments

Heat treatment is the most common method for the decontamination of powder and dried foods. The application of heat presents the advantage of also allowing the inactivation of enzymes responsible for food deterioration. In dry media the heat is transferred differently than in moist products, i.e., in the absence of surrounding water the heat will be transfer by air (dry heat) or by through the addition of steam (moist heat). The treatments based on the use of water vapor are simple and efficient methods of food decontamination and are largely use in industrial scale.

Different industrial methods have been developed to decontaminate dry food using either high-temperature short-time (HTST) or low-temperature long-time (LTLT). Usually, in order to minimize the undesirable changes in food quality the HTST method is favored. The different z -values between unwanted elements (bacterial cells, toxins, enzymes) and desirable ones (nutrients, flavors) need to be exploited so as to destroy pathogens while maintaining the vitamin content, for example (Beney et al. 2003). The application of heat treatment to solid foods presents some problems as the overheating of the product close to the heated surface and the low heat transfer rates to the interior part of the food.

Different forms of steam can be used for the decontamination of dry food: dry steam, saturated steam and superheated steam (Fine and Gervais 2007). Superheated steam is steam that has been given additional sensible heat to raise its temperature above the saturation point at a given pressure. Unlike saturated steam, a drop in temperature will not result in condensation of the steam as long as the temperature is still greater than the saturation temperature at the processing pressure. Cenkowski et al. (2007) processed wheat kernels with supersaturated steam at temperatures varying from 105°C to 175°C and observed changes in color, structure and moisture changes. Despite the high temperatures used, the spore inactivation was limited to around 1.5 log with a treatment at 160°C for 15 minutes.

A HTST treatment was used to decontaminate paprika powder using a gas stream at 160°C for a few seconds and resulted in the reduction of 2 to 3 log of the total bacterial count. The treatment was only effective when high moisture steam was used and color degradation and lipid peroxidation was observed (Almela et al. 2002).

A variation of the steam treatment is the vacuum-steam-vacuum procedure where after a short steam treatment of the product, an abrupt evacuation of the treatment chamber takes place, leading to an intensive re-evaporation of the superficial condensate layer. In this way, the

micro-organisms on the surface are removed from the product, hence this methods combines mechanical and thermal decontamination effects. Spore inoculated in black pepper treated with this method showed an inactivation up to 3 log, but the moisture content increased in all the conditions tested (Lilie et al. 2007).

In LTLT, temperatures below 100°C are applied for durations of hours up to days and generally microbial reduction rates of only 1–3 log are commonly achieved, so this method is not always recommended for highly contaminated products. Dry-heat LTLT (67 and 75°C for 15 days) was used to eliminate bacteria and to improve the gelling and surface properties of dried egg (Baron et al. 2003). Hammershoj et al (2006) obtained a reduction of 3 logs in the total population of dried egg treated at 130°C for 3 hours and they also observed that inactivation was higher when 20% RH was used instead of 2% RH.

2.6 Conclusion and Aim of the Thesis

The literature review aimed to give a general view of spore's properties such as its structure and its resistance to various stresses as well as different decontamination procedures used in the industry to treat dried foods.

The formation of highly resistant spores by stressed cells results in perfect vehicles for the spoilage of food and/or the infection of humans. Some strains, such as *Clostridium botulinum*, can often cause fatal diseases of the nervous system, some *Bacillus cereus* strains can cause serious forms of diarrhea and vomiting and *Bacillus anthracis* that can be used as a bioterrorism weapon.

Several factors are known to be involved in bacterial spore resistance, including a low water content of the protoplast, a low internal pH, a high concentration of DPA, saturation of spore DNA with α/β -type small acid soluble spore proteins, and the low mobility of protoplast contents.

Despite the advances in spore research there are still many unanswered questions about the resistance factors and also the mechanisms of inactivation to different stresses. It is known that moist heat kill spores by inactivating some essential protein however the identity of the protein is unknown as well as the exact moment of spore death. The inactivation of spores by high hydrostatic pressure has received much attention in the last decade and it is now known that spores are only killed when High Pressure is coupled with a mild heat treatment.

Although the mechanisms have been elucidated, the effect of food matrices still needs investigation. These two methods are normally used for the treatment of liquid or high moisture products.

However, the inactivation of dehydrated spores is a major concern and also a challenge for the food industry due to its increased resistance to different treatments compared to most spores. UV radiation and ionization inactivate spores by DNA damage, but the influence of different factors as water content, are not completely understood. Finally, inactivation of spores by dry heat appears to be due to accumulated DNA damage therefore SASP and DNA repair systems play a major role in spore resistance against dry heat. However, many aspects of spore resistance in dried form have not been thoroughly investigated, for example the role of water activity and water content.

There is a lack of studies relating water activity to the heat resistance of dried spores since most of the previous studies did not control this property, i.e., spores were dehydrated using different methods and then heat-treated without measuring or modulating their water content. Another problem often encountered is the gap between the fundamental research about spore inactivation and an actual application of the knowledge obtained. Moreover, there is currently an increased interest in the decontamination of dried foods, as herbs, spices, cereals, powder milk and cocoa after numerous cases of food poisoning including low moisture food products.

In this context, the aim of this thesis was to investigate the role of water content and distribution on the heat inactivation of dried *Bacillus* spores.

The thesis is divided in two parts: a fundamental study and a process development.

In the first part we have used Differential Scanning Calorimetry with an original approach: DSC aluminum pans worked as mini-reactors to perform a controlled heat treatment and simultaneously recover information about heat transitions and weight loss. We were mostly interested in the evaporation of water from the spores, so non-hermetic pans with different resistances were chosen in order to modulate the temperature of evaporation.

The second part of the work consisted in the development of new decontamination procedure using the results obtained from the DSC study. The effect of water activity, temperature and pressure on spore inactivation using this process was studied.

MATERIAL & METHODS

3. MATERIAL AND METHODS

3.1. Biological material

3.1.1 Spore Production

Two *Bacillus* strains were used to allow further comparison of the results obtained: *B. subtilis* ATCC 31324 (DSMZ 704) obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and *B. subtilis* 168 from the BGSC (*Bacillus* Genetic Stock Center, Department of Biochemistry, Ohio State University, USA). The mutant strain of *B. subtilis* without most part of SASP, $\alpha\beta^-$ spores, obtained from the BGSC was also used.

B. subtilis sporulation was induced in a complex medium in a reactor at 37°C, pH 8.0, with an air flow of 4 L/min and under agitation (450 rpm) (Nguyen Thi Minh et al. 2008) and the protocol of preparation is presented in Table 3. Sporulation was estimated by plating on BCP agar (Dextrose Tryptone agar, BIODIAGNOSTICS, Beauvais, France) before and after a heat treatment at 80°C for 10 min. When more than 95% of the population in the sample resisted this heat treatment, the spore suspension was harvested (3–5 days). The spore suspension was washed four times with sterile distilled water, spray-dried (Mini spray dryer B-290, Buchi, France) and stored in powder form in sterile recipients at 4°C until use. The final microbial concentration of the powder was approximately 10^{11} CFU/g. These spores were used in all the DSC and reactor study.

The mutant strain of *B. subtilis* without most part of SASP, $\alpha\beta^-$ spores, and *B. subtilis* 168 spores were produced in little quantity at 37°C on 2 × SG medium agar plates, harvested, cleaned and one part was subsequently freeze-dried (Nicholson and Setlow 1990). All spore preparations used were free of growing cells and germinated spores as observed by phase contrast microscopy. These two samples of freeze dried spores were only used in the test for mutation screening using nalidixic acid medium.

Table 3. Protocol of preparation of the complex sporulation medium

Solution	Component	Quantity (g.L⁻¹)	Sterilization method
1	KH ₂ PO ₄	0.5	Autoclave
	K ₂ HPO ₄	2.5	
2	NaCl	1	Autoclave
	CaCl ₂ .2H ₂ O	0.1	
	CuSO ₄ .5H ₂ O	0.5.10 ⁻³	
	KCl	1	
	ZnSO ₄	5.10 ⁻³	
	MnCl ₂ .4H ₂ O	5.10 ⁻³	
3	FeSO ₄ .7H ₂ O	0.5.10 ⁻³	Filtration
	MgSO ₄ .7H ₂ O	0.5	
4	Glucose	15	Autoclave
5	Yeast extract	32	Autoclave

3.1.2 Equilibration of spores at different water activities

Dried spores with water activities ranging from 0.13 to 0.50 were obtained by placing the spores (± 500 mg) inside 1 L airtight plastic boxes containing saturated salt solutions (solution occupied 1/10 of the box's volume) until equilibrium was reached (at least one week). The saturated salts used are showed in Table 4. The water activity of the saturated solutions and spore powders was determined in triplicate at 25°C using a Decagon-AQUALAB CX-2 osmometer (Pullman, USA).

Table 4. Saturated salt solutions and their corresponding a_w at 25°C.

Salt solutions	a_w
LiBr	0.06
LiCl	0.11
CH₃CO₂K	0.22
NaBr	0.58

The dried spores were also equilibrated at different water activities using glycerol solutions inside the airtight plastic boxes. The use of glycerol solutions allows the equilibration at any given a_w , not only fixed values as with the saturated salt solutions. However, the a_w of the solutions must be controlled periodically since it can change with water absorption or evaporation. This method was chosen for the equilibration of the spores used in the experimental design since spores at water activity of 0.10, 0.20, 0.30, 0.40, 0.50, 0.60 and 0.70 were needed. The solutions were prepared according to the Norrish Equation (Norrish 1966) and the masses are presented in Table 5. These solutions were also used for the treatment in liquid media at different water activities.

Table 5. Glycerol concentrations for the preparations of solutions at different a_w at 25°C.

a_w	Glycerol (g/100g solution)
0.10	96
0.20	92
0.30	88
0.40	84
0.50	77
0.60	72
0.70	64

3.1.3 Spore viability

Heat treated spore samples were serially diluted with physiological water (0.9% NaCl w/v) and 100µl of each dilution was inoculated in triplicate on BCP agar plates (Dextrose Tryptone Agar, Biokar Diagnostics, Beauvais, France). Colonies were counted after 24 hours at 37°C. Control samples contained spores that did not undergo any treatment. All experiments were performed at least in triplicate and the bars in the graphics represent the standard deviation. Spore inactivation was expressed using a logarithmic reduction factor $\log N_t/N_0$, where N_0 was the number of developing spores before treatment and N_t the number of developing spores after treatment.

The spore viability was also assessed in some cases after the treated spores were re-suspended in distilled water and heat treated at 80°C in a water bath during 20 minutes. This test was made to check if there was an increase in the heat sensitivity of spores treated in the

developed reactor described below. One viability experiment was made adding 6% NaCl to the BCP to check if the heat treatment increased spore salt sensitivity. The last test was the isolation of nalr mutants, as 10^6 to 10^7 spores either untreated or treated were spread on $2\times$ YT medium agar plates containing $20\mu\text{g/ml}$ nalidixic acid, incubated at 37°C for 48 h (Huesca-Espitia et al. 2002).

3.2 Differential Scanning Calorimetry

3.2.1 Principle

Differential Scanning Calorimetry (DSC) measures the temperatures and heat flows associated with transitions in materials as a function of time and temperature in a controlled atmosphere. These measurements provide quantitative and qualitative information about physical and chemical changes that involve endothermic or exothermic processes, or changes in heat capacity. During a change in temperature, DSC measures a heat quantity, which is radiated or absorbed excessively by the sample on the basis of a temperature difference between the sample and the reference material (Haines et al. 1998).

Based on the mechanism of operation, DSCs can be classified into two types: heat-flux DSCs and power-compensated DSCs. In a heat flux DSC (Figure 12), the sample material is enclosed in a pan and an empty reference pan are placed on a thermoelectric disk surrounded by a furnace (Intruments 2007).

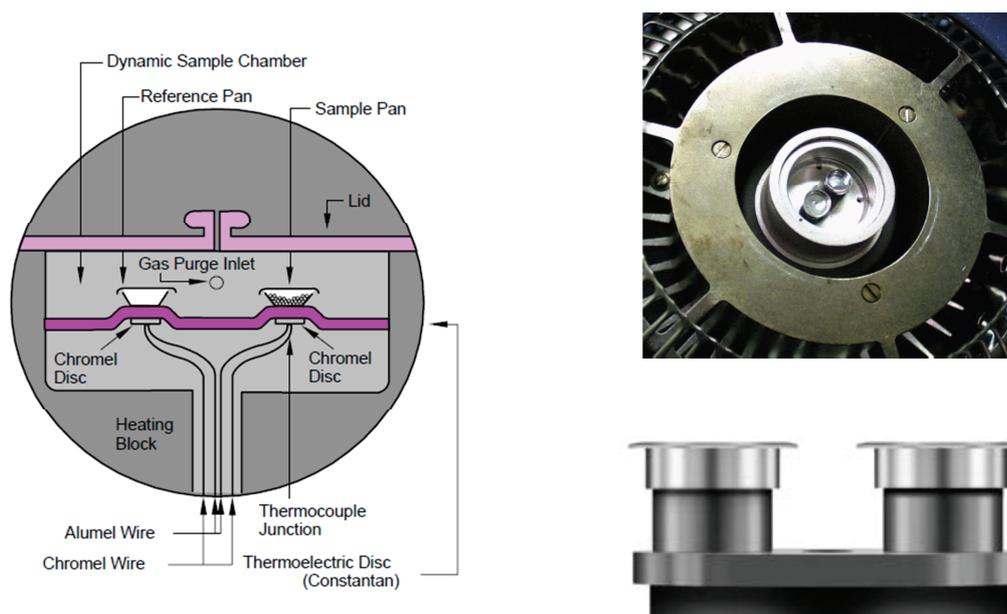


Figure 12. Q-20 DSC (TA Instruments, USA) chamber.

The furnace is heated at a linear heating rate, and the heat is transferred to the sample and reference pan through the thermoelectric disk. In a power-compensated DSC, the sample and reference pans are placed in separate furnaces heated by separate heaters. The sample and reference are maintained at the same temperature, and the difference in thermal power required to maintain them at the same temperature is measured and plotted as a function of temperature or time (Gill et al. 2010). In this study, a heat flux DSC was used. A functional DSC system has three major components: the instrument itself, which contains the system electronics; the cell, which monitors differential heat flow and temperature; and a cooling accessory.

3.2.2 Analysis

The thermal analyses were conducted in a Q-20 DSC (TA Instruments, USA) equipped with a Liquid Nitrogen Cooling System (LNCS). A thermogram with an empty and a reference pan was collected to measure the baseline. The instrument was calibrated for cell constant and temperature using Indium as the reference standard (melting temperature $T_m=156.6^\circ\text{C}$ and melting enthalpy 28.54 J/g). Scans were performed at temperatures ranging from 25° to 220°C at a heating rate of 10°C/min under nitrogen flow (50 ml/ min).

The idea of the experiment is that the spores act as closed pans with a certain resistance to pressure and once there is a pressure buildup inside the spore until the water contained in the core can be vaporized.

Three kinds of experiments were carried out using the calorimeter. The first to assess the pan's characteristics and the others to obtain the thermograms, weight loss and inactivation of spores as presented in Figure 13.

1. The temperature of water evaporation was assessed using a mass of 1 – 3 mg of pure distilled water. Two aluminum pans (TA Instruments, USA) with different pressure resistances were used: Tzero™ with non-hermetic lid (901683.901/901671.901) and Tzero™ with hermetic lid (901683.901/901684.901) and which will be referred to hereinafter as Pan A and Pan B, respectively.
2. Spores with different water activities were heated in the two pans (A and B) to obtain their thermograms. The pans were then rapidly cooled to the initial temperature and immediately rescanned to determine the reversibility of the transitions. Samples were reweighed after DSC measurements to check for weight loss during heating.

3. The third experiment consisted in evaluating spore inactivation after a heat treatment in the calorimeter. Spore samples (about 5 mg) were weighed in the pans (A and B) and sealed using the Tzero DSC Sample Encapsulation Press (TA Instruments, USA).

The weighing of dehydrated spores requires great precaution to avoid re-absorption of water after heating. To avoid this problem the DSC chamber had a constant nitrogen flow to remove all water vapor released during heating. In any case, if some water re-absorption took place it would only have induced an underestimation of the water loss.

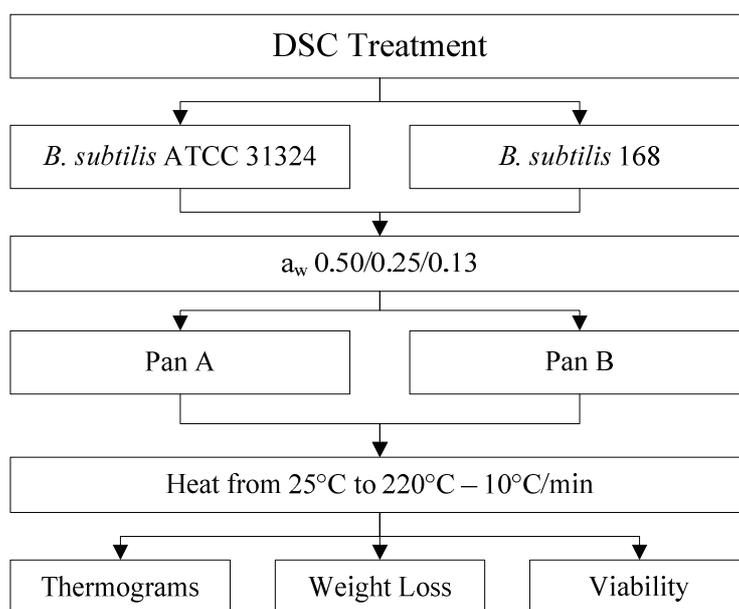


Figure 13. Overview of the DSC experiments.

3.3 Fourier Transform Infrared Spectroscopy

3.3.1 Principle

Infrared (IR) spectroscopy is a reliable method to characterize, identify and quantify many substances, allowing the acquisition of spectra from a very wide range of solids, liquids and gases. The infrared region ($10\text{-}14000\text{ cm}^{-1}$) of the electromagnetic spectrum is divided into three regions: the near-, mid-, and far-IR. The mid-IR ($400\text{-}4000\text{ cm}^{-1}$) is the most commonly used region for analysis as all molecules possess characteristic absorbance frequencies and primary molecular vibrations in this range (Alvarez-Ordóñez et al. 2011). Mid-infrared spectroscopy methods are based on studying the interaction of infrared radiation with samples. As IR radiation is passed through a sample, specific wavelengths are absorbed

causing the chemical bonds in the material to undergo vibrations such as stretching, contracting, and bending (Davis and Mauer 2010).

The technique of Attenuated Total Reflectance (ATR) simplifies the sample preparation process and improves spectral reproducibility. It has been a revolution for solid and liquid sample analyses in recent years.

An infrared beam is directed onto an optically dense crystal with a high refractive index at a certain angle and then extends beyond the surface of the crystal and protrudes only a few microns ($0.5\ \mu\text{m} - 5\ \mu\text{m}$) into the sample held in contact with the crystal. In regions of the infrared spectrum where the sample absorbs energy, the evanescent wave will be attenuated or altered. Since the sample has lower refractive index than the crystal, the infrared beam exhibits total reflection then exits the opposite end of the crystal and is passed to the detector in the IR spectrometer. The detector measures the changes in the totally internally reflected infrared beam and generates an infrared spectrum (Figure 14).

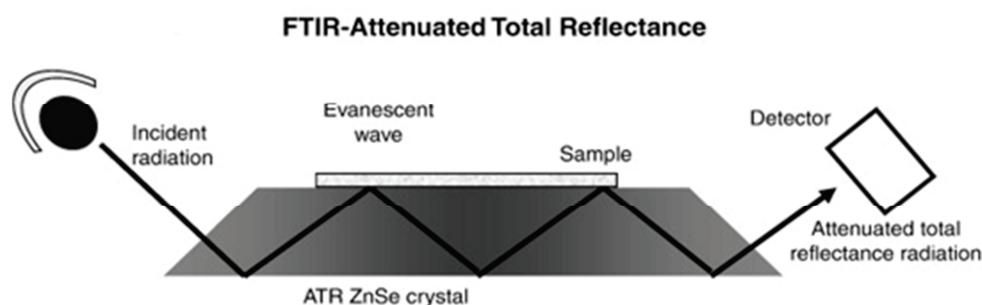


Figure 14. Schematic representation of FTIR-Attenuated Total Reflectance (Alvarez-Ordóñez et al. 2011).

3.3.2 Analysis

FTIR was used to assess structural and biochemical changes in *B. subtilis* 168 spores caused by the heat treatment performed in the DSC. IR spectra were recorded between 4000 and $800\ \text{cm}^{-1}$ at a resolution of $8\ \text{cm}^{-1}$ on a Bruker Vector 22 FT-IR spectrometer (Bruker Optics, Germany) using an Attenuated Total Reflectance (ATR) unit (De Lamo-Castellví and Rodríguez-Saona 2011).

Dried samples were diluted in distilled water (10^8 CFU/ml) and washed four times. A $10\ \mu\text{l}$ aliquot of the spore solution was placed on the ATR cell and dried under nitrogen flow at ambient temperature to form a thin film. Ten spectra were collected for each spore sample. The resulting spectra were checked for major water peaks, and if it appeared that some

solvent remained in the spectrum the film was further dried. The raw spectra were transformed into their second derivatives for analysis to remove baseline shifts, improve peak resolution and reduce variability between replicates (Kansiz et al. 1999). A 17-point Savitsky-Golay smooth was used to obtain the derivative trace. OPUS 3.0 software (Bruker Optics, Germany) was used to control the spectrometer and to acquire and manipulate spectra.

3.4 Treatment at high temperatures and low pressures

The procedure for the inactivation of spores using low pressures and high temperature solves one of the problems found when decontaminating powders: the simultaneous drying of the sample. It allows the sample to be heated without loss of water, maintaining the initial water activity which is not possible with other methods used for dried products.

The reactor developed consisted of a small hermetic stainless steel chamber (1.7 ml of volume) that can be pressurized with gas (Figure 15). Here nitrogen was used to pressurize and once the pressure was set, the reactor's valve was closed maintaining the applied pressure as presented in Figure 16.

Dried spores equilibrated at different water activities were placed in squares of aluminum foil that were folded and put inside the reactor. The reactor was then pressurized between 2 and 7 bar, closed and heated in a oil bath at different temperatures (but always over 100°C). When no pressure was added the valve was left open to allow water evaporation or closed to retain evaporated water. After the treatment, the reactors were cooled in an ice bath and then the valve was open to release the pressure.

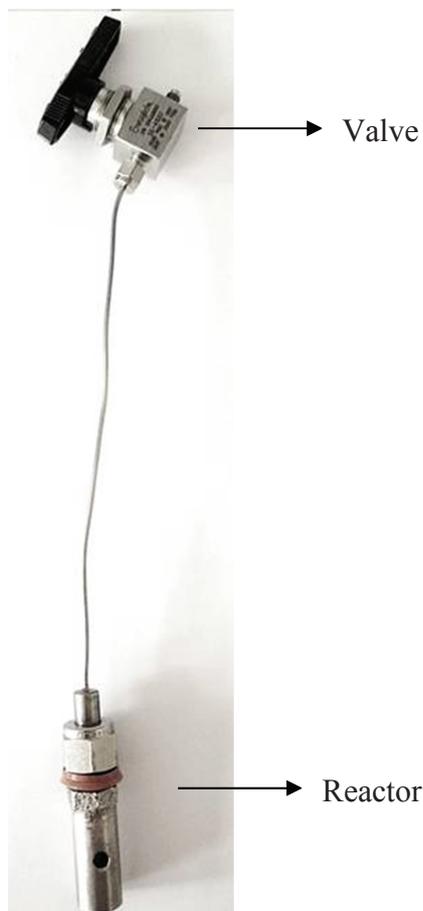


Figure 15. Photo of the reactor composed by a small hermetic stainless steel chamber and a valve to retain pressure.

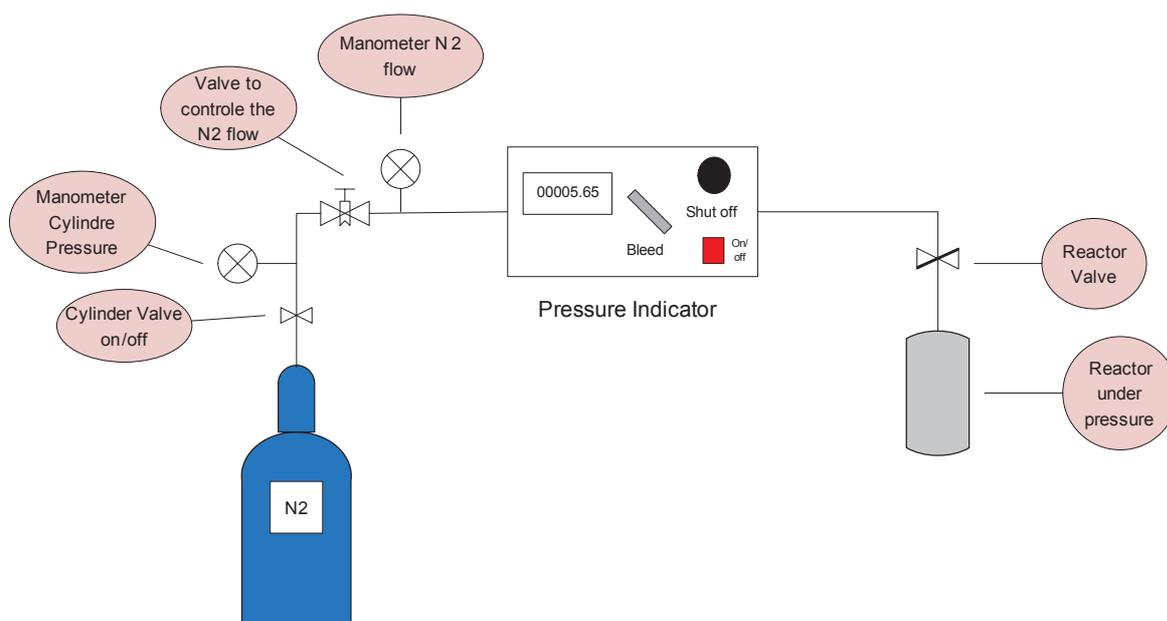


Figure 16. Diagram for the treatment under low pressures and high temperature.

The pressure reported is always the absolute pressure meaning that the nitrogen pressure is added to the atmospheric pressure, giving the total pressure of the system. The different conditions of applied pressure and the coding used in the Results sections are presented in Table 6.

Table 6. Pressure conditions used in the experiments.

Sample name	Treatment condition
Patm open	No nitrogen added, reactor at Patm with the valve open.
Patm closed	No nitrogen added, reactor at Patm with the valve closed.
2 bar	1 bar of nitrogen added
3 bar	2 bar of nitrogen added
4 bar	3 bar of nitrogen added
5 bar	4 bar of nitrogen added
6 bar	5 bar of nitrogen added
7 bar	6 bar of nitrogen added

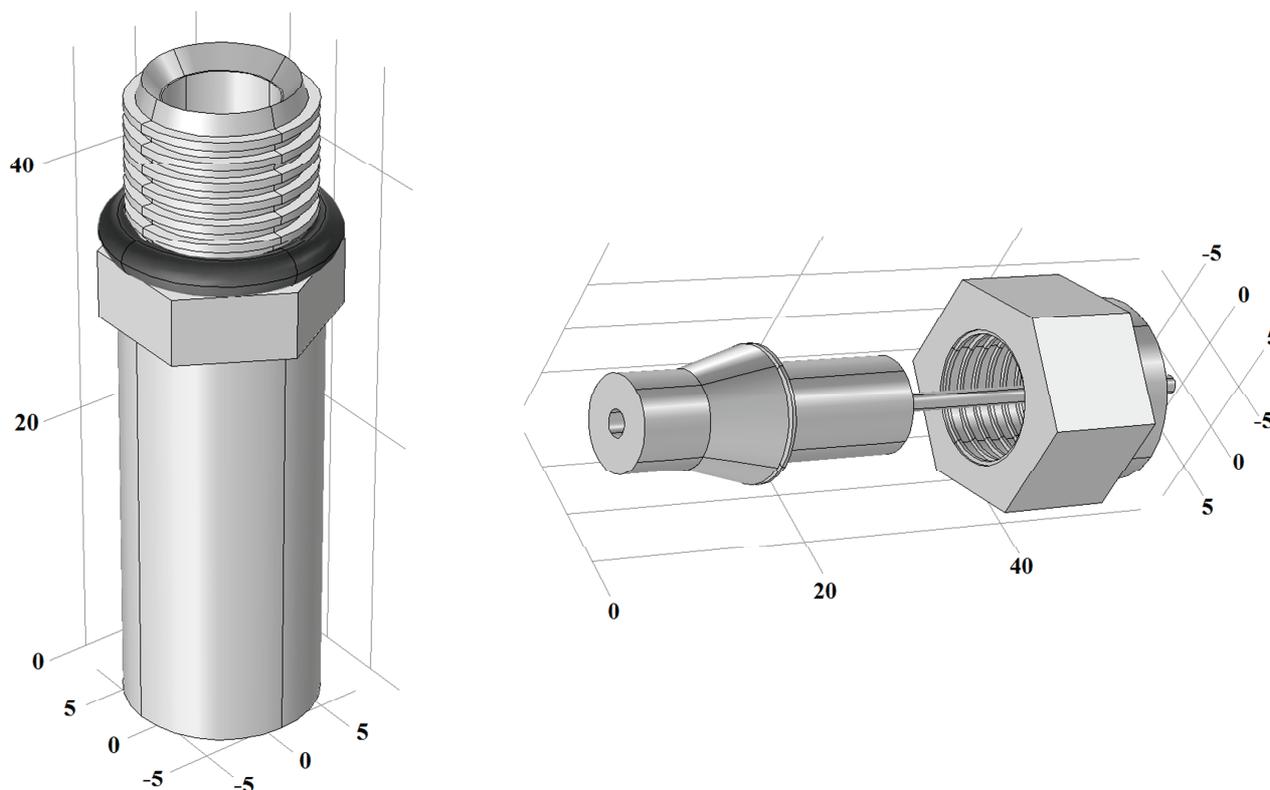


Figure 17. Reactor dimensions (in millimeters).

Figure 18 shows the simulation of the temperature increase in different parts of the reactor. Figure 19 presents the temperature profile with different media (glycerol, water and air). The temperature profiles were obtained by a numerical simulation using the software Comsol 4.2.

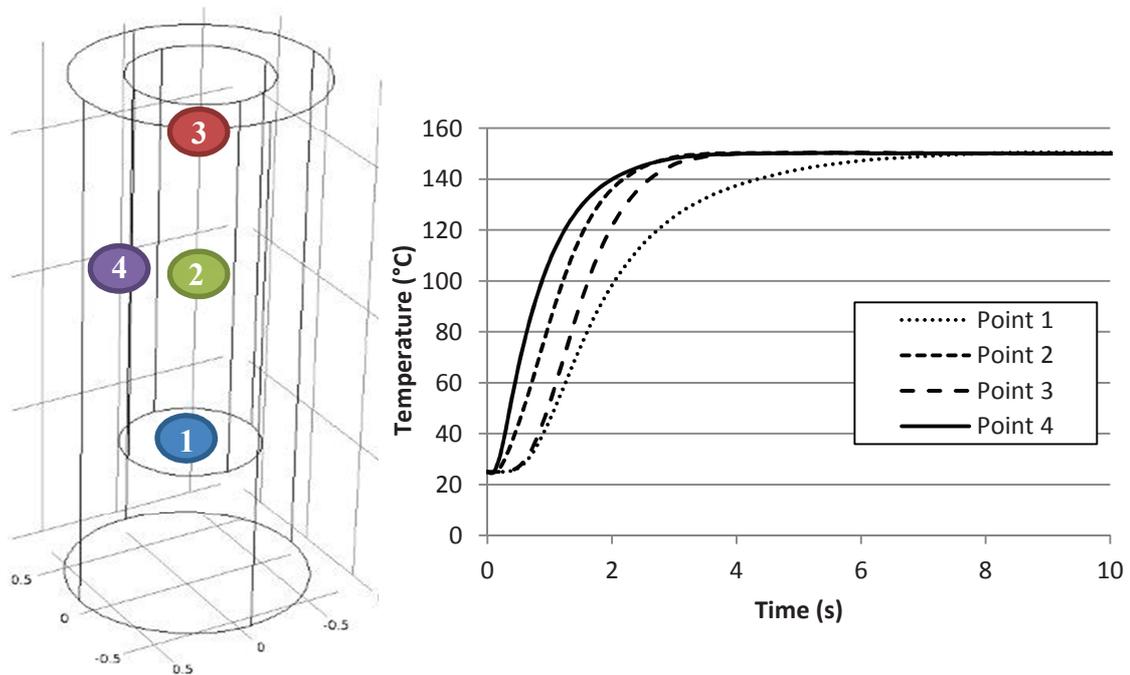


Figure 18. Pressure vessel and temperature profile in different points of the vessel (only transient heating time is shown).

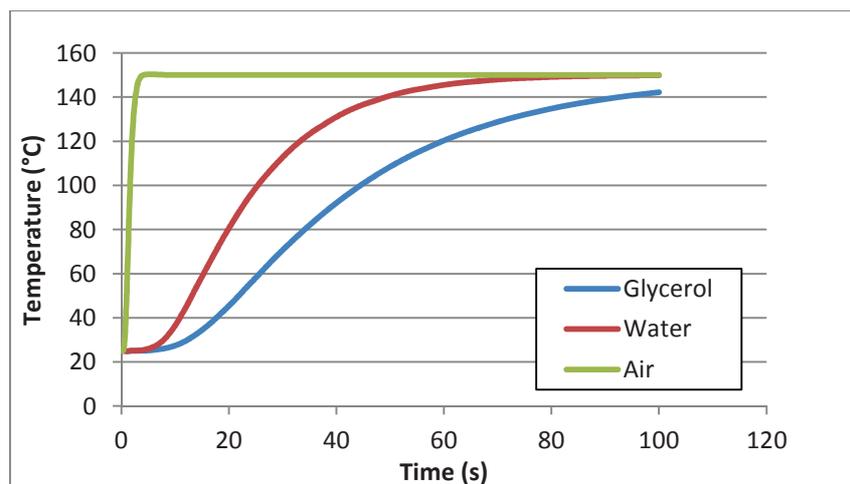


Figure 19. Temperature increase inside the reactor with different media.

3.4.1 Experimental design

Doehlert experimental design (Doehlert 1970) was used to study the effect of treatment variables in the inactivation of dried *Bacillus* spores. Temperature, pressure and water activity were chosen as independent input variables, although they are not independent since pressure will increase inside the reactor upon heating. This design was only intended to give a global view of the system and any statistical conclusions must be carefully examined. Spore inactivation was used as the dependent output variable as shown in Table 7. For statistical calculations the variables X_i were coded as x_i according to Equation 1.

$$x_i = \left(\frac{X_i - X_{oi}}{\Delta X_i} \right) \alpha_i \quad \text{Equation 1}$$

where x_i is the coded value of the i^{th} factor, X_i the natural value, X_{oi} the value at the center point, ΔX_i the step change value, and α_i is the maximum value of the coded factor (i.e. 1.0, 0.866 and 0.816 for five levels, seven levels and three levels, respectively).

Table 7. Doehlert matrix for three variables.

n	A	B	C	T	a_w	P
1	0	0	0	140	0.40	3.5
2	1	0	0	160	0.40	3.5
3	0.5	0.866	0	150	0.70	3.5
4	0.5	0.289	0.816	150	0.50	6
5	-1	0	0	120	0.40	3.5
6	-0.5	-0.866	0	130	0.10	3.5
7	-0.5	-0.289	-0.816	130	0.30	1
8	0.5	-0.866	0	150	0.10	3.5
9	0.5	-0.289	-0.816	150	0.30	1
10	-0.5	0.866	0	130	0.70	3.5
11	0	0.577	-0.816	140	0.60	1
12	-0.5	0.289	0.816	130	0.50	6
13	0	-0.577	0.816	140	0.20	6

The resulting model is described as follows:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{23}x_2x_3 + b_{13}x_1x_3$$

where Y is predicted response, X₁, X₂, X₃ are independent variables, B₀ is offset term, B₁, B₂, B₃ are linear effects, B₁₁, B₂₂, B₃₃ are squared effects and B₁₂, B₂₃, B₁₃ are interaction terms.

RESULTS & DISCUSSION

4. RESULTS AND DISCUSSION

This section is divided in two parts: the first concerns the results obtained with the DSC study followed by a discussion and the second presents the results from the development of a system to inactivate spores using the results found in the DSC study.

4.1 Results and discussion regarding the DSC study

The role of water, its distribution and its implication in the resistance of dried spores was investigated. DSC was used as a tool to perform a heat treatment of linearly increasing temperature on spores of *Bacillus subtilis* equilibrated at different water activity levels. At the same time, data regarding heat-related transitions occurring in the spores and spore viability were recorded. The spores were heat treated in pans with different resistances to pressure, thus modulating the temperature of water vaporization. Moreover the changes in spore structure were evaluated using FTIR.

4.1.1 Physical parameters

4.1.1.1 Determination of pan resistance with pure water

The two pans used presented different behavior regarding the water vaporization temperature as seen in Figure 20.

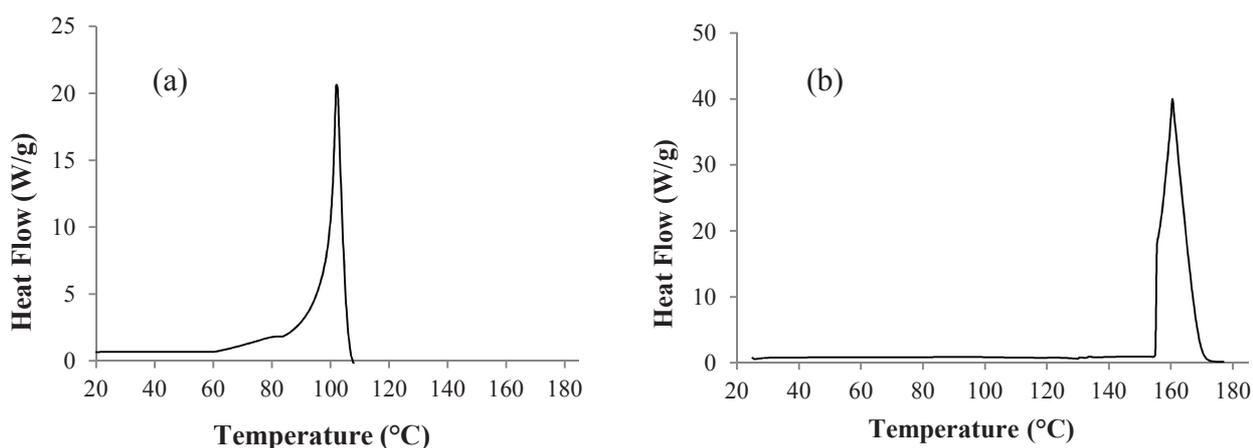


Figure 20. Thermograms of pure water in (a) Pan A and (b) Pan B. Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis).

In Pan A the peak corresponding to water vaporization was broad, ranging from 60 to 110°C, whereas in Pan B the peak was narrow, ranging from 155 to 170°C. This occurs when the vapor pressure of the sample exceeds the total pressure exerted on the sample by its surroundings. For both pans the peaks corresponded to the moment that the pan could no longer withstand the internal pressure buildup caused by water vaporization.

The pressure resistance of the pans was then determined from the vaporization temperature of water in each pan (Figure 21). In Pan B there was a pressure increase of 5 to 6 bars causing water to vaporize at higher temperatures, whereas in Pan A vaporization occurred at atmospheric pressure.

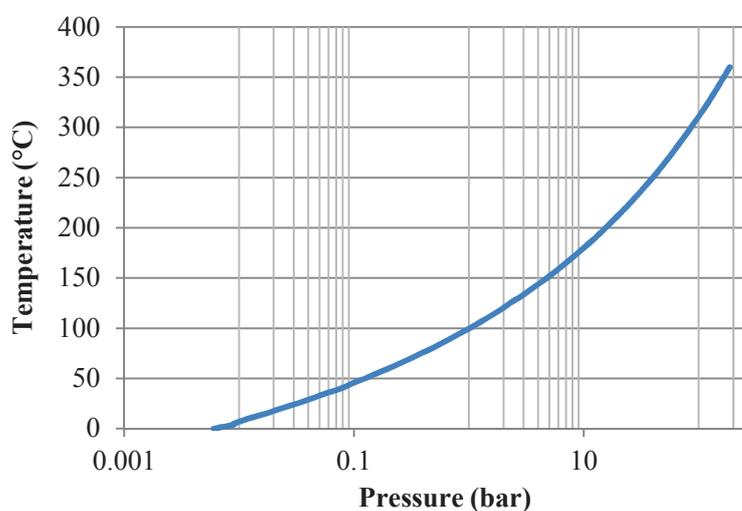


Figure 21. Evolution of water vaporization temperature with pressure. Adapted from Cengel (2011)

The determination of the water boiling point and its enthalpy of vaporization are directly related to the pressure resistance of the pan, as can be seen in Table 8. In Pan A the enthalpy was much lower than the expected value for water at 100°C, which is 2260 J/g. This is a common problem when working with open or non-hermetic pans (Perrenot et al. 1992; Butrow and Seyler 2003) and can be explained by the beginning of water evaporation at relatively low temperatures due to a difference in the vapor pressure between the pan and its surroundings. This evaporation is also amplified by the flow of purge gas used in the DSC measurements causing a small decrease in the specimen before the actual evaporation temperature is reached and consequently leading to a broadening of the peak.

Table 8. Temperature of evaporation of water in the pans studied

Pan	Evaporation temperature range	Pressure	Enthalpic Energy
Pan A	60-110 °C	1 bar	558±48 J/g
Pan B	155-170 °C	5-6 bars	1848±47 J/g

Contrary to the result in Pan A, in Pan B the liquid water and most of the vapor formed remained in the pan (self-generated atmosphere) during heating. This increase in temperature involved an increase in the water vapor pressure in the sealed pan as well as an increase in the water boiling temperature and thus the water remained mainly liquid in the pan above 100°C. Then, as the seal broke the pressure release resulted in an immediate and total vaporization of the water as it suddenly left the pan, thus producing a very narrow DSC peak.

4.1.1.2 Spore Thermograms

Dried spores equilibrated at different water activities (0.13; 0.25; 0.50) were heated up to 220°C in the two pans (A and B) to observe the thermal transitions, then cooled to 25°C and reheated to 220°C to check the reversibility of these transitions. The two pans presented distinctive thermograms (Figures 22 and 23).

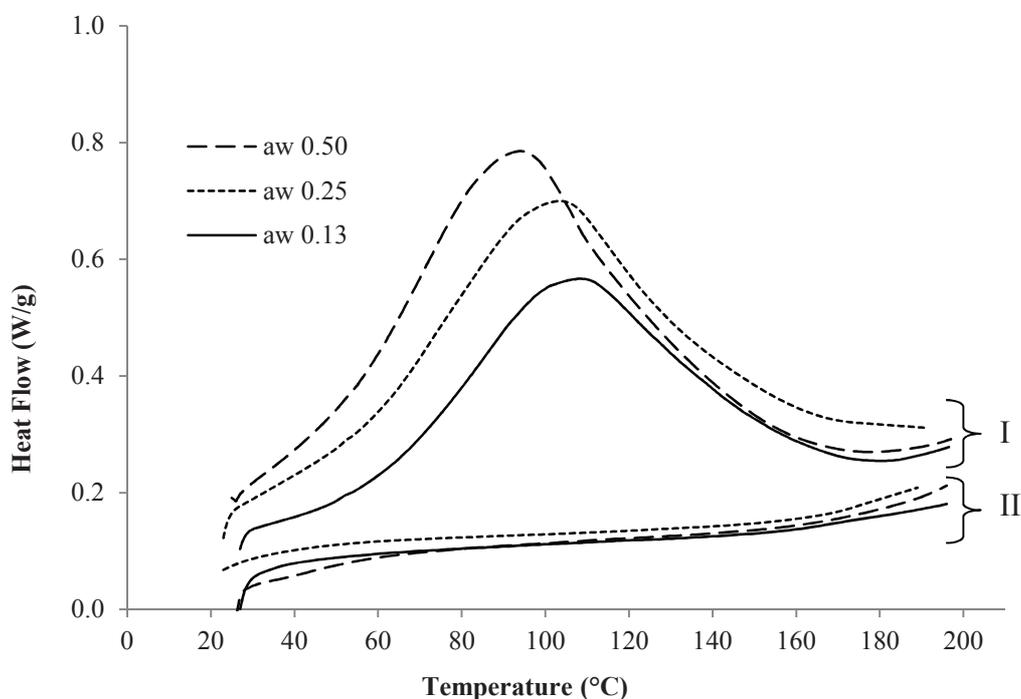


Figure 22. Thermograms of *Bacillus subtilis* ATCC 31324 spores after equilibrium at different water activities in Pan A. I-First heating. II-Second heating. Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis).

The thermograms in Pan A (Figures 22 and 23) showed only one broad endothermic peak, which ranged from 60°C to 160°C and was centered around 100°C. This transition was assumed to be related to the presence of water in the spores since the transition temperature was the same as for pure water and their enthalpies were linearly correlated to the amount of water initially present in the spore sample, as shown in Table 9. No transition was detected in the second heating of the spores in Pan A (See Figure 22) indicating that the reactions were irreversible, which is compatible with water evaporation. In the same way, the broadness of the peak was caused by the continuous evaporation of water at low temperatures as seen for pure water in Fig. 3.1. Maeda et al. (1974) reported the same single broad peak for lyophilized spores of *B. megaterium* with water content around 15% and the peak was also attributed to water evaporation from spores.

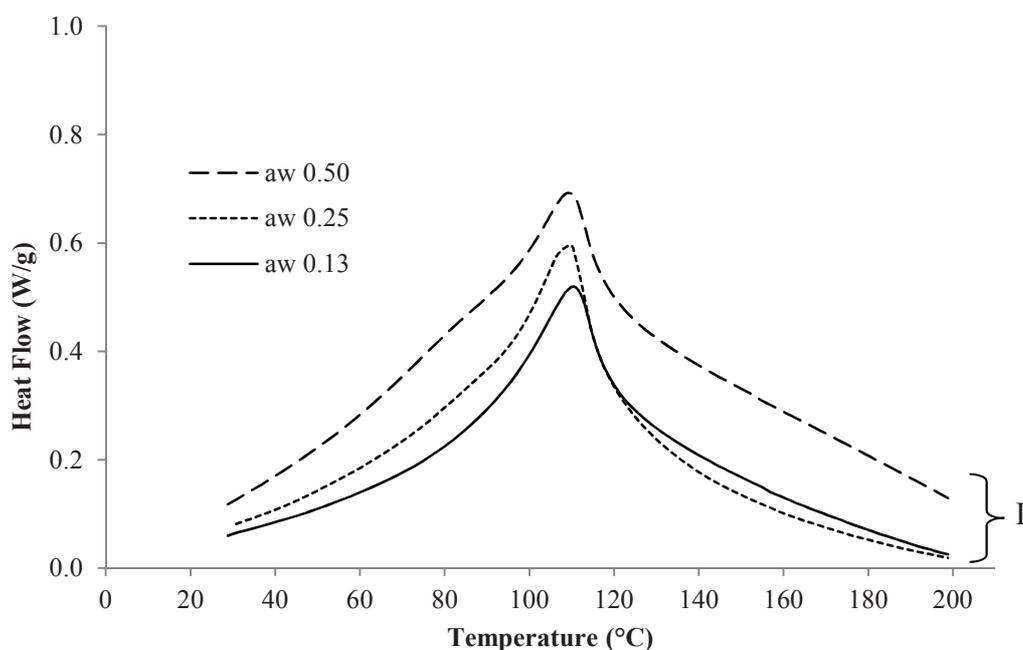


Figure 23. Thermograms of *Bacillus subtilis* 168 spores after equilibrium at different water activities in Pan A. I-First heating. Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis).

By comparing Figures 22 and 23, the same transition was observed for both strains but *B. subtilis* 168 had a higher associated enthalpy than *B. subtilis* 31324 (Table 9). The thermograms also differed in their shape; strain 168 had a sharper peak while strain ATCC 31324 had a rounder peak. The decrease in water activity caused a slight increase in the maximum temperature of the peak for both strains. This difference could be caused by the

small amount of water present in the outermost layers of the spores which did not create enough pressure to break the pan seal at the same temperature.

Table 9. Enthalpic energy of the thermal event observed between 60-160°C in Pan A and between 170-220°C in Pan B.

a_w	Enthalpic Energy (J/g)	
	Pan A <i>B. subtilis</i> ATCC 31324	<i>B. subtilis</i> 168
0.13	121±12	195±11
0.25	131±14	-
0.50	184±5	302±40
a_w	Enthalpic Energy (J/g)	
	Pan B <i>B. subtilis</i> ATCC 31324	<i>B. subtilis</i> 168
0.13	70±9	108±9
0.25	96±10	166±19
0.50	113±5	205±17

In Pan B (Figures 24 and 25) the first thermal event only took place above 150°C (same temperature as vaporization of pure water in pan B, shown in Figure 20b), and the peaks were more defined due to the higher pressure resistance of the pan, consequently not overlapping other minor peaks. The initial scan profile in this pan showed two major events, one at around 150°C and the other at around 200°C. The peak observed at 150°C corresponded to the pan seal breaking as a pressure of 5 bar was reached inside the pan and this was conform with the thermogram found for pure water. Moreover, the enthalpy values related to these transitions were clearly related to the water activity of the sample, as shown in Table 9.

The thermograms of the two strains presented the same transitions in both pans, only differing in the enthalpy associated with the transitions. The difference was particularly marked in Pan B where the peak at 150°C on the *B.subtilis* 168 thermogram had an associated enthalpy inferior to strain 31324 and on the contrary, the peak at 200°C had an enthalpy superior to the latter. These results suggest that water is not equally distributed within the spores in the two strains.

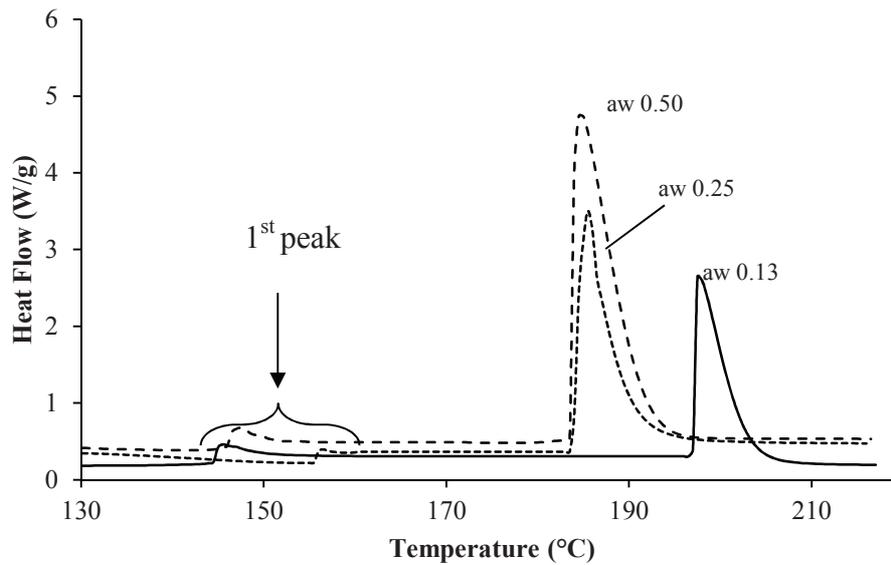


Figure 24. Thermograms of *Bacillus subtilis* ATCC 31324 spores equilibrated at different water activities in Pan B.

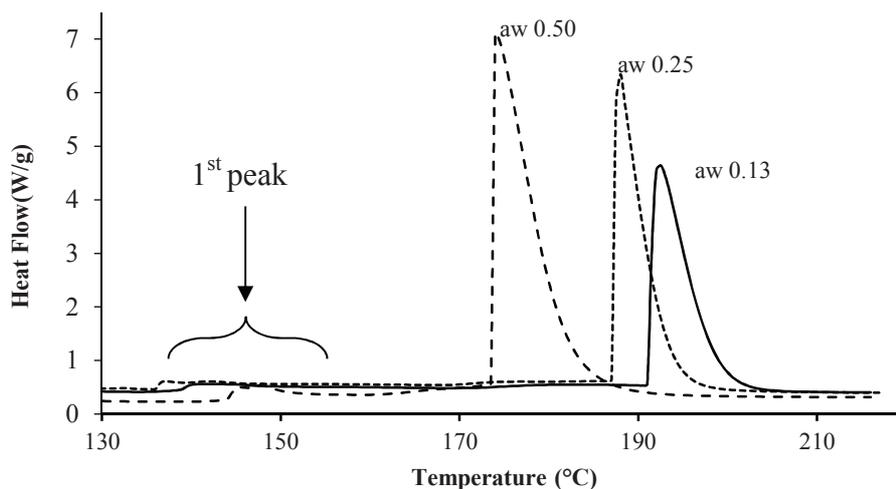


Figure 25. Thermograms of *Bacillus subtilis* 168 spores equilibrated at different water activities in Pan B.

The mechanical characteristics of the pan were crucial in obtaining these results. Considering that the interest was to study water vaporization, it was essential to use a pan with limited pressure resistance that allowed vaporization. Other studies did not report the same results probably because they used completely hermetic pans and stopped the treatment at lower temperatures (Maeda et al. 1974; Leuschner and Lillford 2003). In addition, dried spores with precise water activities were used in this study, differing from studies that used “moist” or “dry” spores.

4.1.1.3 Weight loss after heat treatment

The weight loss during heating of two *Bacillus* strains (ATCC 31324 and 168) at different water activities (0.13; 0.25; 0.50) was evaluated in Pans A and B.

In Pan A, a gradual weight loss was observed (Figure 26) and was assigned to water vaporization, confirming the drying of the sample during the treatment. When the temperature reached 110°C, 5.9 to 9.4% (w/w) of the water had already evaporated and at 200°C around 10 to 16.1% (w/w) of the total weight had been lost, depending on the initial water activity and the strain. Above 220°C there was no further weight loss (results not shown).

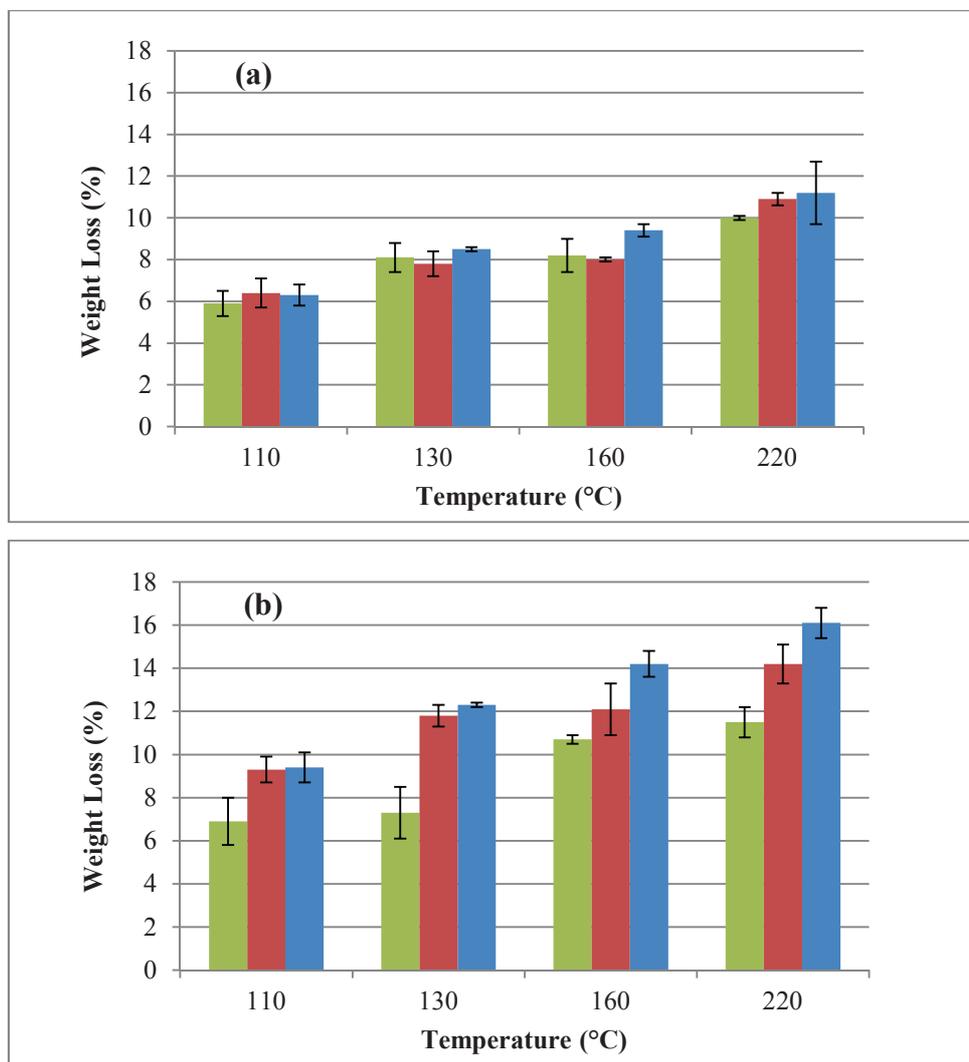


Figure 26. Weight loss after DSC heat treatment in Pan A (a) *B. subtilis* ATCC 31324 and (b) *B. subtilis* 168. (■) a_w 0.13, (■) a_w 0.25, (■) a_w 0.50. Weight loss expressed in g of water/100 g dry matter.

As expected from the thermal transitions, there was no weight loss in Pan B (Figure 27) before the peak at around 150°C, which corresponded to a vapor pressure of 5 bar. After this peak, the weight loss varied from 0.6 to 2.5 % (w/w) depending on the initial water activity and the strain.

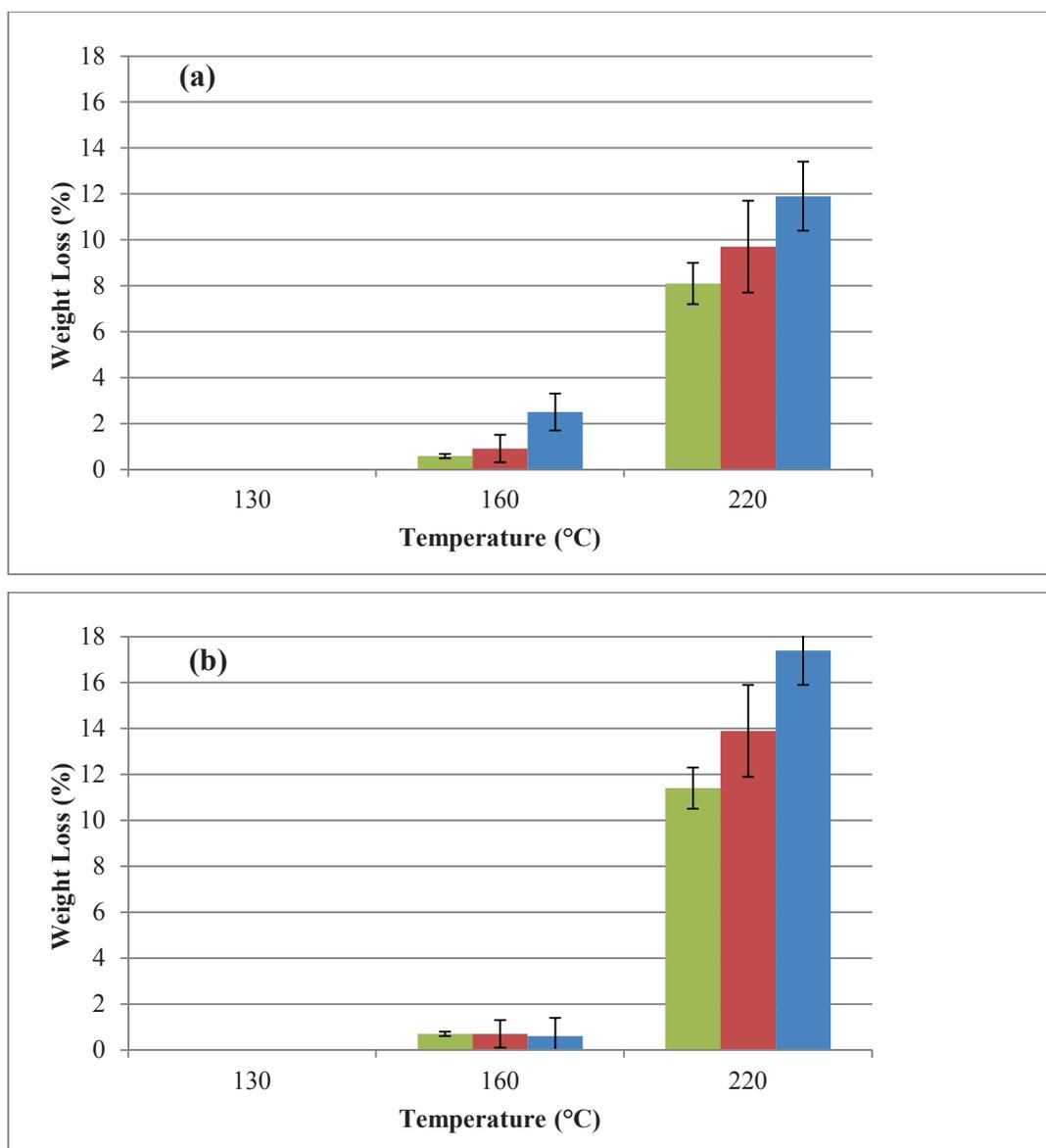


Figure 27. Weight loss after DSC heat treatment in Pan B (a) *B. subtilis* ATCC 31324 and (b) *B. subtilis* 168. (■) a_w 0.13, (■) a_w 0.25, (■) a_w 0.50. Weight loss expressed in g of water/100 g dry matter.

Surprisingly, the greatest weight loss was observed after the second peak, at 200°C, ranging from 8.1 to 17.4% (w/w) of the initial weight depending on the initial water activity and the strain. Considering that the weight loss at 200°C was related to water vaporization, the vapor

pressure thermodynamically associated to this temperature would be around 16 bar suggesting that there is a fraction of water in the spore that needs to surpass this pressure in order to vaporize.

The total weight loss for strain 168 was superior to that of strain ATCC 31324 in both pans and was also distributed differently. The weight loss for strain 168 was lower than for ATCC 31324 after the first peak (0.3-0.5% w/w) and higher after the second peak (12-14% w/w). Hence, this study also showed that it is possible to use this calorimetric technique to study the distribution of water in bacterial spores. These findings are in agreement with the results obtained from the thermograms and peak-related enthalpies and support the following hypotheses: 1) The heat transitions and weight loss are due to water vaporization; 2) There is a drying process in Pan A; 3) Water vaporization occurs in two stages in Pan B (i) the peak at around 150°C corresponds to a fraction of water from the outer layers of the spore that is easily vaporizable and (ii) the peak at 200°C corresponds to water from inside the spore core.

- The temperature of water evaporation and the pressure resistance of the two pans used were determined: in Pan A water evaporates at 100°C and in Pan B at 150°C, consequently, their pressure resistance is 1 and 5 bar, respectively.
- The thermograms of the two strains equilibrated at different water activities, *B.subtilis* ATCC 31324 and *B.subtilis* 168, were obtained for both pans. In Pan A there was a broad peak centered on 100°C whereas in Pan B there were two peaks, the first around 150°C and the second around 200°C. The same transitions were observed in both strains and the enthalpy associated to the peaks was proportional to the initial water activity of the samples. The endothermic transitions observed were irreversible.
- The weight loss during treatment was evaluated; in Pan A there was a constant weight loss however in Pan B weight loss occurred only after the peak at 150°C and 200°C.

4.1.2. Biological Parameters

4.1.2.1 Viability loss after DSC heat treatment

Spore viability was evaluated after heat treatment in the calorimeter. The final temperatures studied were 110, 130, 160 and 220°C (depending on the pan), which corresponded to a treatment duration of 9, 11, 14 and 19 minutes respectively, with a heating rate of 10°C/min and a starting temperature of 25°C.

In Pan A, the *B. subtilis* ATCC 31324 spores were not inactivated below 160°C, regardless of the initial water activity (Figure 28). The water activity only influenced the resistance above 160°C where a remarkable difference in the inactivation between the different water activities was observed. For spores equilibrated at a_w 0.50 a 5 log reduction was observed at 220°C, whereas for spores at a_w 0.13 and 0.25 only a 2 log reduction was observed. As shown in Figure 2, a drying process occurred in Pan A. Water started to evaporate from the spore at around 60°C so the spores had already been further dried when higher temperatures were reached.

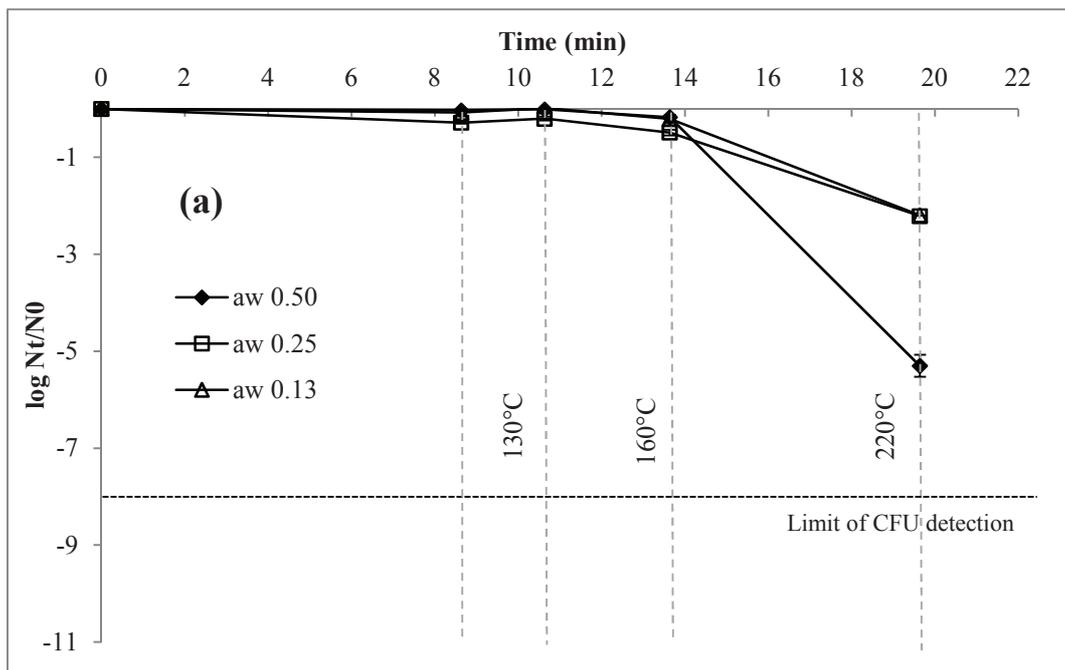


Figure 28. Inactivation of *Bacillus subtilis* ATCC31324 spores after heat treatment using DSC in Pan A.

The *bacillus* spores from strain 168 were less resistant to heat treatment than strain 31324 (Figure 29) in Pan A. For spores at a_w 0.50 a 0.5 log reduction was observed at 130°C and a 1

log reduction at 160°C, while for spores at lower water activities, 0.13 and 0.25, insignificant or no inactivation was observed below 160°C. However, at 220°C no difference in inactivation was observed for the spores according to the water activities.

The inactivation of spores was higher in Pan B, as shown in Figure 30 and 31. The spores from both strains equilibrated at a_w 0.5 were completely inactivated at 160°C, whereas spores at a_w 0.13 and 0.25 presented an inactivation of 5 and 7 log respectively at the same final temperature.

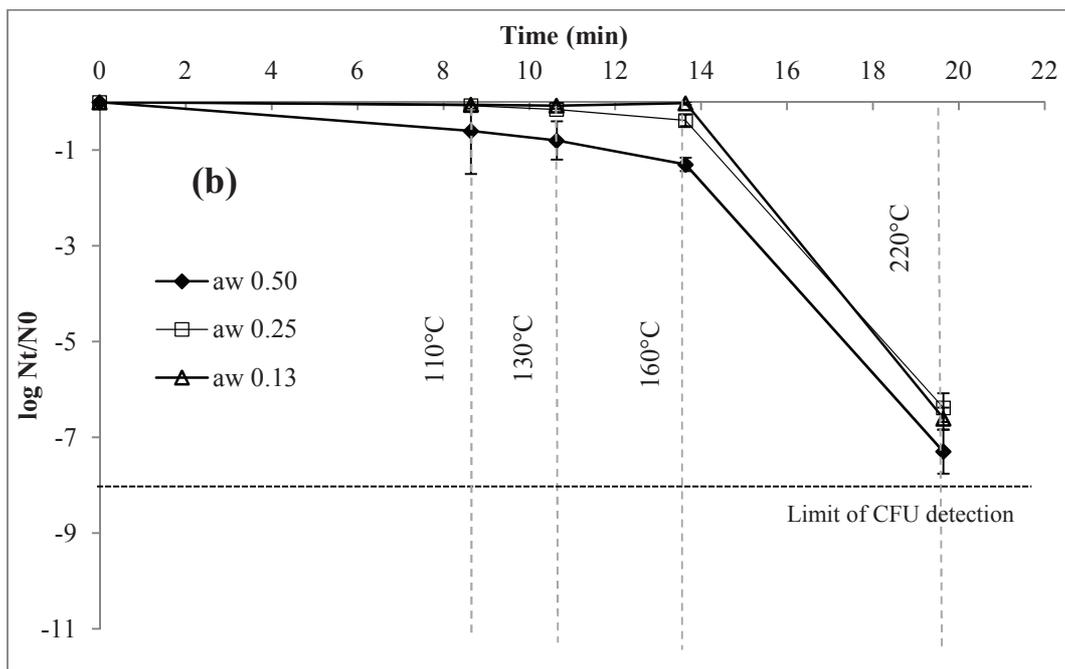


Figure 29. Inactivation of *Bacillus subtilis* 168 spores after heat treatment using DSC in Pan A.

The effect of water activity on the inactivation is very clear in Pan B, showing that under these conditions (pressurized atmosphere) the amount of water in the spore affects its resistance from the beginning of heat treatment and that the destruction is directly related to higher water activities. However, the difference between the strains was not evident in Pan B; both strains showed the same order of inactivation.

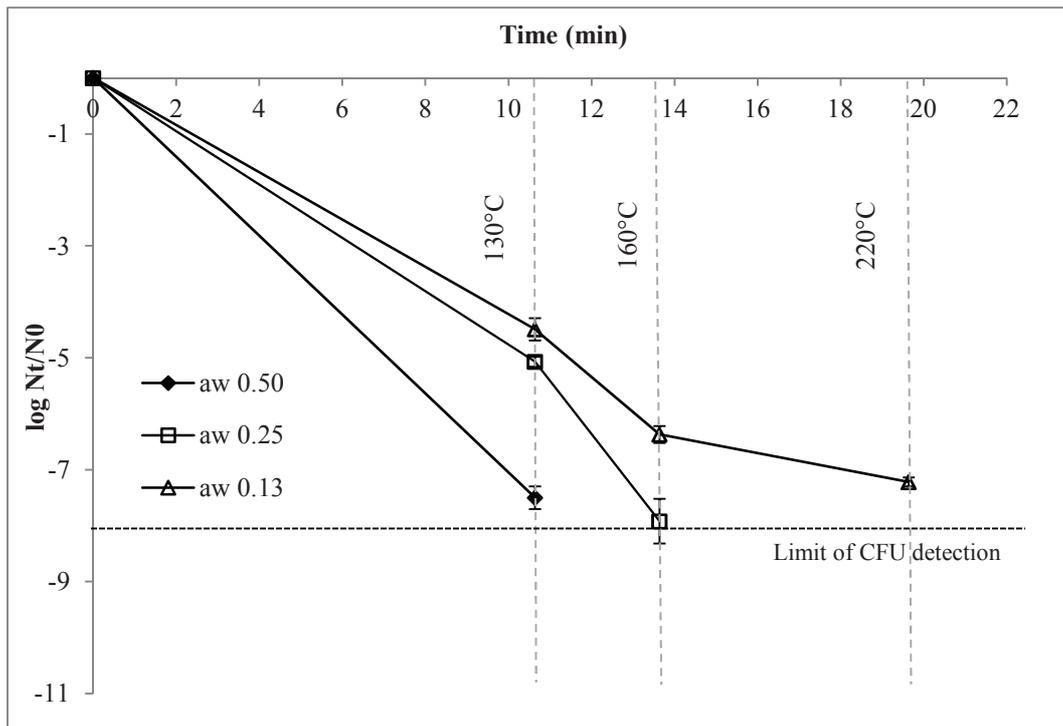


Figure 30. Inactivation of spores after heat treatment using DSC in Pan B for *Bacillus subtilis* ATCC31324.

In Pan B water was just vaporized above 150°C, so the spores retained their original water content during most of the treatment and were more sensitive to heat inactivation. Indeed, it is assumed, as proposed in the previous paragraph, that the water left the spore at two different moments, part at around 150°C and another part at around 200°C.

The results presented in Figures 28 to 31 show that it is possible to maximize the inactivation of dried spores if they are heated without their internal water evaporating. Inactivation with an equivalent heat treatment was found to be superior in Pan B than in Pan A. For instance, spores that had lost almost all water in Pan A (at 220°C) were still able to germinate when put in favorable conditions.

As already shown by Beaman and Gerhardt (1986), these results confirm that spores with a higher core water content, in this case strain 168, are less heat resistant. Moreover, heat resistance is related to the initial water activity of the spores as well as the preservation of this water content throughout the heat treatment. Consequently, when high temperatures are reached and there is no evaporation, the spore is more sensitive to heat.

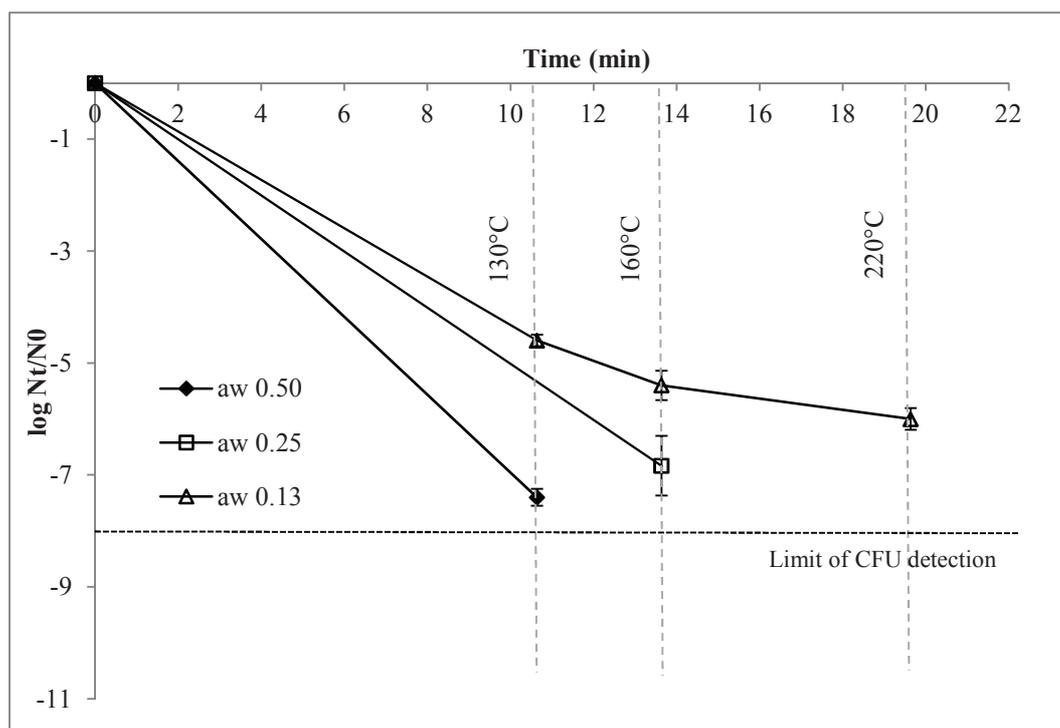


Figure 31. Inactivation of spores after heat treatment using DSC in Pan B for *Bacillus subtilis* 168.

4.1.2.2 FTIR spectrum of spores

FTIR was used to provide an insight into structural and biochemical changes in spores occurring during the heating process by DSC. All the analysis were performed in the strain 168 since different *Bacillus* strains present the same structures and very similar spectrum (Garip et al. 2009). A raw FTIR spectrum of spores is shown in Figure 32 and the general band assignment is given in Table 10.

The spectra of *B. subtilis* spores of strain 168 represent typical mid-infrared endospores spectra. In the region between 2700 and 3500 cm^{-1} we observe broad peaks due to water stretching modes as well as C-H stretches associated with alkane chains of the fatty acids and esters, but these broad bands do not provide much discrimination. For bacterial spores the two strongest bands are the protein amide I and amide II peaks at 1650 and 1540 cm^{-1} , respectively. Indeed, the most sensitive spectral region to the protein secondary structural components is the amide I band which is due almost entirely to the C=O stretch vibrations of the peptide linkages (approximately 80%). The amide II band, in contrast, derives mainly from in-plane NH bending (40–60% of the potential energy) and from the CN stretching

vibration (18–40%) (Krimm 1983; Kong and Yu 2007), showing much less protein conformational sensitivity than its amide I counterpart.

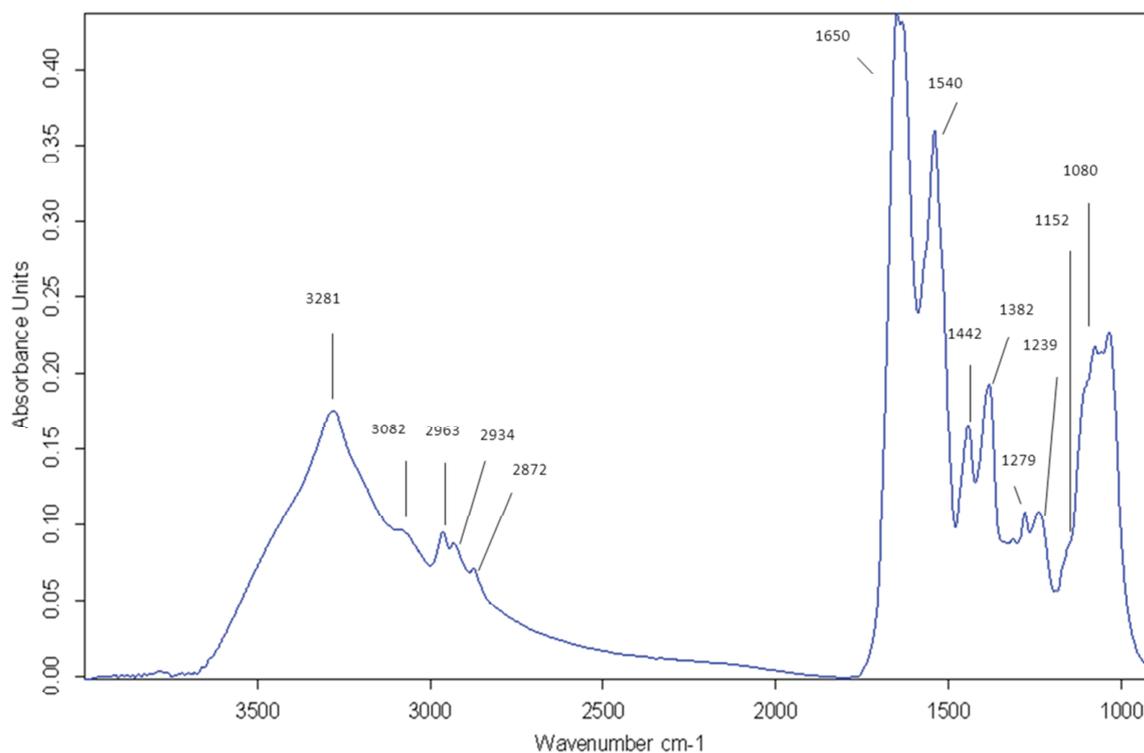


Figure 32. Raw FTIR spectrum of *Bacillus subtilis* spores 168.

The vegetative cell spectrum presents additional peaks including a very strong carbonyl band at 1739cm^{-1} as well as a smaller peak at 1256cm^{-1} . When a small amount of vegetative cells remains in the spore sample, a weak shoulder is observed in Amide I 1650cm^{-1} band (Forrester et al. 2009), however in our spectrum there is no shoulder, indicating that the sample was free, or had an undetectable amount, of vegetative cells.

The second derivative is commonly used since it allows a precise recognition of peak positions and subtle peak features, as shoulders (Kong and Yu 2007; Forrester et al. 2009). The derivatized spectrum of spores, along with the raw spectrum, is presented in Figure 33. Comparing the two spectra it is clear that the second derivative spectrum gives much more information than the raw one.

Table 10. General assignment of the bands found in *Bacillus* spores FTIR spectra*.

Wavenumber	Definition of the spectral assignment
~3300	N–H and O–H stretching vibration of polysaccharides and proteins
~2960	CH ₃ asymmetric stretch: mainly lipids
~2930	CH ₂ asymmetric stretch: mainly lipids
~2870	CH ₃ symmetric stretch: mainly proteins
~1655	Amide I band of α -helical structure of secondary proteins
~1637	Amide I band of β -pleated sheets of secondary proteins
~1616	Stretching bands of COO- group of DPA-Ca chelate
~1570	C-N vibrations of the DPA ring
~1535	Amide II polypeptide structures
~1442	Band of acid peptides/DPA pyridine ring vibration
~1515	“Tyrosine” band
~1467	C-H def of CH ₂ = CH ₂ bending of the acyl chains (phospholipids)
~1410	C-O-H in-plane bending of lipids, carbohydrates and proteins
~1378	Stretching bands of COO- group of DPA-Ca chelate
~1280	Amide III bands of proteins/DPA band
1236	PO ₂ asymmetric stretching: mainly nucleic acids with the little contribution from phospholipids
1150–900	Asymmetric and symmetric stretching of PO ₂ – and P(OH) ₂ in phosphates; vibrations of C–OH, C–O–C, and C–C of polysaccharides

*Adapted from Naumann (2000), Perkins et al. (2004), Forrester et al. (2009), Garip et al. (2009) and Bombalska et al. (2011).

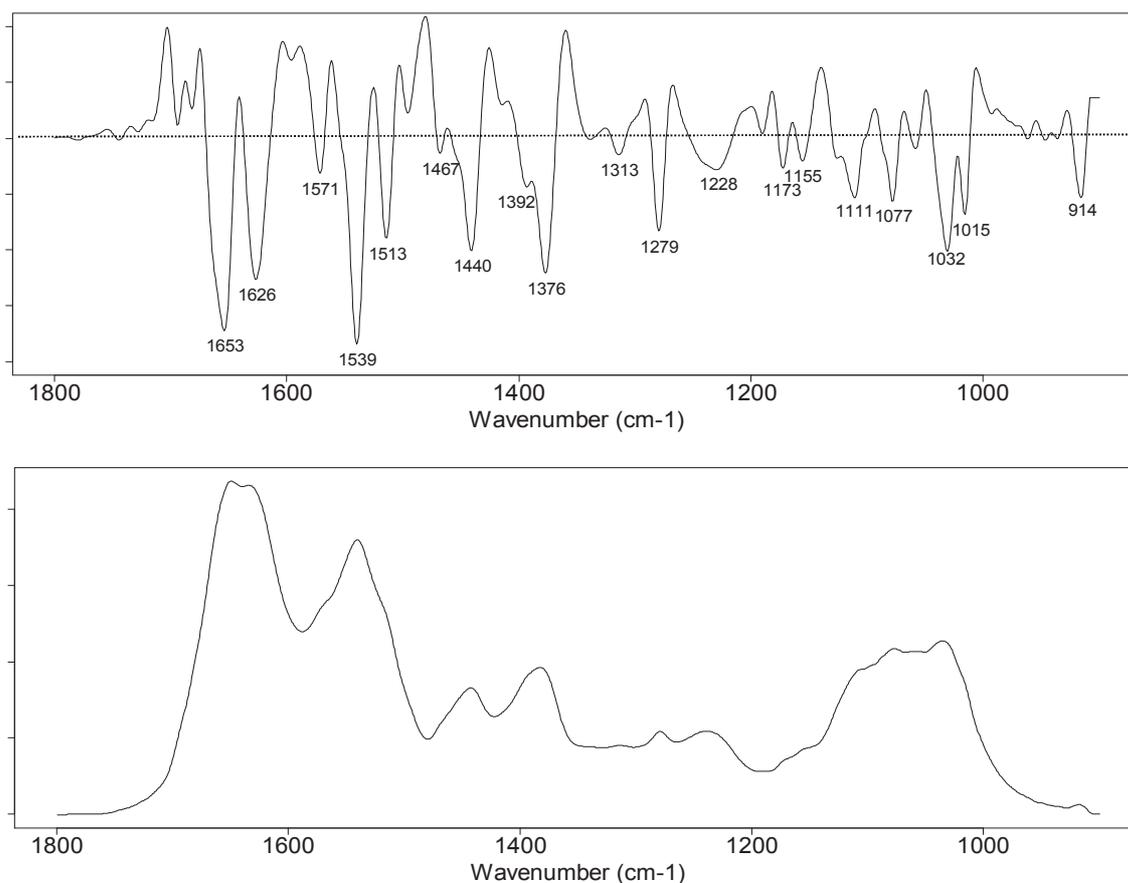


Figure 33. Comparison between the raw and the 2nd derivative spectra of *B. subtilis* 168 spores

4.1.2.3 Changes in spore structure after DSC heat treatment

The spectral range between 1800 and 1200 cm^{-1} has been reported to contain almost all signals of interest in studying spore inactivation treatments (Perkins et al. 2004; Subramanian et al. 2006; Trevisan et al. 2012). Consequently, we have used this range to evaluate changes in spore structure after treatment.

As presented before in Figures 28 to 31, spore inactivation was stronger when water did not evaporate before 150°C (Pan B) consequently forming a moist atmosphere that increased spore mortality. Therefore, to evaluate the difference in spore structure after the heat treatment in the two pans we have compared the spectra of spores heated until 220°C in each pan as shown in Figure 34.

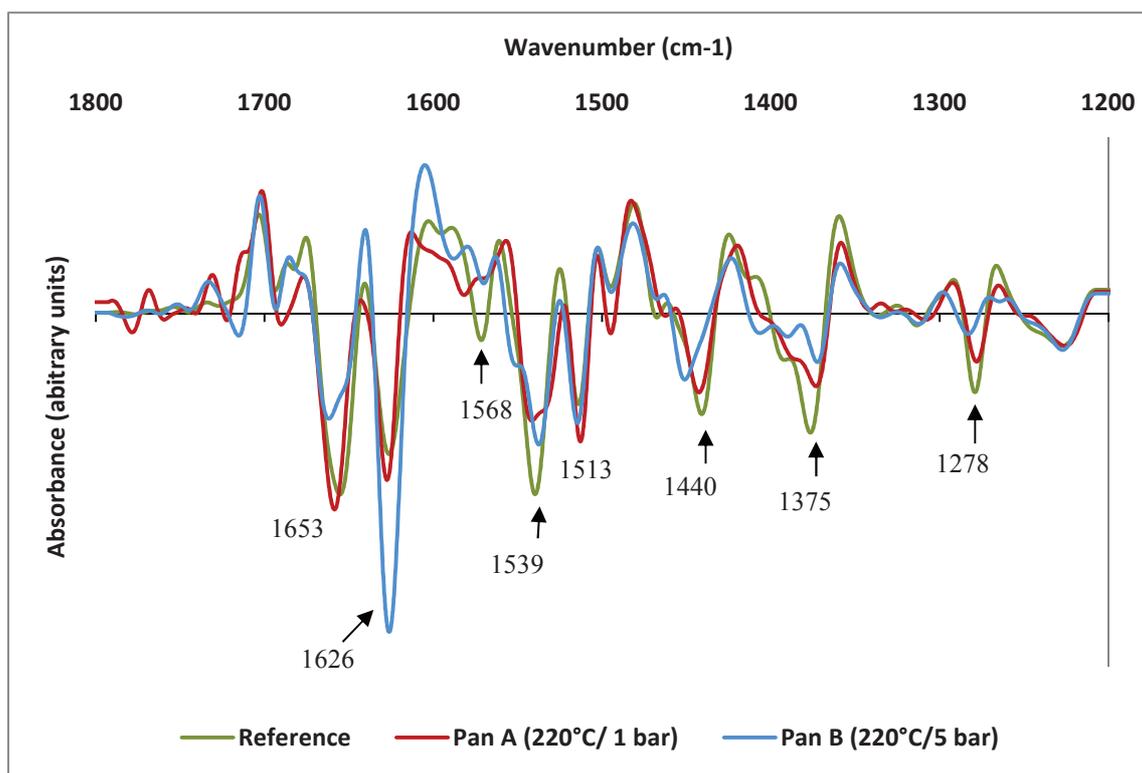


Figure 34. Changes in composition of *Bacillus subtilis* 168 spores (a_w 0.50) during heat treatment performed by DSC. Derivative (2nd) FTIR spectra of untreated spores (Reference) and treated spores (Pan A and Pan B).

Comparison of the derivative spectra of heat treated spores with those of untreated spores showed various differences in the regions associated to DPA (1568, 1440, 1375 and 1278 cm^{-1}) and secondary proteins (1653 and 1626 cm^{-1}). The changes observed in spores treated in Pan A included loss of intensity in peaks at 1539, 1440, 1375 and 1278 cm^{-1} and a loss of signal at 1568 cm^{-1} . No changes were observed in the peaks related to amide I. These same changes were observed for spores treated in Pan B, however the loss of intensity was more pronounced than in Pan A. Additionally, major changes were observed for the peak at 1626 cm^{-1} , which is associated with amide I bands corresponding to β -pleated sheets of secondary proteins, and the peak at 1655 cm^{-1} , which is related to amide I bands corresponding to the α -helical structure of proteins (Naumann 2000).

In Pan B, as shown in Figure 34, spore proteins underwent structural modifications from α -helices to β -sheets after the treatment, which is evidence of protein aggregation that can lead to biological inactivation of the proteins (Subramanian et al. 2006). There was also a peak shift from 1655 cm^{-1} to 1661 cm^{-1} . Such variation could be related to the severity of the heat

treatment since those spectral shifts are known to be related to protein denaturation and subsequent aggregation during autoclaving (Perkins et al. 2004). In Pan A however, there was only a small shift from 1655cm^{-1} to 1658cm^{-1} .

This complete loss of signal for the peak at 1568 cm^{-1} suggests that either the majority of the spore's DPA compounds were released or that DPA underwent an undetermined transformation. The decrease in intensity of the peaks related to DPA was proportional to the pressure under which the spores were treated. In Pan A (1 bar) there was an intermediate decrease in intensity while in Pan B there was a drastic decrease in intensity.

In order to check at which point of the heating the modifications occurred, we have analyzed the spectra of spores at different times of treatment, or, as it will be called here, at different final temperatures of treatment. These temperatures were the same ones used in the viability and weight loss analysis, so as to do a comparison between those phenomena and the structural changes observed by FTIR.

In Figure 35 we observe the spectra of spores at a_w 0.50 heat treated in Pan A. As observed in Figure 34, the changes in this pan were less pronounced, the major changes being related to the peaks of DPA (1568 , 1440 , 1375 and 1278 cm^{-1}). The intensity of the peaks decreased gradually with treatment but there was no significant event that could explain the mortality observed between 160°C and 220°C (Figure 29).

On the contrary, spores treated in Pan B (Figure 36) went through numerous changes regarding the protein structure and also on DPA. The most remarkable modification is the conversion of α -helices to β -sheets that was observed already at 130°C , continued until 160°C and then increased abruptly after 220°C . Comparing the two pans at each final temperature, we observe that at 130°C in Pan A there is only a slight intensity loss on DPA related peaks but in Pan B besides the changes in secondary proteins, some other modifications have already occurred that allowed DPA to leave the spores. The inactivation results showed that these spores (a_w 0.50/Pan B/ 130°C) had already lost 7 logs of their initial viability, therefore it is possible to assume that the damage that caused DPA to leave, or the DPA release itself, is related to spore inactivation.

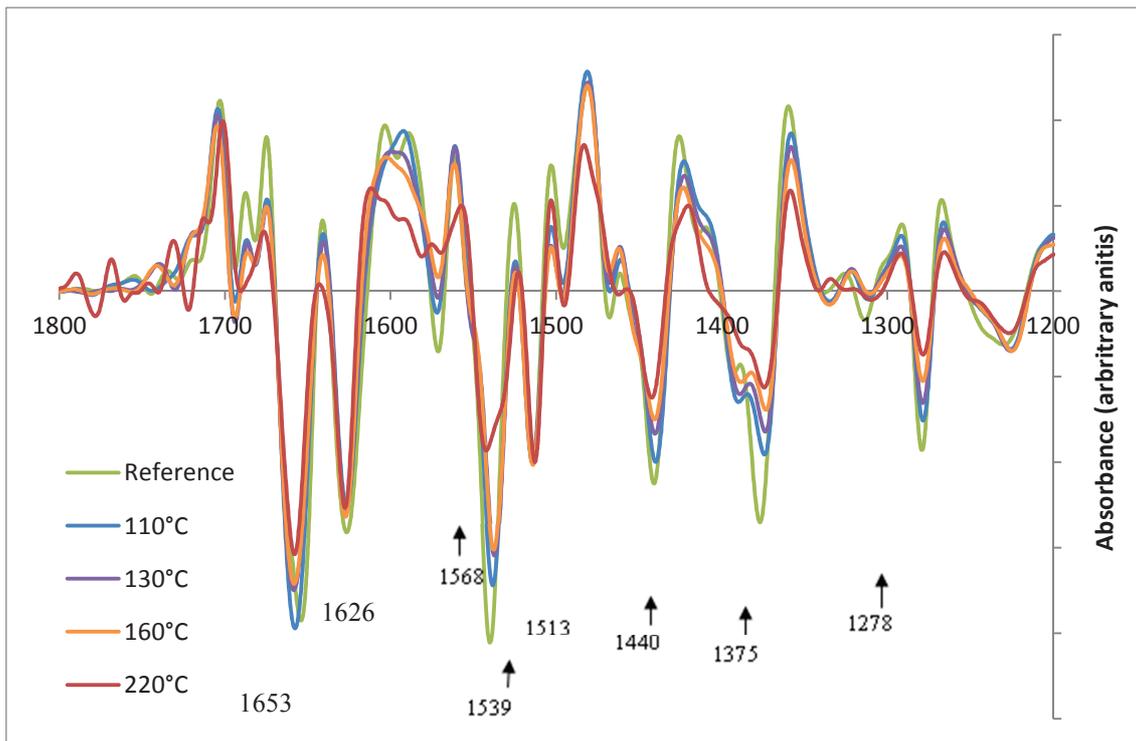


Figure 35. Changes in *B. subtilis* 168 spores (a_w 0.50) composition during treatment in Pan A at different final temperatures.

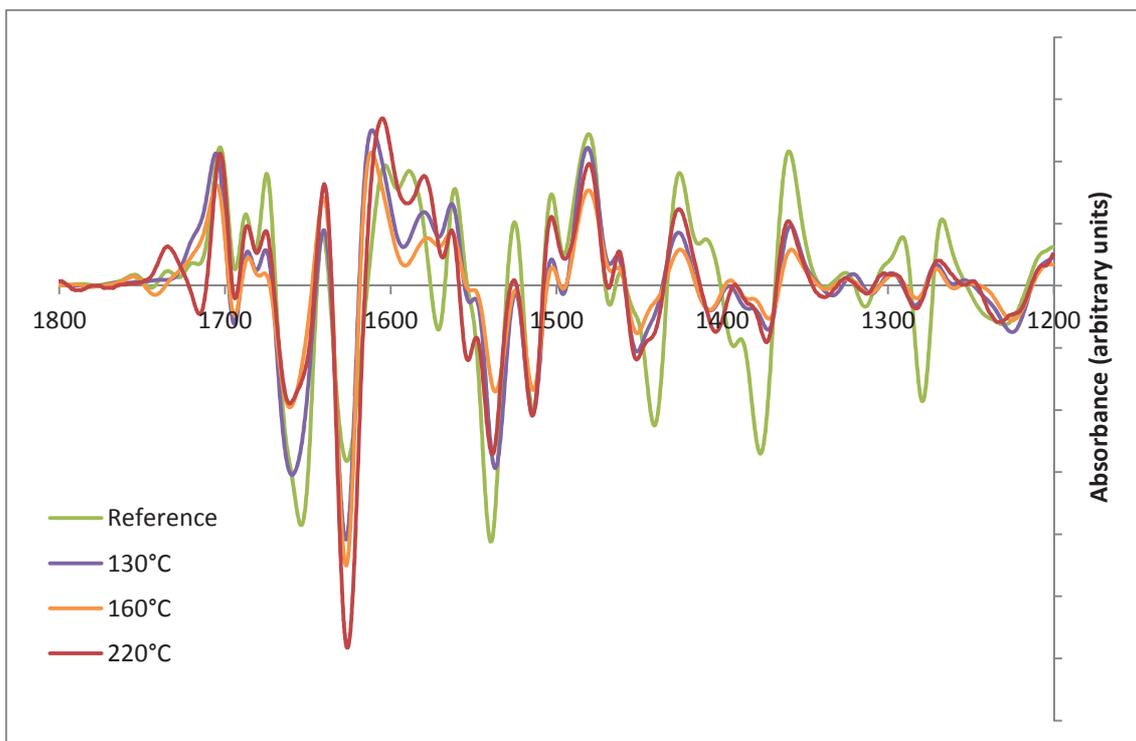


Figure 36. Changes in *B. subtilis* 168 spores (a_w 0.50) composition during treatment in Pan B at different final temperatures.

Last, to see if there were differences between spores treated at a_w 0.50 and those at a_w 0.13, the spectra of spores treated in Pan A at a_w 0.13 were analyzed as presented in Figure 37. Comparing the spectra we observe that the spores that were treated at a_w 0.13 present less modifications in their structure, the DPA peaks are mostly conserved and the alterations in amide I and amide II are intermediary between the control sample and spore at a_w 0.50. These results confirm those from inactivation, where it was found that dryer spores are more resistant to heat treatment. In addition, it also shows that their structure is mainly conserved indicating that the killing mechanisms might be related to DNA mutation, which cannot be observed in FTIR analysis.

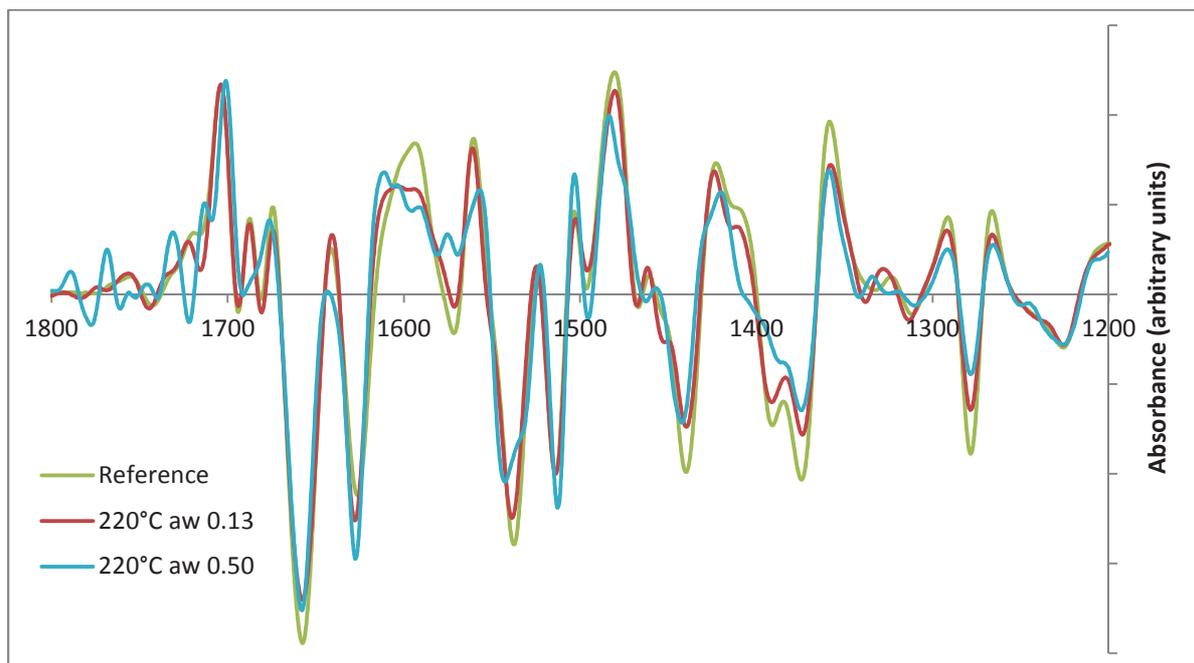


Figure 37. Changes in *B. subtilis* 168 spores (a_w 0.13) composition during treatment in Pan A at different final temperatures.

- Spore inactivation increased with the increase in water activity in both pans and both strains. Inactivation was higher in Pan B.
- The use of a method that prevents the spores from drying during heat treatment has shown that the water conservation in spores is directly related to its destruction.
- In Pan B spore proteins underwent structural modifications from α -helices to β -sheets.
- The high inactivation in Pan B can be explained by the extensive changes in protein structure and DPA release as observed by FTIR.
- In Pan A the structure of spore proteins was essentially preserved which correlates to a lower inactivation.

4.1.3 Discussion

The transitions observed are related to water vaporization

Apart from water vaporization, other transitions that could normally occur when spores are heated are related to protein denaturation, alterations of complex cellular structures and denaturation-induced aggregation of macromolecules (Belliveau et al. 1992). However, Snyder et al (2005a; 2005b) used Thermogravimetric Analysis coupled with Mass Spectrometry or Pyrolysis Bioaerosol Detection and showed that spore components begin to undergo thermal decomposition only above 250°C and the same was found regarding dipicolinic acid and proteins. In addition, in our study the transitions studied occurred in the same temperature range as pure water vaporization; they were highly correlated with the amount of water present in the sample, judging by the enthalpy that is proportional to the initial water activity and they were also irreversible. Therefore the weight loss observed in this study could not be related to the decomposition of spore components and can be attributed to water evaporation from the spore.

A fraction of spore water localized in the core seems to be isolated by the low water permeability of the inner membrane

One important finding in this work was that spores equilibrated at a very low water activity of 0.13 still had a water content of 8.1 to 11.5% (w/w) (Figures 26 and 27) considering that all the weight loss was the result of water evaporation and depending on the strain. This value is significantly higher than those found for the sorption isotherm obtained for *Bacillus subtilis* ATCC 31324 spores (Nguyen Thi Minh et al. 2010). It was observed that the weight loss was always superior to the total water content estimated by the water activity measured in the spores. One hypothesis to explain this paradox takes into account that the water activity only measures the water in equilibrium with the surroundings and not the water in the core, which is slowly affected by the changes in the atmosphere but does not contribute to the water activity measured. This can be explained by the low water permeability of the inner membrane which is consistent with the high degree of lipid immobilization in this membrane (Cowan et al. 2004).

Two different evaporation events were observed in Pan B and could be related to water located in different parts of the spore (core and non-core).

Considering these results, presented in Figure 38, we believe that the water left the spore at two different moments, once at around 150°C and then at around 200°C. One hypothesis is that the external water, or noncore water (from the cortex and coat), of the spore evaporates easily when the pan seal breaks at 150°C. The second evaporation event may be related to the water that is inside the spore core and because of this it needs to surpass the pressure exerted by the spore cortex and the internal membrane in order to evaporate. These results confirm that water is compartmentalized in the spore and that a significant fraction of the total water is located in the core (See Figures 39 and 40).

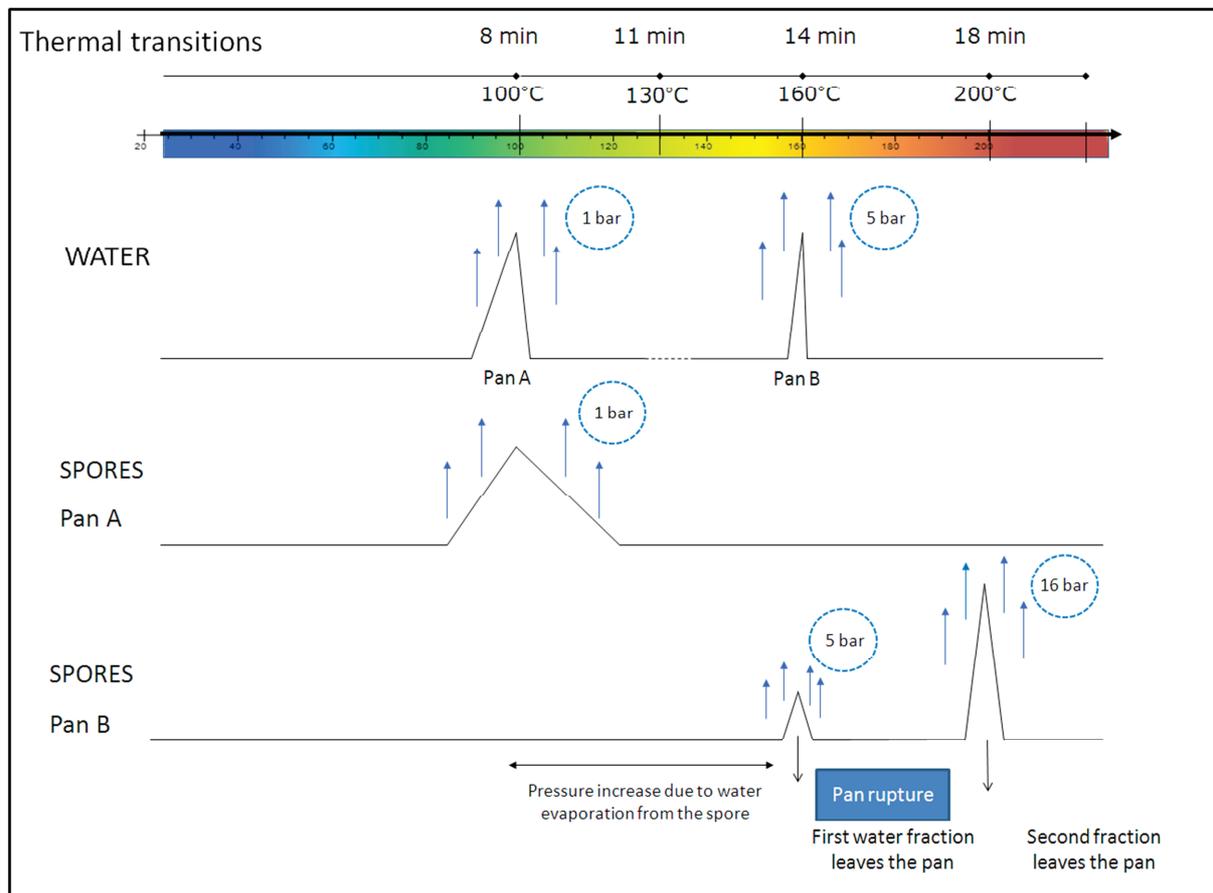


Figure 38. Thermal transitions related to water in the spores.

Spore Inactivation and changes in their structure after heat treatment

In order to understand why more hydrated spores are less resistant to dry heat treatment as observed in this study, we can examine the FTIR spectra of the spores. From the infrared spectra it is possible to obtain information about the type of damage suffered by the spores during heat treatment. DNA is usually the target of spore inactivation in dry heat treatment (Nicholson et al. 2000) but interestingly many of the observed changes in Pan B were also found in autoclaved spores (Subramanian et al. 2006). The loss of intensity or absence of peaks related to DPA (1568, 1450, 1378 and 1279 cm^{-1}) in Pan B suggests that there was a rupture of the cortex and the inner membrane allowing DPA leakage from the core. These findings are consistent with our hypothesis of water vaporization from the core at higher temperatures since the vaporization can only take place if a specific pressure is surpassed then causing the collapse of the inner membrane and the cortex.

The peaks related to the structure of proteins were also affected by the heat treatment in Pan B, which could indicate that a key germination protein or enzyme was denatured and lost its activity. In contrast, in Pan A no major modification was observed in the protein structure suggesting that the dehydration protected the proteins from heat.

The inactivation of one or more key spore proteins is believed to be important in spore heat killing (Coleman et al. 2010). When the heat surpasses a threshold level, changes in the structure of vital growth proteins become irreversible causing spore inactivation (Grinshpun et al. 2010). It is known that in low moisture states the temperature of protein denaturation increases (Sochava 1997) making it more difficult to inactivate these key proteins. In this context, the high spore heat resistance at low water content can be explained by the low mobility and changes in the conformation of proteins and enzymes. Indeed, most spore proteins are rotationally immobilized, which may contribute to heat resistance by preventing heat-denatured proteins from aggregating irreversibly (Sunde et al. 2009). Consequently, when the original water content of the spore is maintained, damage to proteins is increased during heat treatment and such damage is certainly related to the difference in spore inactivation found in our experiments. Another factor that could influence this low spore inactivation is the local decrease in temperature caused by the continuous evaporation of water, i.e. the evaporative cooling that occurs since evaporation is an endothermic reaction.

All these results allow the proposition of a scenario for the evolution of water content in the spore and the corresponding mortality.

These results show that *Bacillus subtilis* spores contain two different water fractions: one easily available, certainly from the coat and cortex and a second located in the spore core in which the changes with the exterior are limited by the internal membrane (Figure 39). This fraction is not considered in the water activity measurements, where the equilibrium time is not very long.

In Pan B, where the pan breaks at 5 bar (150°C), spores stay hydrated until this temperature and therefore they are very sensitive to heat (Figure 39). This sensitivity is related to the initial water content of the spore, increasing with high water contents. Between 150°C and 200, the pan is open, available water fractions evaporates progressively but one important fraction of internal water remains in the spore and leaves as vapor at around 200°C. After 200°C spores become extremely dry and, if they have survived treatment, they will become very heat resistant.

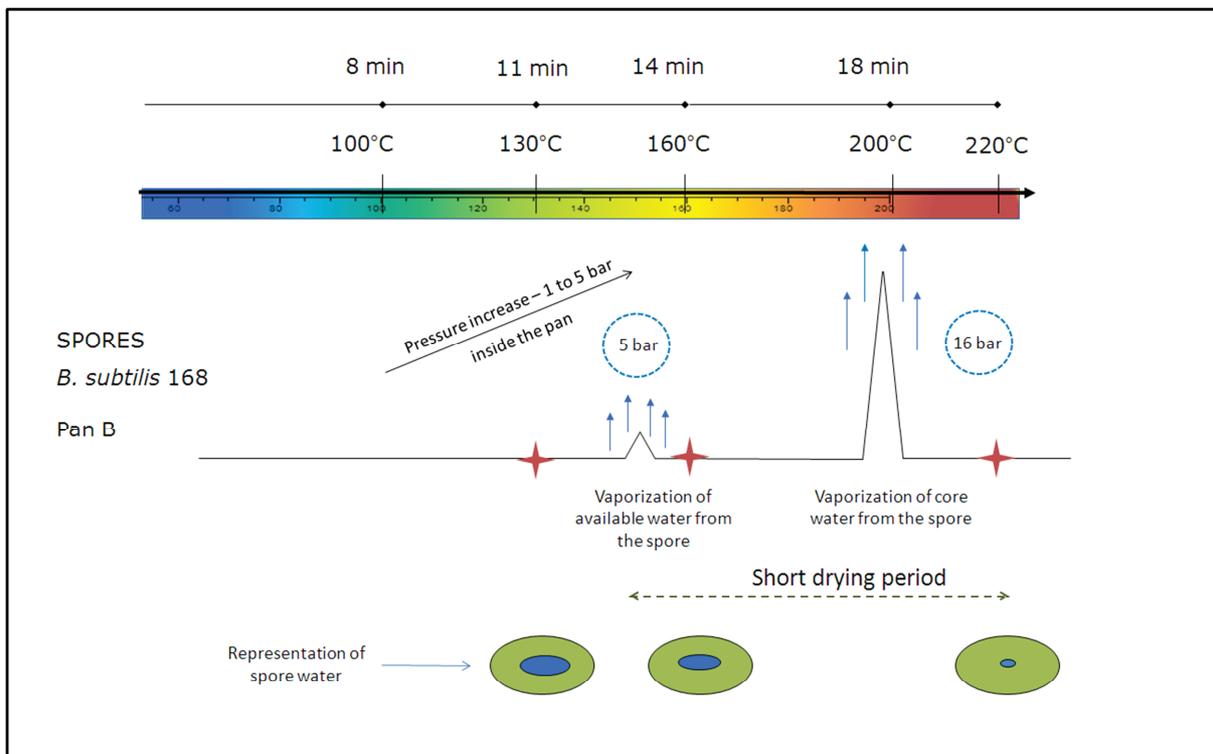


Figure 39. Focus on Pan B: scenario proposal for water in the spores.

In Pan A, at atmospheric pressure, there is a long drying phase during which even internal water evaporates progressively as showed in the diagram in Figure 40. Below 100°C, the water present in the spores will slowly evaporate due to water gradient difference and also the high flow of nitrogen in the furnace. Between 100°C and 130°C, it can be assumed that water will vaporize, especially water located in the spore core. Consequently the spore becomes extremely dry and thus very heat resistant as shown in Figure 42.

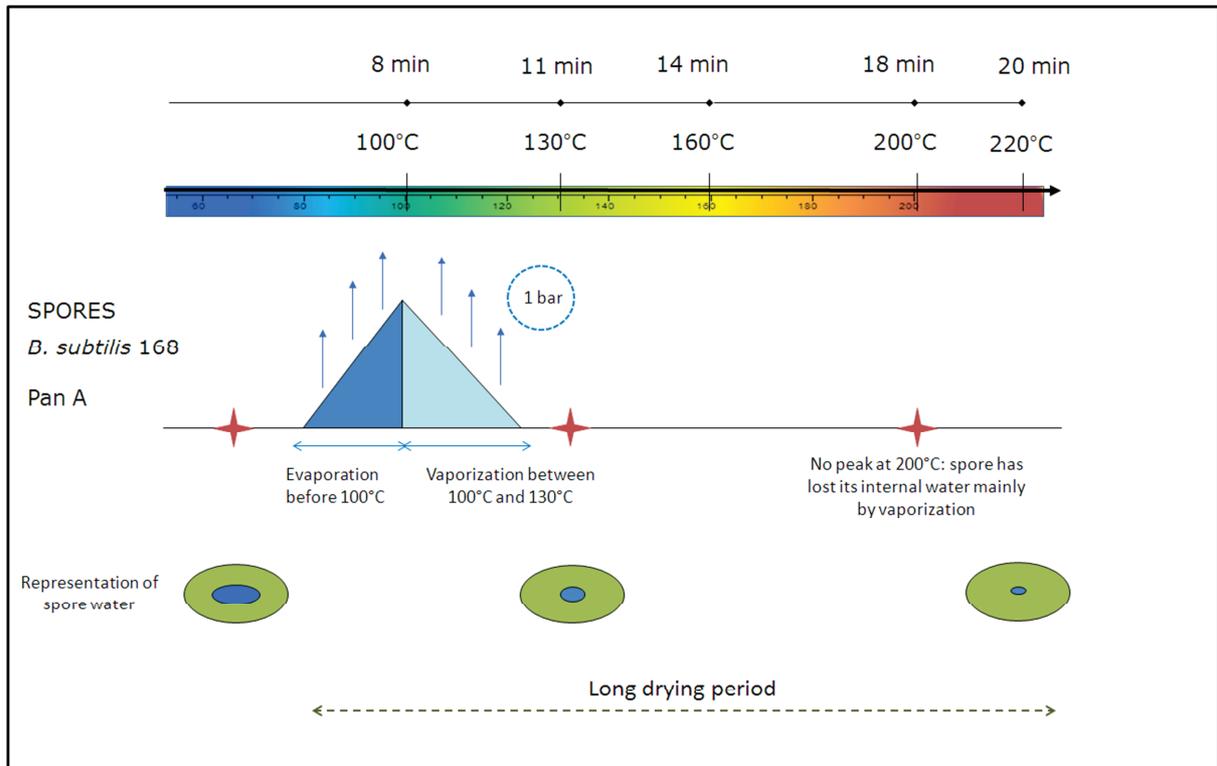


Figure 40. Focus on Pan A: scenario proposal for water in the spores

Concerning the spore inactivation in Pan B (Figure 41), in which the seal breaks at 5 bar (150°C), spores stay hydrated until this temperature and therefore, they are very sensitive to heat, especially at high initial water content. Between 150°C and 200, the pan is open, thus available water fractions will evaporate progressively but one important fraction of internal water will remain in the spore, leaving as vapor at around 200°C. After 200°C the spore becomes extremely dry and (if it has survived treatment), becomes very heat resistant.

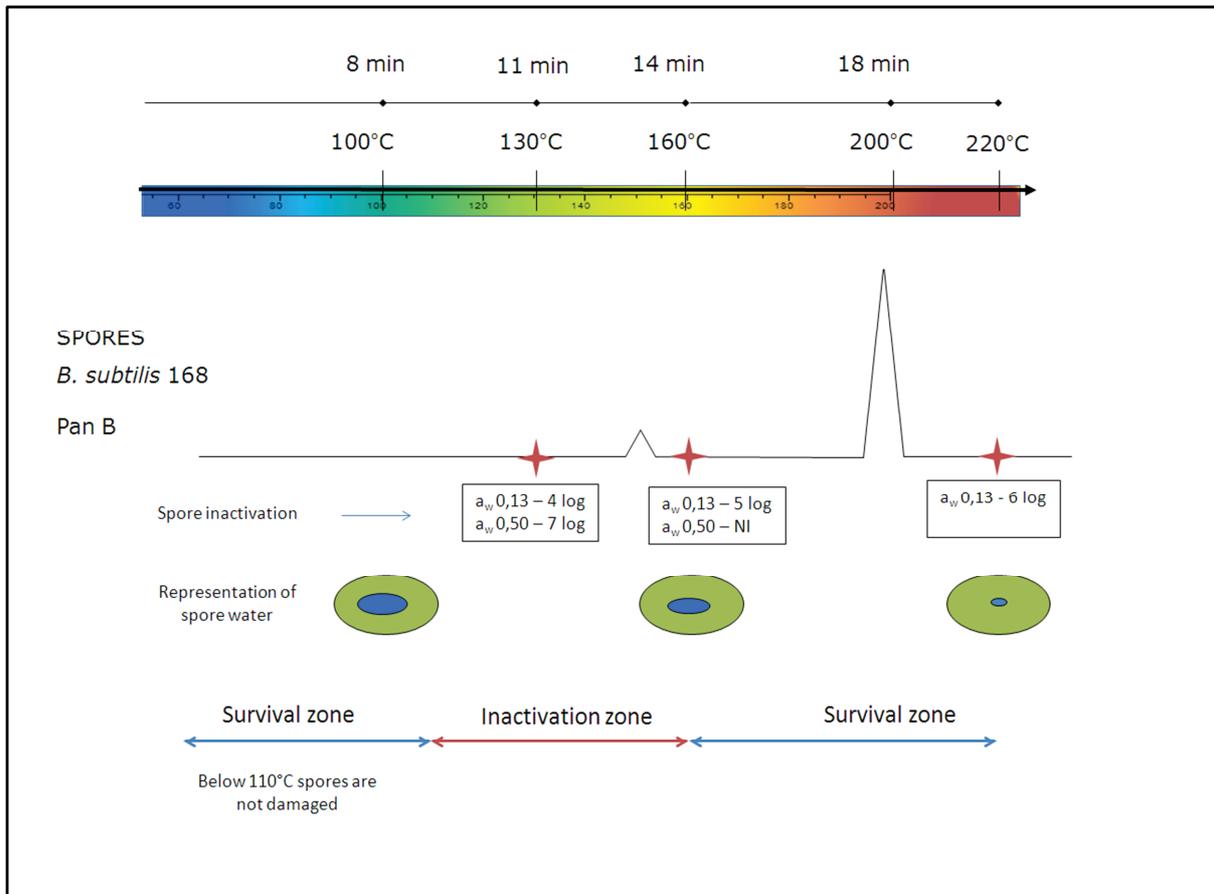


Figure 41. Focus on Pan B: Spore inactivation.

However, in Pan A, when spores reach lethal temperatures ($>110^\circ\text{C}$) they have already lost most of their initial water, making them more resistant to heat. Consequently there is almost no decrease in spore population before 160°C .

This analysis has shown that dry spores resist much better than hydrated spores to the same heat treatment and more importantly, considering the presence of internal water closely bound to the spore. One hypothesis is that some macromolecules (proteins, DNA) are much more sensitive to denaturation in aqueous phase which has been confirmed by the FTIR results.

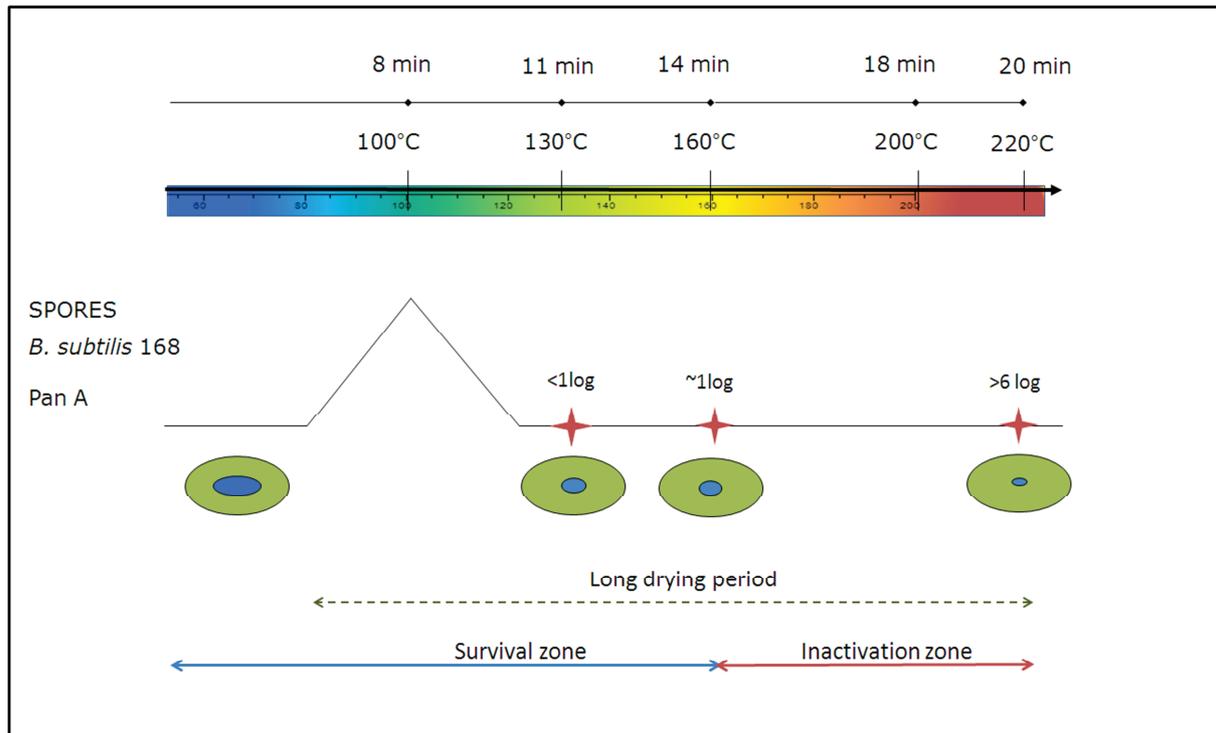


Figure 42. Focus on Pan A: Spore inactivation.

One scientific article presenting some of the results above was published in the journal Food Biophysics and can be seen in the ANNEX section of this thesis.

4.2. Inactivation of dried spores of *Bacillus* by a treatment combining high temperatures and pressure

The results of the DSC study indicate that it is possible to maximize inactivation if the heat treatment is applied without the evaporation of water. In order to apply these findings for the decontamination of dried spores we have developed a small system to validate the results obtained. This system allows us to heat the samples in a hermetic container under a few bars of pressure. The applied pressure prevents water evaporation from the sample allowing the maintenance of its initial water content. The effect of pressure level and initial water activity were investigated.

The different steps of the heat treatment performed at high temperatures and under pressure are presented in Figure 43.

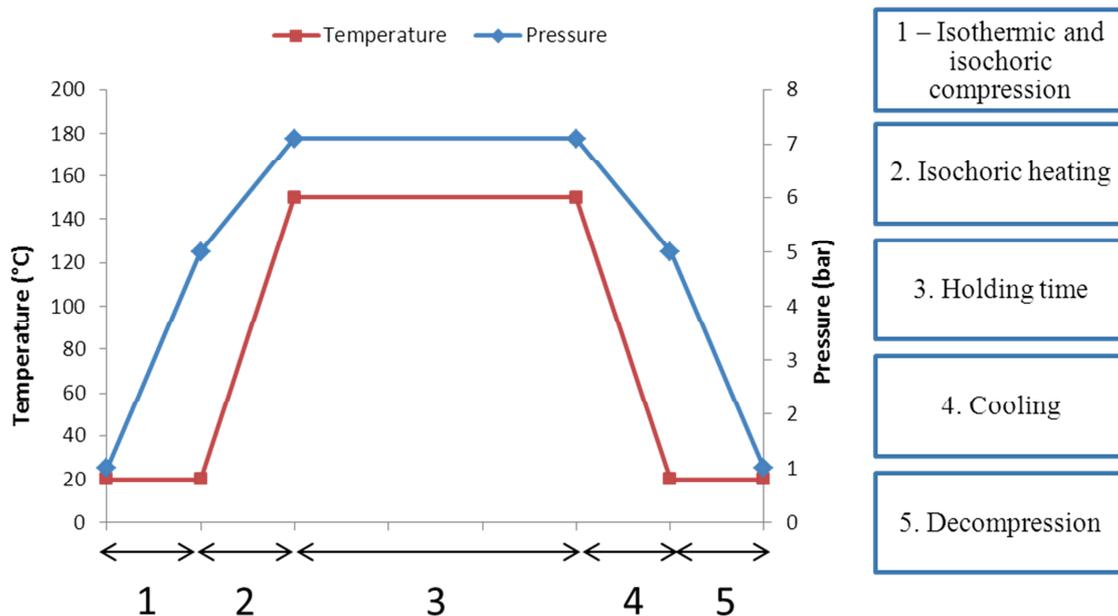


Figure 43. Steps of the heat treatment under pressure considering an added pressure of 5 bar, (total pressure of 6 bar) and a treatment temperature of 150°C.

4.2.1 Global analysis of the system

In order to give a general view of the system, an experimental design was designed *a posteriori*. The influence of three treatments parameters T, P and a_w , in the inactivation of *B. subtilis* spores was assessed. However, P and T are not really independent since the system pressure will increase with an increase in temperature. An experimental design domain was

defined over temperature of treatment, 120 to 160°C, a_w of spores, 0.10 to 0.70 and pressure of treatment, 1 to 6 bar. The Doehlert design allows the description of the region around an optimal point and contains k^2+k+1 points for k variables (Ferreira et al. 2004). For 3 variables, a set of 13 experiments was required. The experimental response was the spore inactivation after a 3 minute treatment. The objective was to study the efficiency of the heat treatment using short treatment times in order to minimize undesirable reactions in a final food product. The multiple regression analyses based on the least square method was performed and the significant variables (p -value < 0.01) were temperature, a_w , pressure and the interaction between temperature and a_w .

The regression coefficient, r^2 , was equal to 0.90, therefore about 90% of the total variation could be explained by the model. The greater the absolute value of the linear coefficients (b_i), the more important was the variable influencing the response. Therefore, the influence of temperature ($b_t=3.65$) was greater than the influence of a_w ($b_{aw}=1.29$) and pressure ($b_p=0.92$) and spore inactivation increased with the increase of the three variables. The quadratic coefficients were not significant however, a significant interaction between T and a_w was observed. The negative value of the coefficient ($b_{t*aw} = -2.03$) suggested that at low water activities the temperature effect is stronger than at high water activities.

The effect of temperature and a_w in spore inactivation in the different pressures tested (1, 3.5 and 6) are presented in Figure 44. It can be seen that the reduction of *B. subtilis* spores increased with increase in a_w and temperature, but the slope at low water activity is steeper than the one at high water activity, confirming the interaction between the two variables. At low a_w and lower temperatures we observe almost no inactivation but at low temperature and high a_w there is already a 5 log reduction in spore load. Therefore, it can be concluded that spores with higher water content can be inactivated at lower temperatures but extremely dry spores can only be inactivated at higher temperatures. When no pressure is added, i.e., at P_{atm} , the maximal inactivation was 4 logs and as the pressure is increased this value increased to 5 and 6 logs, at 3.5 and 6 bar, respectively.

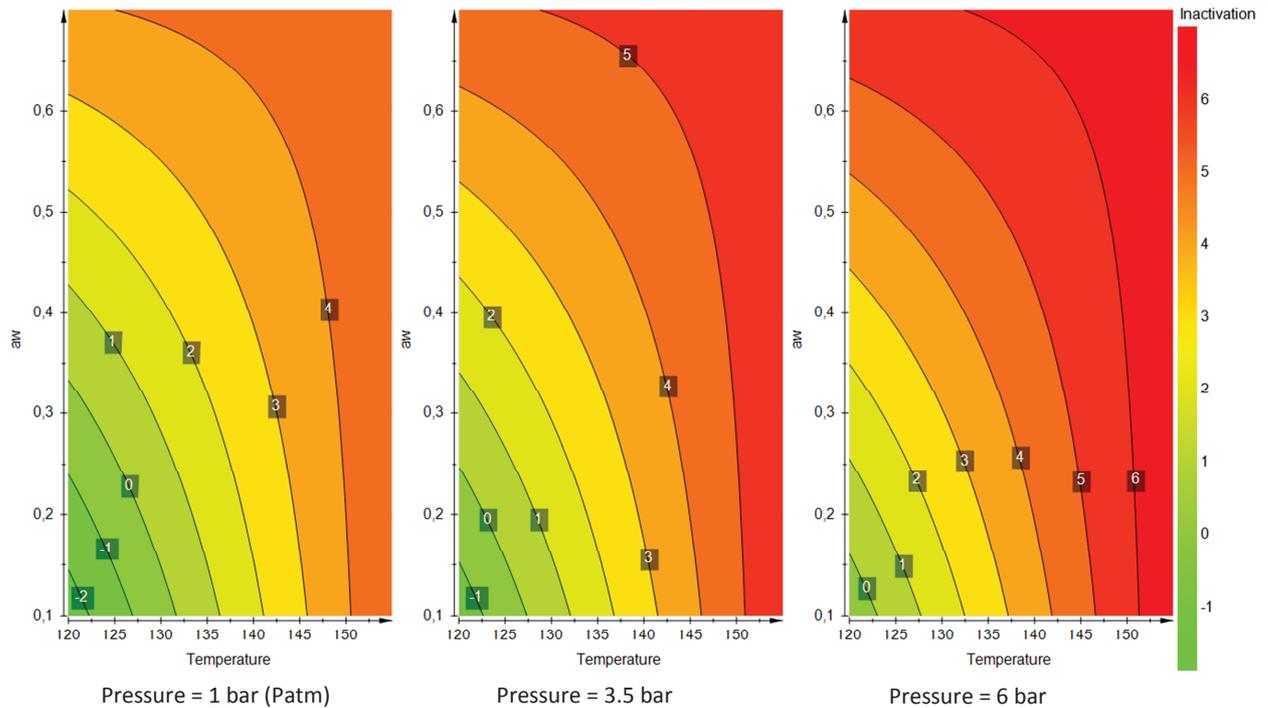


Figure 44. Contour plot of the effect of temperature and a_w on *B. subtilis* spores at different pressures and treatment time of 3 minutes.

Observing the contour plots (Figure 44), it is clear that at Patm is not possible to achieve a destruction higher than 4 log unless temperatures over 155°C were used or longer treatment times, both which could increase undesirable reactions in a final food product. If the pressure is increased to 3.5 bar, the zone of presenting an inactivation higher than 4 log increased but except at higher water activities (>0.60), temperatures above 150°C or longer times were still needed. Finally, with a pressure of 6 bar it was possible to achieve 5 log inactivation at lower temperatures, especially at higher water activities. Examining the region where most part of dried products normally fall in, a_w between 0.40 and 0.60, we observe that a high inactivation can be achieved with lower temperatures, in the range of 126°C-141°C.

From these results, it can be assumed that the three variables tested influence the inactivation of dried spores. The effect of pressure and a_w of the spores is more deeply investigated in the following paragraphs.

4.2.2 Effect of pressure application

Pressures between 1 and 7 bars were used to study the effect of the pressure combined with heat in the inactivation of *Bacillus* spores (Figure 45). The maximum initial pressure was 7 bar in function of the resistance of the chamber that did not support additional pressure

without gas leaking. Spores were equilibrated at a water activity of 0.50 which is the usual a_w of dried foods as flours, cereals and spices.

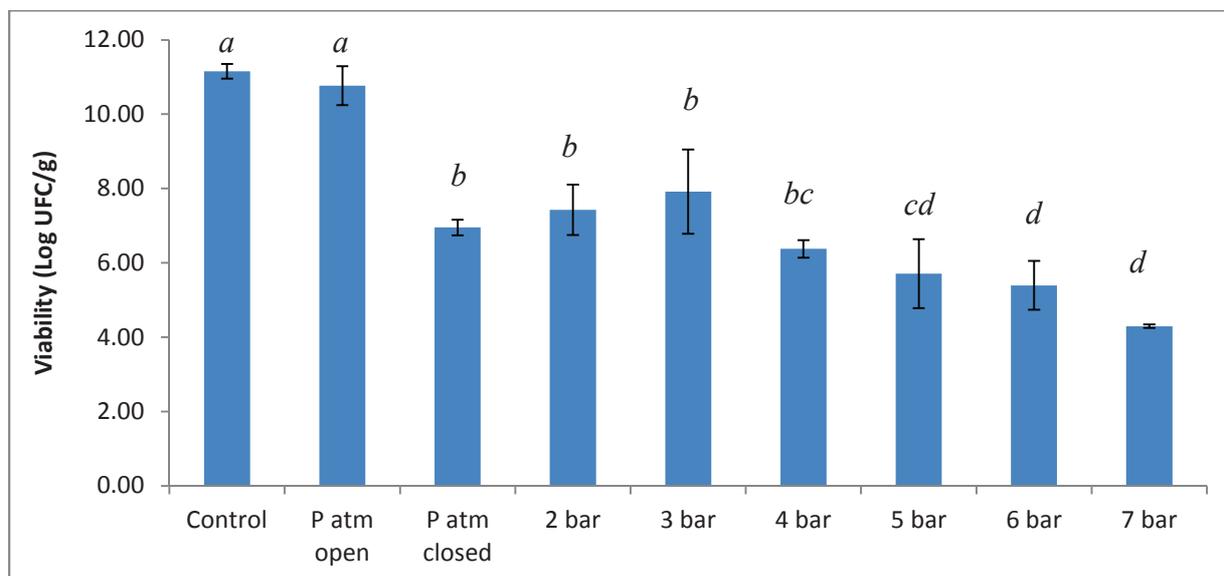


Figure 45. Survival of *B. subtilis* spores equilibrated at a water activity of 0.50 after a 120s treatment at 150°C with different pressures applied. $P > 0.05$, analysis of variance and Tukey's test; same letter indicates no significant difference between treatments.

In Figure 45 we observe that when the applied pressure is increased the spore inactivation is also increased. The treatment performed at atmospheric pressure did not differ from the control sample, however at 2 bar (Patm + 1 bar of nitrogen) there was a 3 log reduction on spore population. The inactivation was higher with a pressure of 7 bar, where around 7 log of spores were killed. However, there was no statistical difference between spores treated at 2, 3 and 4 bar and those treated at 5, 6 and 7 bar.

Comparing the two treatments at Patm, a remarkable difference in viability is observed, reminding that the difference between them is only the position of the valve (open or closed). This variation can be explained by the atmosphere created inside the reactor. When the valve is left open there is no increase in the pressure inside and as the spores are heated, the water present will evaporate and leave the reactor in the same way as Pan A of the DSC study. However, when the valve is left closed, the heating will cause the interior pressure to increase and also water will evaporate and will stay inside the reactor, increasing the specific humidity (mass water vapor/mass of air) inside the reactor and consequently the final pressure. This treatment is similar to Pan B of the DSC study where there was a self-formed pressure from water evaporation.

To study the kinetics of spore inactivation with the treatment combining heat and pressure, spores with the same water content (a_w of 0.50) were treated with or without applied pressure. The pressure used was of 6 bar. The inactivation of spores with time is showed in Figure 46. Those spores heated without pressure showed only a slight inactivation after 240 s, in contrast, spores put under pressure showed a 5 log inactivation after only 120 s. Examining data from Figure 45 and 46 it is clear that the application of pressure has a remarkable effect in the destruction of *B. subtilis* spores at a_w of 0.50.

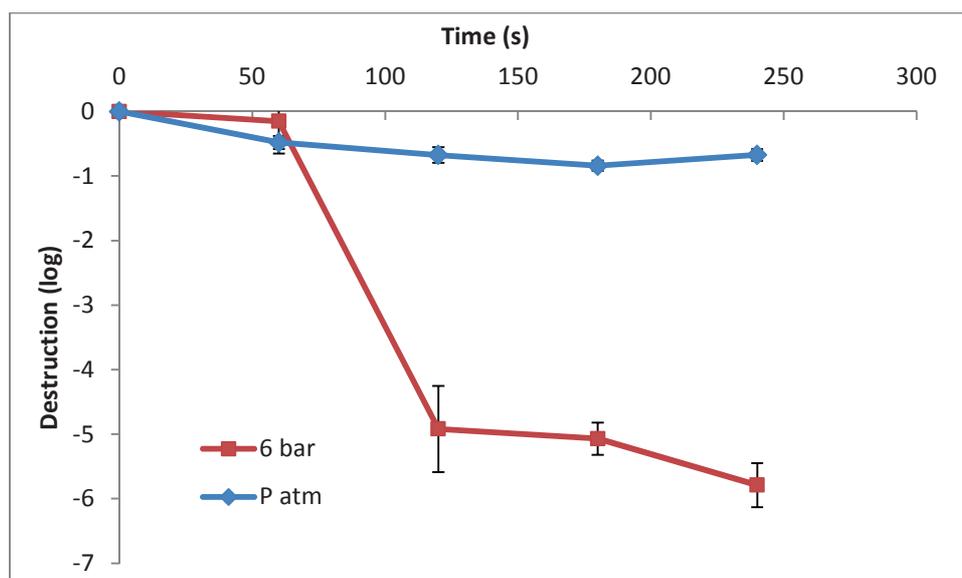


Figure 46. Inactivation of spores of *B.subtilis* 168 equilibrated at a water activity of 0.50 treated at 150°C and (■) 6 bar or (◆) P_{atm} .

4.2.3 Thermodynamic characterization of the system and the effect on spore inactivation

The final pressure inside the reactors is determined by two factors: pressure increase due to temperature increase and due to water evaporation from the sample.

4.2.3.1 Pressure increase due to temperature increase

Internal pressure will increase with the temperature increase, following the ideal gas law ($PV = nRT$), since the heating is performed in a closed vessel and there is no change in volume. The final pressure in each treatment was calculated, considering that the initial

temperature was 25°C and the temperature if treatment was 150°C, and is presented in Table 11.

In closed systems the vapor pressure increases until the pressure reaches a certain point that inhibits further evaporation. In this case, if the final pressure is lower than the P_{vap} at 150°C (5 bar), water vaporization will take place until P_{vap} is reached. On the other hand, if final pressure is higher than P_{vap} at 150°C, there will be no vaporization of the water present in the spores.

Table 11. Final pressure inside the reactor after heating from 25°C to 150°C.

Initial Pressure (T _i =25°C)	Final Pressure (T _f =150°C)
Patm closed = 1 bar	1.42
2 bar	2.84
3 bar	4.26
4 bar	5.68
5 bar	7.10
6 bar	8.52
7 bar	9.94

4.2.3.2 Pressure increase due to water evaporation

To evaluate the amount of water that will vaporize we have to consider the evolution of internal pressure during heating and the number of molecules in gas phase. In the cases of initial pressure of 1, 2 or 3 bar, the final pressure will be lower than 5 bar (Table 11) so water will evaporate inside the reactor. However, from 4 bar on there will be no evaporation since the heating of the reactor causes the final pressure to exceed 5 bar.

Atmosphere inside the reactor

The n total inside the reactor at $t=0$ depends on the amount of nitrogen added and the amount of air that remained inside (Equation 2). The variation of the mass fractions of air and nitrogen with initial pressure is presented in Figure 47.

$$n_{total_i} = n_{moist\ air} + n_{N_2} = (n_{dry\ air} + n_{H_2O\ air}) + n_{N_2} \quad \text{Equation 2}$$

However n_{H_2O} will be neglected since the amount of water present in the air is very low (in the order of 1×10^{-2} mg), representing only 0.5 and 2.0% of spores' water content when water activity is 0.70 and 0.10, respectively.

When the temperature of evaporation is reached the n inside the reactor is described by Equation 3:

$$n_{total_f} = n_{moist\ air} + n_{N_2} + n_{H_2O\ spore} = (n_{dry\ air} + n_{H_2O\ air}) + n_{N_2} + n_{H_2O\ spore}$$

Since the n_{H_2O} from air was neglected, all the increase of n will be due to water evaporation from the sample. Considering the volume of the reactor (1.7×10^{-3} l), every milligram of water evaporated will increase the internal pressure by 1.135 bar.

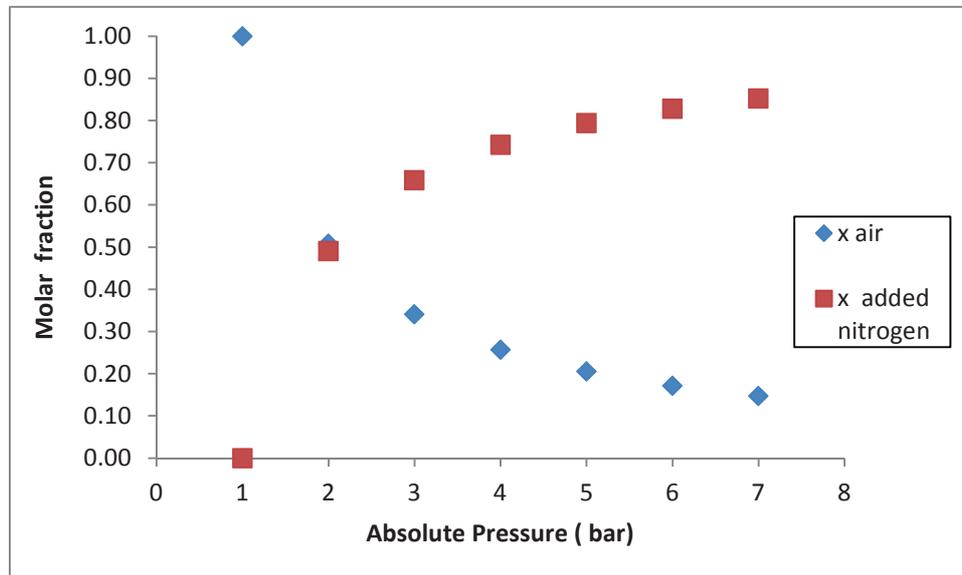


Figure 47. Evolution of the molar fraction of air and gaseous nitrogen inside the reactor. ($x_i = n_i/n_t$).

Using spores equilibrated at a_w 0.50 (17% water content – from the weight loss observed in the DSC study) and a sample size of 10 mg (1.7 mg water) as an example, the evaporation of this amount of water would increase the pressure by 1.92 bar. Therefore, when the initial pressure is 1 or 2 bar all the water contained in the spore can evaporate because the final pressure will still be under 5 bar. However, when the initial pressure is 3 bar there is a special situation since the pressure will be under 5 bar after heating but the evaporation of all water would cause the pressure to pass 5 bar. In this case only a fraction of the water can evaporate until the pressure reaches 5 bar.

Calculation of the amount of water evaporated at $P_i = 3$ bar

$$P_{vap} = 5 \text{ bar and } P_f = 4.26 \text{ bar} \rightarrow \Delta P = 0.74$$

If pressure increase due to 1 mg of water evaporated is 1.135, then by cross multiplication the amount of water that can evaporate is 0.65 mg. Following the example, this would mean that 38% of total water content of the spore can vaporize in these conditions.

4.2.3.3 Changes in temperature of vaporization

The pressure increase will cause the temperature of vaporization to increase and consequently, the water in spores will then vaporize at higher temperatures. To find the initial temperature of vaporization for each given initial pressure, the intersections between the curve relating pressure and temperature of vaporization and the curves of pressure increase with temperature in the reactor were determined using Matlab® as shown in Figure 48. The temperatures of evaporation given by the intersection of the curves are presented in Table 12.

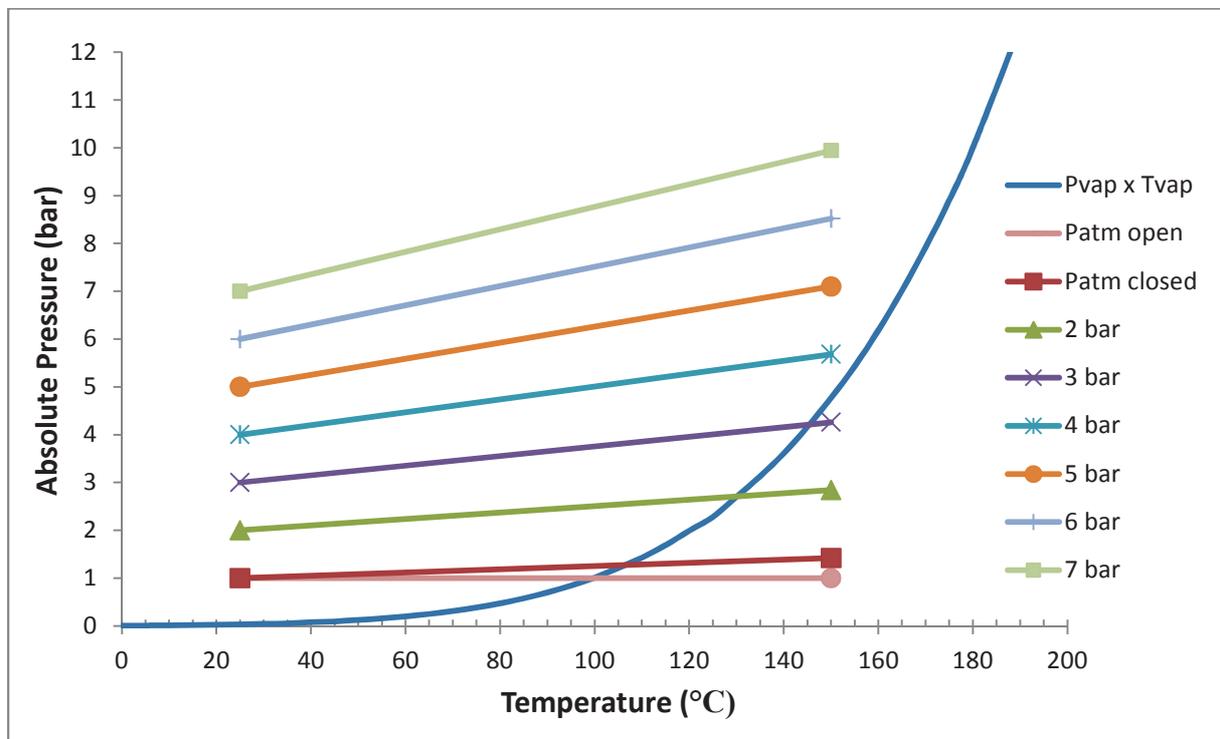


Figure 48. Determination of vaporization temperatures according to the initial pressure inside the reactor.

Figure 48 clearly shows that when P_i is higher than 3 bar, water will not vaporize inside the reactor. However, when the initial pressure is 1, 2 or 3 bar the spores will heat until 107°C, 130°C and 145°C, respectively, without any evaporation. The water from the spores will be able to vaporize only when the new vaporization temperature is reached. In the case of initial pressure of 1 and 2 bar, all the water (1.7 mg from the example above) will evaporate which will cause the final pressure inside the reactor to reach 3.34 and 4.76 bar, respectively.

Table 12. Temperature of evaporation according the initial pressure. Values from the intersection of the curves of Figure 48.

Initial Pressure	Temperature of vaporization
Patm open	100°C
Patm closed	107°C
2 bar	130°C
3 bar	145°C
4 bar	157°C
5 bar	167°C
6 bar	176°C
7 bar	183°C

Figure 49 shows the evolution of temperature in a sample of spores inside the reactor at atmospheric pressure or with different applied pressures. At atmospheric pressure, spores will heat in a given time and when they reach 100°C the water will start to vaporize and the temperature will not change during this state change. When the vaporization stops then spores' temperature will continue increase until the temperature of the oil bath. In Figure 49 only pressures allowing some water vaporization were presented, i.e, until 3 bars.

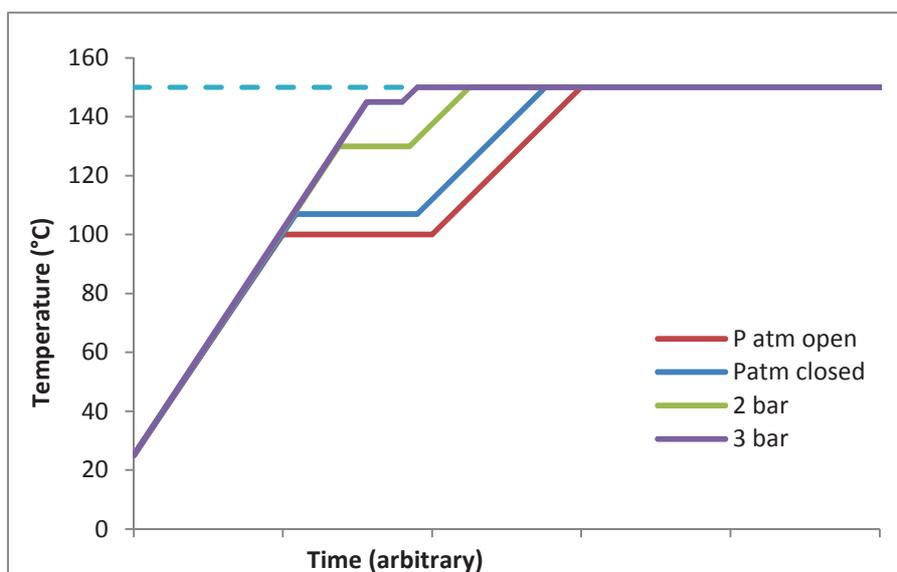


Figure 49. Temperature evolution in the spores during heating and phase changes.

The effect of pressure in spore inactivation is related to the inhibition of evaporation of initial spore water. As demonstrated in the DSC study, maintaining the spore water greatly increases the efficiency of a heat treatment. Unlike other treatments, as steam application, where water is added, in the proposed technique the water from spore itself is used to maximize the destruction without any increase in moisture. This is an important aspect since often dried food products need to go through a drying step after decontamination, increasing the costs and the damages to the product.

4.2.4 Effect of a_w on spore destruction

4.2.4.1 Dried media

Dried food products and food powders have normally a water activity ranging from 0.20 to 0.70 so it is important to investigate the effect of this variable on the proposed treatment.

The influence of water activity on the inactivation of spores is presented in Figure 50. It is clear that the water activity and consequently the water content of the spores play a fundamental role in dried spores' resistance.

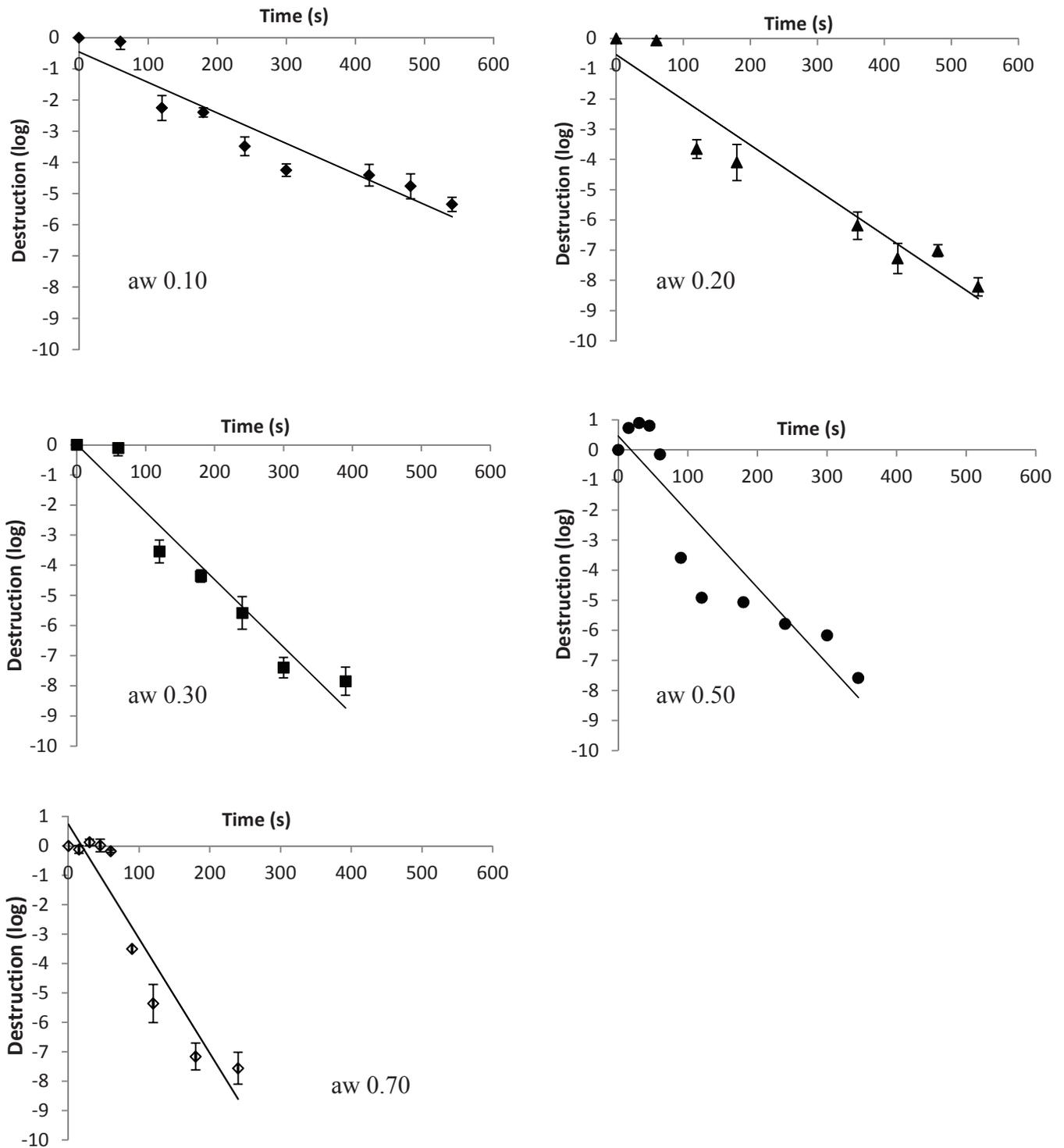


Figure 50. Destruction of *B. subtilis* spores equilibrated at different water activities (0.10, 0.20, 0.30, 0.50 and 0.70) after a treatment at 150°C and 6 bar.

Spores equilibrated at a_w of 0.10 presented an inactivation of 5 logs after 560s against 8 logs from spores at a_w of 0.20 at the same time. When water activity was increased there was an increase in the inactivation of spores that can be easily seen by the reduction of the D-values

(Table 13). The same increase in water activity does not result in a proportional increase in the spore inactivation, for example there is only a small difference between the spores at a_w 0.30 or 0.50, but the further increase to a_w of 0.70 resulted in a complete inactivation after only 240s. Consequently, it can be assumed that the same order of inactivation can be reached when spores are equilibrated in water activities between 0.30 and 0.50.

It is important to emphasize that with the application of pressure the treatment times are greatly reduced, even in low water activities, 0.10 and 0.20 it is possible to reduce 5 logs in around 9 and 6 minutes, respectively.

Table 13. Decimal reduction times (in minutes) for *B. subtilis* spores according to their water activity

a_w	D (min)
0.10	1.70
0.20	1.12
0.30	0.74
0.50	0.67
0.70	0.43

4.2.4.2 Liquid media

The presence of solutes in the treatment medium decreases the a_w of the medium and therefore protects spores from heat. However, solutes also have specific effects on spores, sometimes reducing the thermal resistance of spores. This antagonism makes it difficult to predict the effect of the addition of a solute in spore inactivation (Mazas et al. 1999).

As shown in Figure 51, the water activity of the treatment medium had a major effect in spore inactivation. When treated in higher water activities, spores were more sensitive to heat. Spores treated at a_w 0.10 or 0.20 presented a 2 log reduction after 240 s whereas spores at a_w 0.30, 0.40 and 0.50 presented a 6 log inactivation after 180 s.

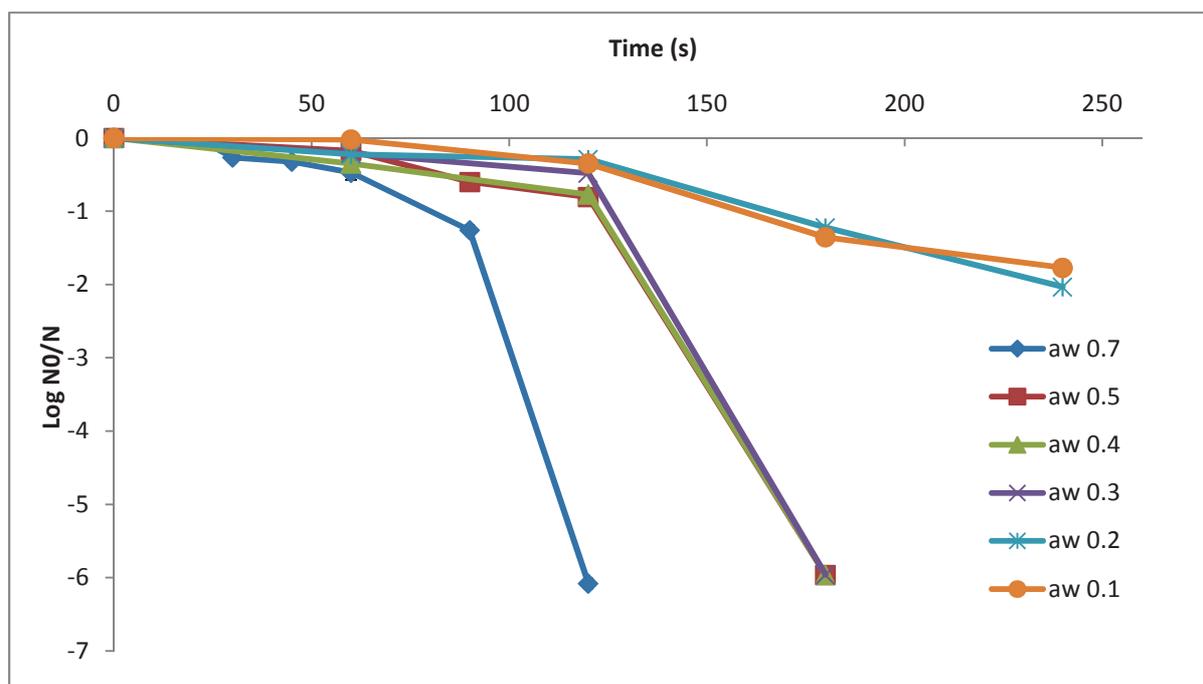


Figure 51. Spore inactivation at different water activities in liquid media (water/glycerol) after a treatment at 150°C and 6 bar.

Spore inactivation has begun at different treatment times depending on the a_w of the medium, and later then the observed for spores treated in dried form. This can be explained by the heating curves inside the reactor, as presented in the Results section (Figure 18). The temperature inside the reactor reached the oil bath temperature in less than 10 s when air/nitrogen was the transmitting media, however more than 100 s were needed for pure glycerol to reach the same temperature. Water heating was faster, the coldest part of the reactor reached 150°C after approximately 60 s. Observing Figure 51, it is apparent that at a_w 0.7, where there is a higher amount of water, inactivation was noticeable between 60 and 90 s but in lower a_w inactivation started only after 120 s of treatment. It would be possible to reduce the treatment time if the heating period was shorter; one solution is the addition of an agitation system inside the reactor to increase the heat transfer coefficient.

Comparing these results with those from dried spores, we observe that at the same water activity and treatment time, the inactivation was similar. However, the dried spores reached the treatment temperature faster than those in liquid media so, if this difference is considered, the treatment in liquid media at the same water activity resulted in higher inactivation.

4.2.5 Mechanisms of inactivation

Some tests were performed in order to elucidate the mechanisms of inactivation of spores during the treatment at high temperatures and under pressure. It is assumed that spores that underwent heat treatment in the reactor suffered the same damage to spore proteins as already demonstrated by FTIR.

We chose to study mutagenesis of wild-type spores and $\alpha\beta^-$ spore mutants by this heat treatment because dry heat have been shown to be mutagenic for both wild and $\alpha\beta^-$ spores, while wet heat treatment will cause mutations only in $\alpha\beta^-$ spores. We have investigated the presence of nalidixic acid-resistant (nalr) mutants *gyrA* gene that can be used as an estimation of the overall frequency of mutation in the spore (Huesca-Espitia et al. 2002). The spores used in this experiment were lyophilized, which can by itself induce some level of mutation, especially in the absence of SASP in the spore core. In Table 14 the frequency of mutation in non-treated and treated spores is presented.

Table 14. Frequency of nalr mutations in spores of *B.subtilis*

Spore	Control	After 5 min/150°C/6 bar
Wild-type <i>B.subtilis</i> 168	2.6×10^{-7}	2×10^{-6}
<i>B. subtilis</i> $\alpha\beta^-$ spores	3.1×10^{-5}	1.5×10^{-4}

It was observed the mutation frequency increased in both wild-type and $\alpha\beta^-$ spores which suggests that the treatment in question has an effect in spore's DNA. It was concluded that the inactivation of spores treated in a moist atmosphere (Pan B) is related to protein denaturation and here it was demonstrated that the equivalent treatment performed in the reactor causes DNA mutation. However, DNA mutations are not observed in wild spores treated with moist heat, therefore, from these results it can be suggested that the mechanisms of spore inactivation in the reactor combine protein denaturation and DNA damage.

Spore recovery following some heat treatments is also often lower when spores are further stressed by the addition of high salt to recovery media (Adams 1978; Russell 1982; Hurst 1983). Heat treated spore consistently showed a poor recovery (1 log reduction) when plated in a medium containing 6% NaCl which suggests inner membrane damage that is usually found in spores treated with moist heat.

We have also tested the spore resistance to a subsequent mild heat treatment (80°C/20 min) to check for germination during treatment or decreased heat resistance. However no difference in viability was found for wild spores treated at 150°C, 5 min at 5 bar, thus spore inactivation cannot be attributed to germination during or after the treatment in the reactor.

- Spores were treated in high temperatures under a few bars of pressure to avoid water evaporation and its viability was assessed.
- A global analysis of the system showed that an increase in pressure, temperature or a_w will increase spore inactivation.
- The treatment performed at $P_{atm}/150^\circ\text{C}/2 \text{ min } 0/a_w 0.50$ did not differ from the control sample, however at 2 bar there was a 3 log reduction on spore population and at 7 bar around 7 log of spore were destroyed.
- Spores equilibrated at $a_w 0.30$ and 0.50 and heat treated at 6 bar/150°C/3 min showed an inactivation of 4.5 and 5.5 log, respectively.
- The pressure inside the reactor will increase as the reactor is heated and also due to water evaporation from the sample when $P_f < P_{vap}$.
- Higher initial water activities lead to a higher inactivation in both dry and liquid media.

4.2.6 Discussion

Nowadays, commercial and legal requirements regarding the safety, quality and storage of food products are focused on the development and improvement of decontamination methods. However the destruction of bacterial spores is rather difficult and most of the techniques commonly used to treat dry foods result in a very low spore inactivation.

The most often used decontamination techniques for dry products are steam thermal processing, irradiation and fumigation. However, steam adds undesirable moisture to dry foods and changes in color, fumigation has been banned in most countries due its toxicity and irradiation is strongly rejected by the consumers. In addition, these decontamination techniques cause loss of quality and sometimes low inactivation rates. As a consequence, there is a need for the development of innovative technologies for the production of high quality and safe dry food ingredients, as herbs and spices for example.

Even though spices are non-perishable ingredients, once they are put in water rich food products, their natural or contaminant flora can quickly develop and multiply consequently

putting in risk the health of consumers. This is an important issue especially when spices are added to ready-to-eat foods which are not subjected to further heat treatments (Van Doren et al. 2013).

Different techniques have been studied for the decontamination of dry foods, including spices. Staack et al. (2008) investigated the effect of infrared heating on the inactivation of *B. cereus* in paprika powder and showed that the spore reduction was dependent of a_w but spores were only significantly inactivated at very high a_w (>0.80). Nevertheless the maximal reduction was less than 2 log and this low number was attributed mainly to spore drying during the treatment. The use of pulsed light also showed very low microbial reduction on the treatment of spices, even for vegetative cells of *Bacillus* (~1 log) (Nicorescu et al. 2013). Other techniques as microwaves, high pressure CO₂ and high pressure were shown to have little effect on microbial inactivation in low moisture media. As a result, their use would be conditioned to a previous increase of moisture content of the food to be treated which would lead to the same problem found with the use of steam.

The technique developed in this work presents some advantages regarding other procedures as:

- High microbial destruction even at low a_w : the inactivation is dependent of the initial a_w but even in low a_w levels (<0.30) there is a considerable spore reduction and at normal a_w levels for food powders (0.30-0.50) we observed an inactivation superior to 5 log in short treatments (3 to 4 minutes).
- No increase in water content: despite its efficiency, steam treatment adds water to the product as a result of vapor condensation thus a subsequent drying step must be performed to avoid mould growth and product spoilage. Our technique works by keeping the original water content in order to increase spore destruction.
- No simultaneous drying of the product: common dry heat and infrared heat treatments result in the evaporation of the water initially present in the product, therefore decreasing spore inactivation. In our case, the use of a counter pressure increased the temperature of water vaporization in the system and prevented the drying of the spores.

The last item was considered to be the key of spore inactivation in dry media as shown both in the DSC study as in the results from the reactor. The treatment must be performed under

pressure in order to avoid drying, but as the interior pressure increases upon heating the initial pressure level can be lower than the level required to avoid water vaporization. For example, at 150°C the P_{vap} is 5 bar but, as the thermodynamic analysis showed (Table 11), a P_i of 4 bar is enough to prevent drying since at 150°C the P_f will be 5.68 bar, i.e., higher than P_{vap} . For treatments at different temperatures the same considerations must be made.

However the current study has its limitations. First, the treatments were made using only dried spores and not real food products. Therefore tests must be conducted using different food matrices inoculated with *Bacillus* spores to check the levels of inactivation achieved and also the possible changes in food quality parameters (color, aroma, volatile compounds, and functionality, etc.).

Another limitation is the use of only one bacterial spore specie, even if two strains were used. *B. subtilis* is normally used as a model for other pathogenic spores but different species and even different strains can present a higher or lower resistance to the treatment. In addition, the use of different *B. subtilis* mutants could provide a deeper knowledge of the mechanisms involved in their destruction in this reactor.

Finally, the system was developed in a laboratory scale and consequently it is not optimized. In order to treat food matrices, a larger volume reactor must be constructed with an agitation system to ensure a homogeneous heating and avoid over processing of the product closer to the walls.

CONCLUSION & PERSPECTIVES

CONCLUSION AND PERSPECTIVES

The focus of this study was the comprehension of the factors and mechanisms involved in the heat resistance of dried spores. The dissertation was divided in two main parts: first, a fundamental study of spore's water content and distribution and its effect on spore heat resistance followed by a second part that describes an application of these findings. The originality of the work lies on the method chosen to analyze dried spores (Differential Scanning Calorimetry) and in the development of an application from the fundamental results. This conclusion will reiterate the main findings and point out future directions and perspectives regarding the developed heat treatment system.

DSC

Even though DSC had already been used for the analysis of spores, the focus of these works was mainly the glass transition, i.e., a classical application of calorimetry. In this study, the DSC pan was thought of as a reactor that allowed the heat treatment in extremely controlled conditions and the recovery of different data on the state of the spores during and after treatment, especially the relation between spore viability results and spore's thermograms and weight loss. Moreover, the use of two pans with different pressure resistances made possible the modulation of the temperature of water evaporation from the spores, creating consequently, two very distinct heat treatments.

The first important result is the remarkable water content in extremely dry spores ($a_w = 0.13$) and also that most part of the water is probably located in the core. In the experiment using a pressure resistant pan (Pan B), two different events related to water evaporation were observed and they were associated with external and internal water. Interestingly, the second event represented a fraction of water that evaporated around 200°C which is the equivalent of a vapor pressure of 16 bar.

It was estimated that in fully hydrated spores 30% of water is located in the core and 70% in the outer layers (Kong et al. 2012). However when spores are dried water from the outer layers will leave before there is any change in core water (Maeda and Koga 1981; Ishihara et al. 1994; Kong et al. 2013) due to the low inner membrane permeability (Cowan et al. 2004). Consequently, as water transfer is very low, the equilibrium time will be very long and thus spores will have a higher proportion of water inside the core at low water contents. Moreover,

we have observed that at low water activities (<0.3) the amount of water evaporated in the DSC was higher than the value given by the sorption isotherm of the same strain (Nguyen Thi Minh et al. 2010). This result suggests that the outer layers and the core have different water activities, and the value obtained is actually the external water activity. Therefore, although being an important process parameter, water activity does not represent the best estimation of spore water content.

This study also showed the crucial effect of the water content on spore heat resistance, spores equilibrated at different water contents presented distinct inactivation profiles. Spores with higher water contents presented a higher inactivation for the same heat treatment. Furthermore, the two pans produced different treatments since in Pan A there was a long drying phase starting at low temperatures whereas in Pan B this drying phase was short and begun at very high temperatures. Consequently, spores that were heat treated and dried simultaneously (Pan A) were much more heat resistant than those that kept the initial water content until high temperatures were reached (Pan B).

The difference between those treatments was explained based on the FTIR spectra of treated spores. The spores that lost their water (Pan A) maintained its protein structure almost intact, little protein damage was observed, especially when very dry spores were used. However, spores that kept its initial water content (Pan B) showed major modifications in protein structure and they lost all the DPA from the core suggesting that spore conformation was greatly affected by this treatment. These results confirm that simply by keeping spore's own water during the dry heat application can completely change the mechanisms of spore inactivation. Therefore, the low inactivation associated with the minimal protein modification confirms that low water contents protect spores from heat by minimizing protein denaturation.

Dry heat vs. moist heat

Until now it was well known that moist heat was more efficient than dry heat in the destruction of bacterial spores. However, considering all these findings we think that this hypothesis must be re-discussed and we bring a different view regarding the inactivation of bacterial spores. We propose that it is in fact the level of hydration of the spore that determines its destruction by heat at temperatures over 100°C. It is reasonable to think that the treatment by moist heat allows the hydration of spores, which contributes to spore destruction,

whereas the conventional dry heat treatment causes the spore to dehydrate thus making spores less sensitive to the treatment.

To verify these hypotheses, two different experiments could be proposed. In the first one, very dry spores (<10% water dry weight) are submitted to a very short moist heat treatment. Since the time necessary to hydrate spores is sufficiently long we can assume that they will remain “dry” during treatment, and as it was shown, their resistance increases remarkably at very low water contents. Consequently, this treatment is expected to result in a very low mortality rate. In this case it would be demonstrated that a moist heat treatment by itself will not destroy dry spores.

The second experiment would be the opposite: fully hydrated spores are to be treated with dry heat at high temperatures and short time. Hydrated spores are more sensitive to heat treatments, and as said before, the time to equilibrate spores at low water contents is quite long hence the treatment time should be longer than the time necessary to increase spores temperature. Briefly, as long as the treatment has a short duration and does not affect spore’s water content, they will be very sensitive to heat and very high mortality rates are expected. Thus in this case it would be demonstrated that dry heat can inactivate moist spores.

Reactor

From the interesting results obtained with the DSC study, we have developed a Proof of Concept in order to verify the findings and test its feasibility. Therefore, to avoid water evaporation from the spores a counter pressure was used with the addition of gaseous nitrogen. The new system proved to be very efficient in the inactivation of dried *Bacillus* spores as a high inactivation was achieved with quite short treatment times (5 to 6 log in a few minutes).

Steam treatment is extensively used nowadays for microbial decontamination of dry foods but it present problems since there is water condensation on the product that needs to be removed to avoid subsequent mould growth and spoilage. Moreover, other treatments available in aqueous medium are not efficient in low water activities as high pressure and pulsed electric fields. So new procedures for the decontamination of dried foods that are able to destroy spores are needed and our system is a good candidate.

The model system described here can be used for the treatment of low water content foods, it has the advantage of not adding moisture to the product and it results in high spore destruction rates (< 5 log). The next step would be to develop a scale-up version of the system to perform test in real food products in order to evaluate both microbiological decontamination and changes in quality parameters.

This pilot could then be used to treat products like spices for example. The a_w normally found in spices is between 0.30 and 0.50 and as it has been showed, an inactivation higher than 5 logs could be expected with a 3 minute treatment at these water activities. Therefore if the spices are already around this a_w no previous equilibration is needed, however if the product to be processed has a very low a_w , an equilibration step would be necessary. In this case, the spices would be stored in an atmosphere with a relative humidity of 50% for a few weeks and then they would be treated. Certainly, different conditions of time, temperature and pressure need to be tested to optimize this new decontamination procedure

In this way, we are currently working on the deposit of a patent of this miniature system which will be achieved by the end of the year.

ANNEX

Water Distribution in Bacterial Spores: A Key Factor in Heat Resistance

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Abstract The role of water, its distribution and its implication in the heat resistance of dried spores was investigated using DSC (Differential Scanning Calorimetry). *Bacillus subtilis* spores equilibrated at different water activity levels were heat treated under strictly controlled conditions. The temperature was increased linearly in pans with different resistances to pressure. Data from the heat-related transitions occurring in the spores were recorded and spore viability was assessed at different stages during DSC. The thermodynamic transitions observed were related to the water status in the spores and spore survival. The results demonstrated that water still remained in the spore core when water activity was as low as 0.13. The first transition occurred at around 150 °C and was assumed to be related to a mobile fraction of water from the outer layers of the spore. The second occurred at around 200 °C, which could correspond to a fraction of water embedded in the spore core. Moreover, the results showed that spore destruction during heating was favored by the amount of water remaining in the spore. The changes in their structure were also evaluated by FTIR (Fourier Transform Infrared Spectroscopy). This work offers new understanding about the distribution of water in spores and presents new elements on the heat resistance of spores in relation to their water content.

Keywords Spore water content · Inactivation · Heat treatment · Calorimetry

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Introduction

Bacterial endospores are resistant to multiple forms of stress such as heat (wet and dry), pressure, UV radiation, chemicals and desiccation that would normally kill vegetative bacteria [1–3]. Consequently, they can survive in dried foods and can be found in quite high concentrations (exceeding 10^2 – 10^5 spores/g) in some dried ingredients such as spices, cocoa powder, milk powders and cereals [4–6]. Their survival represents a problem when dried products are added to high-moisture foods which may cause them to sporulate leading to the deterioration of food or food-borne diseases.

The factors involved in spore resistance have been partially explained by its structure and the presence of specific internal components. Endospores present a multilayered structure very different from that of growing cells [1] and different layers play a role in spore resistance. The spore's coat protects it from chemicals and lytic enzymes and also acts as a permeability barrier [7]. The cortex and the underlying germ cell wall are essential in maintaining the integrity of the spore inner membrane. The inner membrane exhibits low permeability to small molecules, perhaps even water, which is one factor involved in the resistance of spores to some chemicals, in particular DNA damaging chemicals. The final layer is the core that contains the spore DNA, RNA and most enzymes. The high level of Ca-DPA (dipicolinic acid) (25 % of core dry weight), the saturation of DNA with a/b-type small, acid-soluble spore proteins and the low water content in the core (25–50 % of wet weight depending on the species) all contribute to spore resistance properties [8].

It is believed that the remarkable resilience of spores to severe stress is a direct consequence of the low water content within the spore core which in aqueous medium contains only a fraction of the water found in the respective vegetative cell [9]. The core water content is also inversely related to the heat resistance of the spores; spores with less water in the core are more heat resistant than those with higher water levels [10]

11]. Nevertheless, such comparisons have not been made on dried media.

Some authors have suggested that the spore core water is in a glassy state, which would explain the protection of proteins against heat denaturation and its consequent resistance [12, 13]. Several attempts to detect and prove the glass-like state have been unsuccessful [14, 15] until a recent report showed that the spore core water mobility level is too high for such a state to occur [16].

Some of the methods that have already been used to characterize spore water include refractive index measurements [17], water sorption and desorption [18–21], wet and dry density [22], nuclear magnetic resonance spectroscopy (NMR) [12, 16, 21, 23, 24], Time Domain Reflectometry (TDR) [25, 26] and DSC [12, 13, 15, 27].

DSC has been used to determine the thermally induced transitions with the purpose of evaluating the relationship between the stability of spore components and cell injury or death [28–30]. However, none of these studies have investigated the effect of water content on the thermal transitions and neither have they associated water content with spore inactivation using DSC. Another limitation of the previous studies is that they analyzed mostly fully hydrated spores or the water activity of the spores was not controlled during treatment [12, 15, 27].

FTIR is a useful technique to identify and characterize vegetative cells and spores based on their chemical composition and also to point out structural and biochemical changes in the spore after an inactivation treatment [31]. FTIR combined with multivariate analysis was already used to identify biochemical changes in spores treated by autoclaving and pressure-assisted thermal processing [32, 33].

Bacillus spores are extremely resistant to inactivation treatments, especially when they are in a dry state. Therefore, the aim of this work was to better understand the role of water on spore resistance to heat. We have used a calorimetric technique to analyze water evaporation from dried spores and the corresponding spore inactivation at different water activities. The structural modifications on spore were evaluated in the different treatment conditions.

Material and Methods

Bacterial Strains and Growth Conditions

Two *bacillus* strains were used to allow further comparison of the results obtained: *B. subtilis* ATCC 31324 (DSMZ 704) obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and *B. subtilis* 168 from the BGSC (Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, USA).

B. subtilis sporulation was induced in a complex medium in a reactor at 37 °C, pH 8.0, with an air flow of 4 L/min and under agitation (450 rpm) [34]. Sporulation was estimated by plating on Bromocresol Purple agar (BCP) (Dextrose Tryptone agar, BIOKAR Diagnostics, Beauvais, France) before and after a heat treatment at 80 °C for 10 min. When more than 95 % of the population in the sample resisted this heat treatment, the spore suspension was harvested (3–5 days). The spore suspension was washed four times with sterile distilled water, spray-dried (Mini spray dryer B-290, Buchi, France) and stored in powder form in sterile recipients at 4 °C until use. The final microbial concentration of the powder was approximately 10¹¹ colony-forming units per gram (CFU/g) and the water activity of the spore powder was around 0.55.

Equilibration of Spores at Different Water Activities

Dried spore powders with water activities ranging from 0.13 to 0.50 were obtained by placing the spores (±500 mg) inside 1 L airtight plastic boxes containing saturated salt solutions (salt solution occupied 1/10 of the box's volume) until equilibrium was reached (at least 1 week) [19]. No difference in spore viability or change from phase bright to phase dark was observed after the equilibration of water activity. The water activity of the saturated solutions and spore powders was determined in triplicate at 25 °C using a Decagon-AQUALAB CX-2 osmometer (Pullman, USA). Lithium bromide, lithium chloride, potassium acetate and sodium bromide were used to equilibrate the spores at water activities of 0.06, 0.11, 0.22, and 0.58, respectively [35].

Differential Scanning Calorimetry

The experiment was based on the thermal treatment of spores in closed pans with different pressure resistances. Three kinds of experiments were carried out using the calorimeter. Firstly, the temperature of water evaporation was assessed using a mass of 1–3 mg of pure distilled water. Two aluminum pans (TA Instruments, USA) with different pressure resistances were used: Tzero™ with non-hermetic lid (901683.901/901671.901) and Tzero™ with hermetic lid (901683.901/901684.901) and which will be referred to hereinafter as Pan A and Pan B, respectively. Secondly, spores with different water activities were heated in the two pans to obtain their thermograms. The pans were then rapidly cooled to the initial temperature and immediately rescanned to determine the reversibility of the transitions. Samples were reweighed after DSC measurements to check for weight loss during heating. The third experiment consisted in evaluating spore inactivation after a heat treatment in the calorimeter. Spore samples (about 5 mg) were weighed in the pans and sealed using the Tzero DSC Sample Encapsulation Press (TA Instruments, USA).

The thermal analyses were conducted in a Q-20 Differential Scanning Calorimeter (TA Instruments, USA) equipped with a Liquid Nitrogen Cooling System (LNCS). A thermogram with an empty and a reference pan was collected to measure the baseline. The instrument was calibrated for cell constant and temperature using Indium as the reference standard (melting temperature $T_m=156.6\text{ }^\circ\text{C}$ and melting enthalpy 28.54 J/g). Scans were performed at temperatures ranging from 25° to $220\text{ }^\circ\text{C}$ at a heating rate of $10\text{ }^\circ\text{C/min}$ under nitrogen flow (50 ml/min).

The weighing of dehydrated spores requires great precaution to avoid re-absorption of water after heating. To avoid this problem the DSC chamber had a constant nitrogen flow to remove all water vapor released during heating. In any case, if some water re-absorption took place it would only have induced an underestimation of the water loss.

Spore Viability

Heat treated spore samples were serially diluted with physiological saline ($0.9\text{ }\%$ NaCl w/v) and $100\text{ }\mu\text{l}$ of each dilution was inoculated in triplicate on BCP agar plates. Colonies were counted after 24 h at $37\text{ }^\circ\text{C}$. Longer incubations times (48 h – 72 h) did not lead to an increase in CFU/g. Control samples contained spores that did not undergo any treatment and all experiments were performed at least in triplicate. Spore inactivation was expressed using a logarithmic reduction factor $\log N_t/N_0$, where N_0 was the CFU/g before treatment and N_t the CFU/g after treatment.

Fourier Transform Infrared Spectroscopy

FTIR was used to assess structural and biochemical changes in *B. subtilis* 168 spores caused by the heat treatment performed in the DSC. IR spectra were recorded between $4,000$ and 800 cm^{-1} at a resolution of 8 cm^{-1} on a Bruker Vector 22 FT-IR spectrometer (Bruker Optics, Germany) using an Attenuated Total Reflectance (ATR) unit [36]. Spores heat treated in the two different pans and untreated samples were stored under refrigeration and analyzed within 2 days. Dried samples were diluted in distilled water (10^8 CFU/ml) and washed four times. A $10\text{ }\mu\text{l}$ aliquot of the spore solution was placed on the ATR cell and dried under nitrogen flow at ambient temperature to form a thin film. Ten spectra were collected for each spore sample. The resulting spectra were checked for major water peaks, and if it appeared that some solvent remained in the spectrum the film was further dried. The spectral range between $1,800$ and $1,200\text{ cm}^{-1}$ has been reported to contain almost all signals of interest in studying spore inactivation treatments [33], so only this region was analyzed. The raw spectra were transformed into their second derivatives for analysis to remove baseline shifts, improve peak resolution and reduce variability between replicates

[37]. OPUS 3.0 software (Bruker Optics, Germany) was used to control the spectrometer and to acquire and manipulate spectra.

Results

Determination of Pan Resistance with Pure Water

The two pans worked as reactors enabling a strictly controlled heat treatment. They presented different behavior regarding the water vaporization temperature as shown in Fig. 1. In Pan A the peak corresponding to water vaporization was broad, ranging from 60 to $110\text{ }^\circ\text{C}$, whereas in Pan B the peak was narrow, ranging from 155 to $170\text{ }^\circ\text{C}$. The pressure resistance of the pans was then determined from the vaporization temperature of water in each pan and they are presented in Table 1. In Pan B there was a pressure increase of 5 to 6 bars causing water to vaporize at higher temperatures whereas in Pan A vaporization occurred at atmospheric pressure.

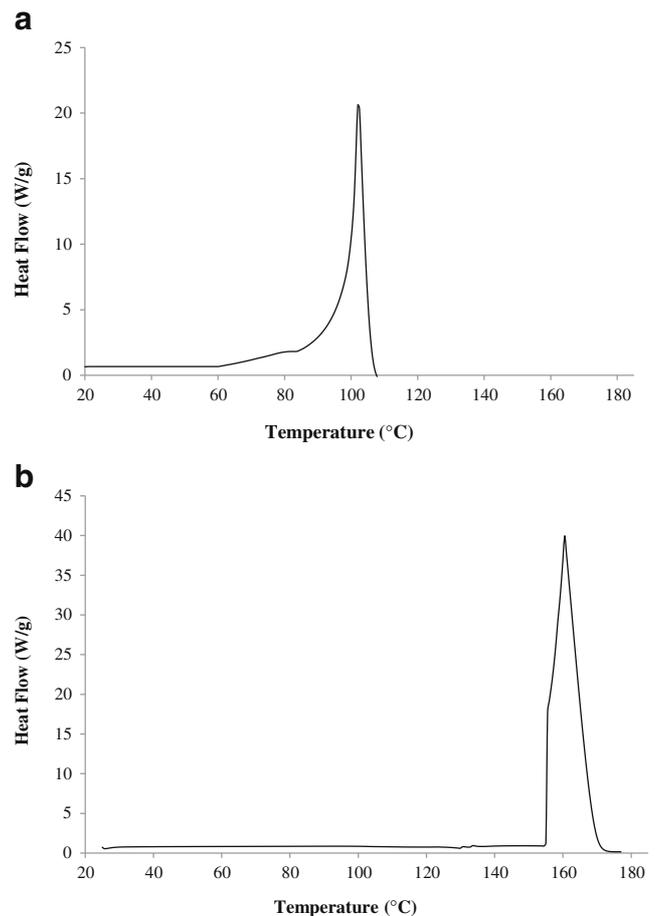


Fig. 1 Thermograms of pure water in **a** Pan A and **b** Pan B. Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis)

Table 1 Temperature of evaporation of water in the pans studied

Pan	Evaporation temperature range	Pressure	Enthalpic energy
Pan A	60–110 °C	1 bar	558±48 J/g
Pan B	155–170 °C	5–6 bars	1,848±47 J/g

The determination of the water boiling point and its enthalpy of vaporization are directly related to the pressure resistance of the pan, as can be seen in Table 1. In Pan A the enthalpy was much lower than the expected value for water at 100 °C, (enthalpy of water vaporization=2,260 J/g). This is a common problem when working with open or non-hermetic pans [38, 39] and can be explained by the beginning of water evaporation at relatively low temperatures due to a difference in the vapor pressure between the pan and its surroundings.

Contrary to the result in Pan A, in Pan B the liquid water and most of the vapor formed remained in the pan (self-generated atmosphere) during heating. This increase in temperature involved an increase in the water vapor pressure in the sealed pan as well as an increase in the water boiling temperature and thus the water remained mainly liquid in the pan above 100 °C. Then, as the seal broke the pressure release resulted in an immediate and total vaporization of the water as it suddenly left the pan, thus producing a very narrow DSC peak.

Spore Thermograms

Dried spores were heat treated in the two pans (A and B) and their thermograms are presented in Figs. 2 and 3.

The thermograms in Pan A (Fig. 2a) showed only one broad endothermic peak, which ranged from 60 °C to 160 °C and was

Fig. 2 Thermograms of the two strains of spores after equilibrium at different water activities in Pan A. **a** *Bacillus subtilis* ATCC 31324, **b** *Bacillus subtilis* 168. *I*—First heating. *II*—Second heating (not shown in **(b)**). Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis)

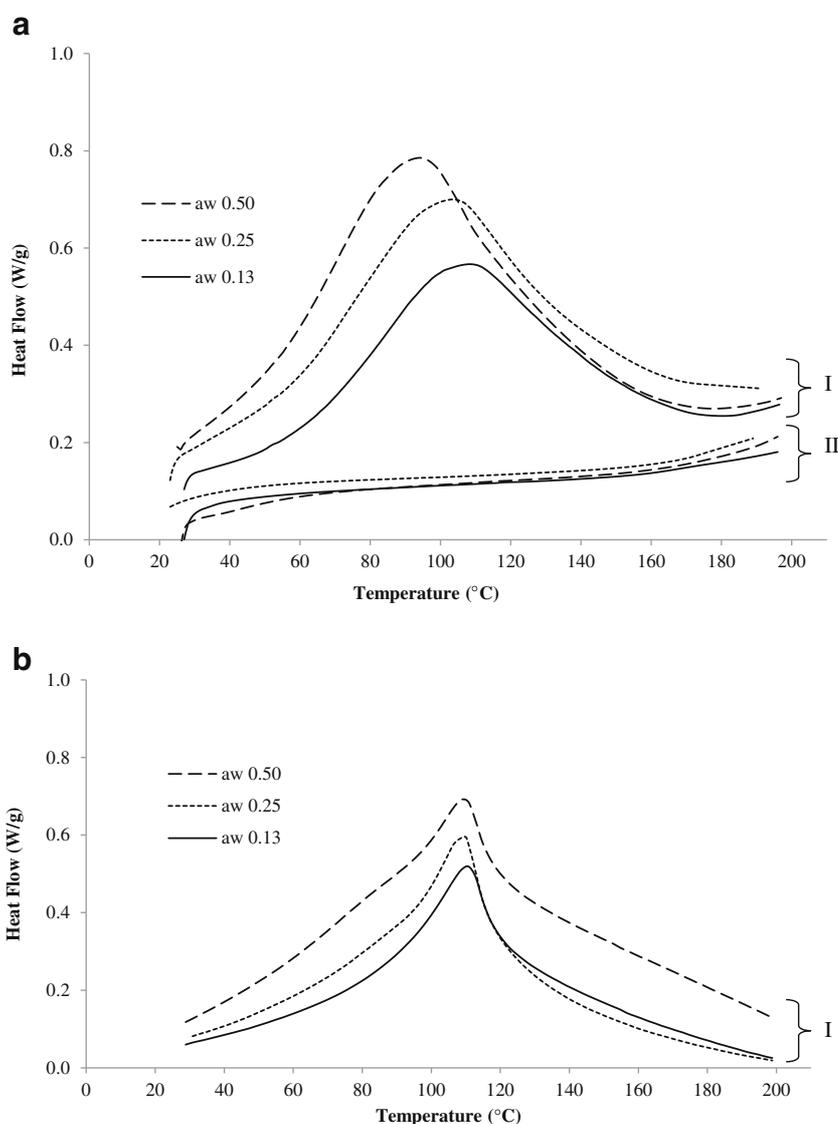
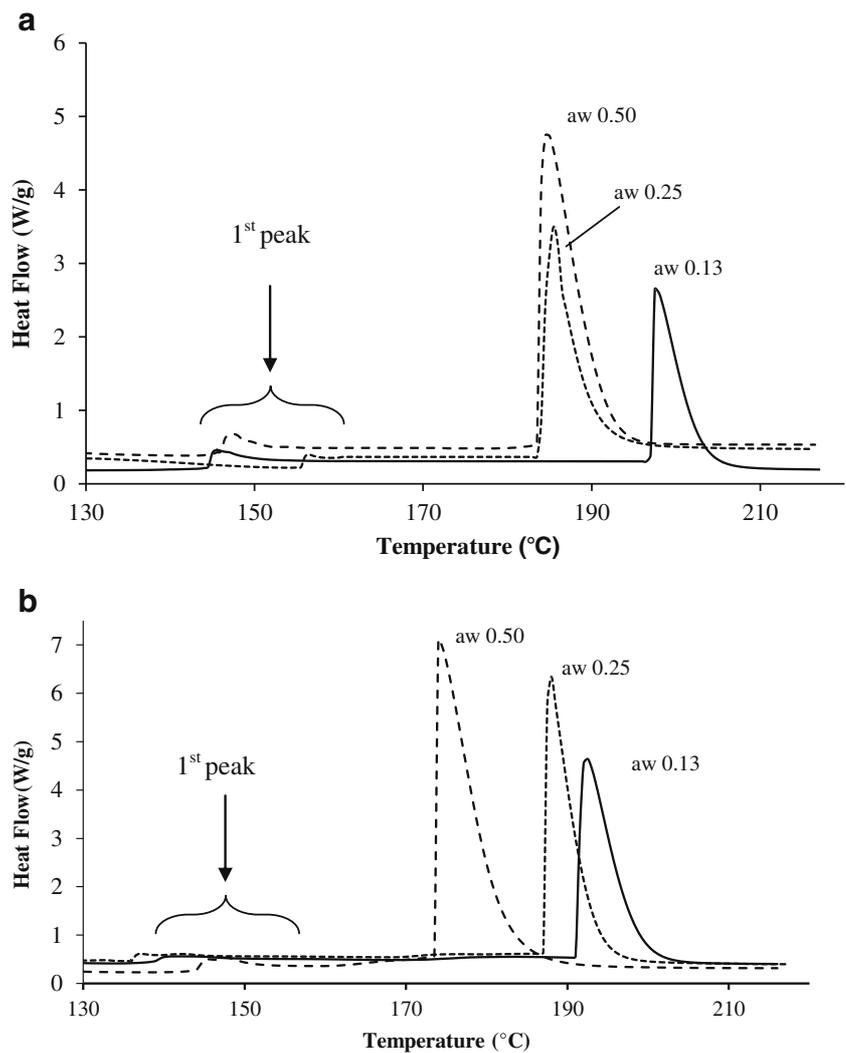


Fig. 3 Thermograms of **a** *Bacillus subtilis* ATCC 31324 spores and **b** *Bacillus subtilis* 168 spores equilibrated at different water activities in Pan B. Endothermic events are equivalent to increasing heat flow and exothermic events to decreasing heat flow (y-axis)



centered around 100 °C. No transition was detected in the second heating of the spores in Pan A (see Fig. 2a) indicating that the reactions were irreversible. By comparing Fig. 2a and b, the same transition was observed for both strains but *B. subtilis* 168 had a higher associated enthalpy than *B. subtilis* 31324. The thermograms also differed in their shape; strain 168 had a sharper peak while strain 31324 had a rounder peak. The decrease in water activity caused a slight increase in the maximum temperature of the peak for both strains. In Pan B (Fig. 3) the first thermal event only took place above 150 °C (same temperature as vaporization of pure water in pan B, shown in Fig. 1), and the peaks were more defined, consequently not overlapping other minor peaks.

The initial scan profile in Pan B showed two major events, one at around 150 °C and the other at around 200 °C. The peak observed at 150 °C corresponded to the pan seal breaking as a pressure of 5 bar was reached inside the pan. Moreover, the enthalpy values related to these transitions were clearly related to the water activity of the sample, as shown in Table 2. It can

therefore be assumed that these transitions observed are related to water evaporation in the same way as in Pan A. As with

Table 2 Enthalpic energy of the thermal event observed between 60 and 160 °C in Pan A and between 170 and 220 °C in Pan B

aw	Enthalpic energy (J/g)	
	<i>B. subtilis</i> ATCC 31324	<i>B. subtilis</i> 168
Pan A		
0.13	121±12	195±11
0.25	131±14	–
0.50	184±5	302±40
Pan B		
0.13	70±9	108±9
0.25	96±10	166±19
0.50	113±5	205±17

pan A, no peaks were observed in the second heating (data not shown).

The thermograms of the two strains presented the same transitions in both pans (see Fig. 3a and b), only differing in the enthalpy associated with the water thermal transitions. The difference was particularly marked in Pan B where the peak at 150 °C on the *B.subtilis* 168 thermogram had an associated enthalpy inferior to strain 31324 and on the contrary, the peak at 200 °C had an enthalpy superior to the latter.

The mechanical characteristics of the pan were crucial in obtaining these results. Considering that the interest was to study water vaporization, it was essential to use a pan that allowed that, i.e. a pan with limited pressure resistance.

Weight Loss After Heat Treatment

In Pan A, a gradual weight loss due to water evaporation was observed (Table 3), confirming the drying of the sample during the treatment. When the temperature reached 110 °C, 5.9 to 9.4 % (w/w) of the water had already evaporated and at 200 °C around 10 to 16.1 % (w/w) of the total weight had been lost, depending on the initial water activity and the strain.

Table 3 Weight loss after heat treatment using DSC

aw	Pan	Temperature	<i>Bacillus subtilis</i> ATCC 31324 Weight loss ± SD	<i>Bacillus subtilis</i> 168
0.13	A	110	5.9±0.6	6.9±1.1
		130	8.1±0.7	7.3±1.2
		160	8.2±0.8	10.7±0.2
		220	10.0±0.1	11.5±0.7
	B	130	0	0
		160	0.58±0.1	0.7±0.2
		220	8.1±0.9	11.4±1.1
		220	10.9±0.3	14.2±0.9
0.25	A	110	6.4±0.7	9.3±0.6
		130	7.8±0.6	11.8±0.5
		160	8.0±0.1	12.1±1.2
		220	10.9±0.3	14.2±0.9
	B	130	0	0
		160	0.9±0.6	0.7±0.3
		220	9.7±2	13.9±0.9
		220	11.2±1.5	16.1±0.7
0.50	A	110	6.3±0.5	9.4±0.7
		130	8.5±0.1	12.3±0.1
		160	9.4±0.3	14.2±0.6
		220	11.2±1.5	16.1±0.7
	B	130	0	0
		160	2.5±0.8	0.6±0.2
		220	11.9±1.5	17.4±0.3
		220	11.9±1.5	17.4±0.3

Weight loss expressed in g of water/100 g dry matter

Pressure in each pan: A=1 bar and B=5 bar

Above 220 °C there was no further weight loss (results not shown).

As expected from the thermal transitions, there was no weight loss in Pan B before the peak at around 150 °C (Fig. 3). After this peak, the weight loss varied from 0.6 to 2.5 % (w/w) depending on the initial water activity and the strain. Surprisingly, the greatest weight loss was observed after the second peak, at 200 °C, ranging from 8.1 to 17.4 % (w/w) of the initial weight depending on the initial water activity and the strain.

The total weight loss for strain 168 was superior to that of strain ATCC 31324 in both pans and was also distributed differently. The weight loss for strain 168 was lower than for ATCC 31324 after the first peak (0.3–0.5 % w/w) and higher after the second peak (12–14 % w/w).

Viability Loss After DSC Heat Treatment

Spore viability was evaluated after heat treatment in the calorimeter. The final temperatures studied were 110, 130, 160 and 220 °C (depending on the pan). In Pan A, *B. subtilis* ATCC 31324 spores were not inactivated below 160 °C, regardless of the initial water activity (Fig. 4a). The water activity only influenced the resistance above 160 °C where a remarkable difference in the inactivation between the different water activities was observed.

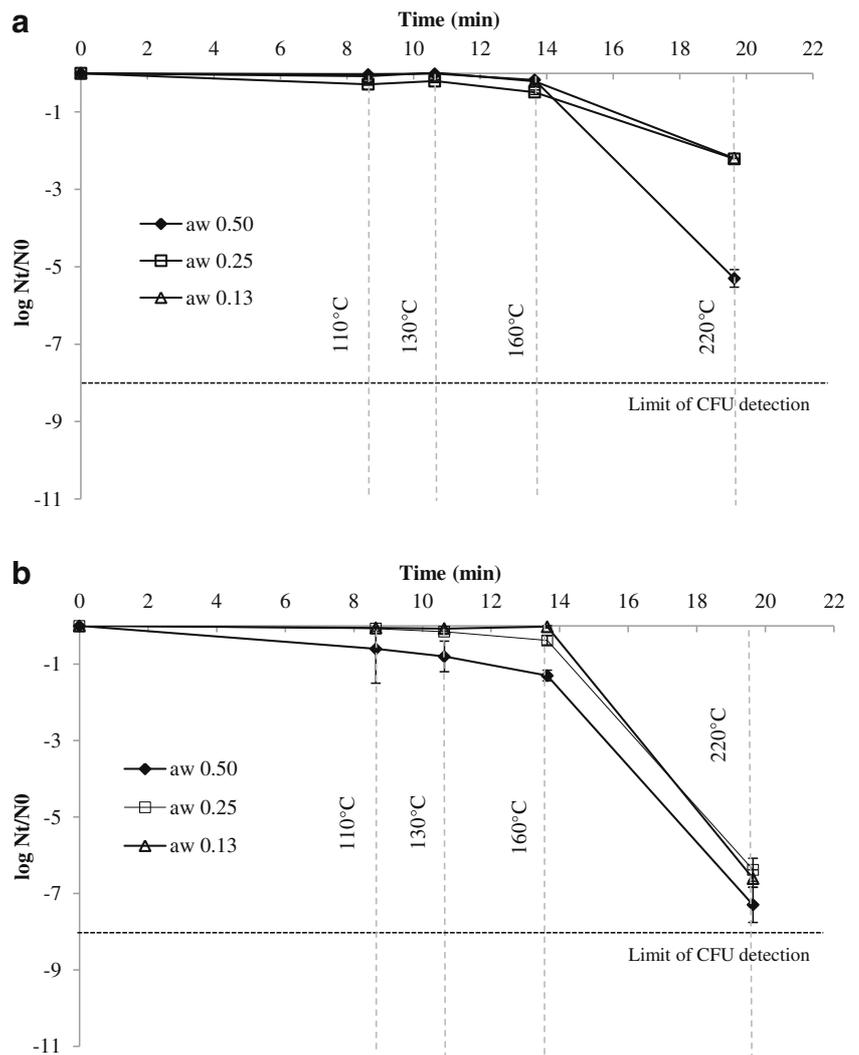
The bacillus spores from strain 168 were less resistant to heat treatment than strain 31324 (Fig. 4b) in Pan A. However, at 220 °C no difference in inactivation was observed for the spores according to the water activities.

The inactivation of spores was higher in Pan B, as shown in Fig. 5. The spores from both strains equilibrated at a_w 0.50 were completely inactivated at 160 °C, whereas spores at a_w 0.13 and 0.25 presented an inactivation of 5 and 7 log respectively at the same final temperature. The respective treatments in Pan B produced plates with no detectable colony growth for both strains in the lowest dilution: a_w 0.50 at 160 °C and 220 °C and a_w 0.25 at 160 °C. The effect of water activity on the inactivation is very clear in Pan B, showing that under these conditions (pressurized atmosphere) the amount of water in the spore affects its resistance from the beginning of heat treatment and that the destruction is directly related to higher water activities. However, the difference between the strains was not evident in Pan B; both strains showed the same order of inactivation.

Changes in Spore Structure After Heat Treatment

The infrared spectra of the spores before and after heat treatment in the two pans are presented in Fig. 6. Comparison of the derivative spectra of heat treated spores with those of untreated spores showed various differences in the regions corresponding to DPA and secondary proteins.

Fig. 4 Inactivation of spores after heat treatment using DSC in Pan A **a** *Bacillus subtilis* ATCC31324 and **b** *Bacillus subtilis* 168



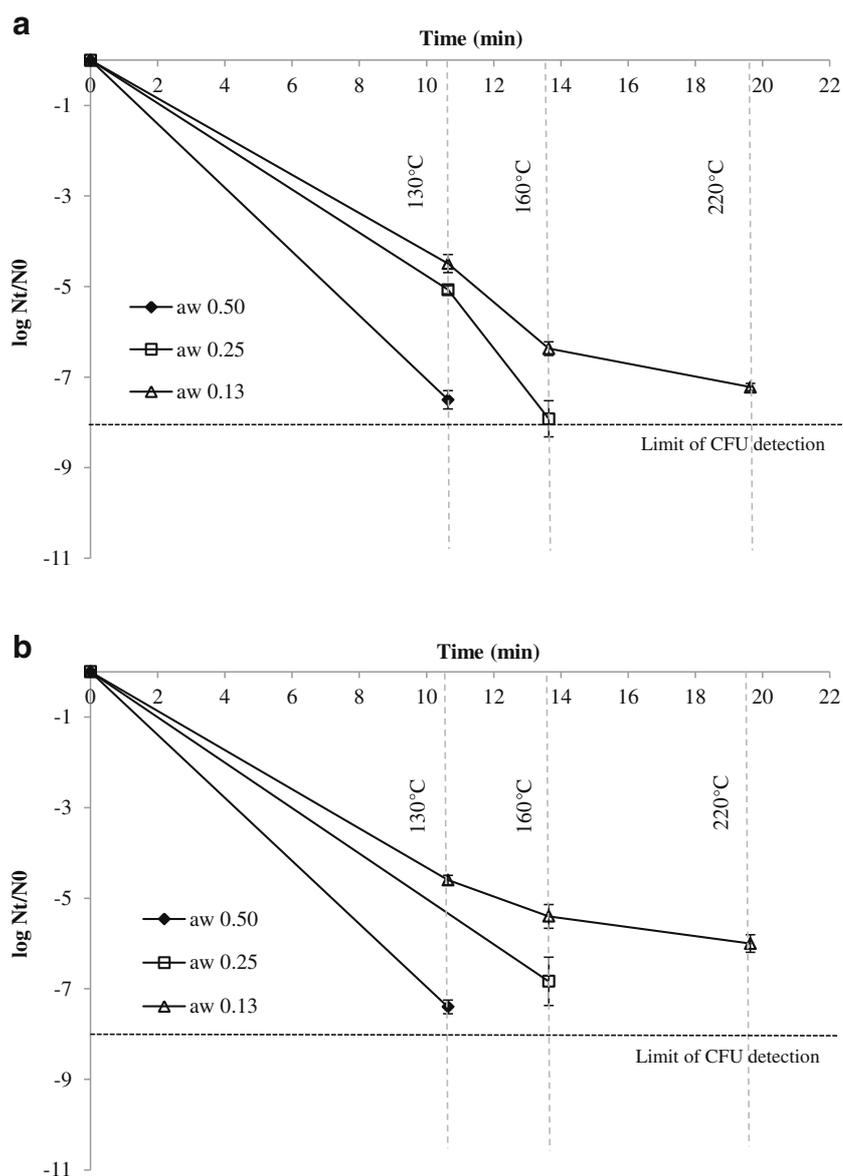
Regarding protein structure, major changes were observed for the peak at $1,626\text{ cm}^{-1}$, which is associated with amide I bands corresponding to β -pleated sheets of secondary proteins, and the peak at $1,655\text{ cm}^{-1}$, which is related to amide I bands corresponding to the α -helical structure of proteins [40]. In Pan B, as shown in Fig. 6, spore proteins underwent structural modifications from α -helices to β -sheets after the treatment. There was also a peak shift from $1,655\text{ cm}^{-1}$ to $1,661\text{ cm}^{-1}$. In Pan A however (Fig. 6), there was no significant modification in protein structure, only a small shift from $1,655\text{ cm}^{-1}$ to $1,658\text{ cm}^{-1}$. Peaks corresponding to DPA ($1,568$, $1,450$, $1,378$ and $1,279\text{ cm}^{-1}$) showed significant changes after the treatment for both pans, with either a complete loss of signal ($1,568\text{ cm}^{-1}$) or decrease in intensity and shift ($1,450$, $1,378$ and $1,279\text{ cm}^{-1}$). This complete loss of signal for the peak at $1,568\text{ cm}^{-1}$ suggests that either the majority of the spore's DPA compounds were released or that DPA underwent an undetermined transformation. The decrease in intensity of the DPA peaks was proportional to the pressure under which the spores were treated.

Discussion

The Transitions Observed are Related to Water Vaporization

Apart from water vaporization, other transitions that could normally occur when spores are heated are related to protein denaturation, alterations of complex cellular structures and denaturation-induced aggregation of macromolecules [28]. However, Snyder et al. [41, 42] showed that spore components begin to undergo thermal decomposition only above $250\text{ }^{\circ}\text{C}$ and the same was found regarding dipicolinic acid and proteins. In addition, in our study the transitions studied occurred in the same temperature range as pure water vaporization; they were highly correlated with the amount of water present in the sample, judging by the enthalpy that is proportional to the initial water activity and they were also irreversible. Therefore the weight loss observed in this study could not be related to the decomposition of spore components and can be attributed to water evaporation from the spore core.

Fig. 5 Inactivation of spores after heat treatment using DSC in Pan B **a** *Bacillus subtilis* ATCC31324 and **b** *Bacillus subtilis* 168



A Fraction of Spore Water Localized in the Core Seems to be Isolated by the Low Water Permeability of the Inner Membrane

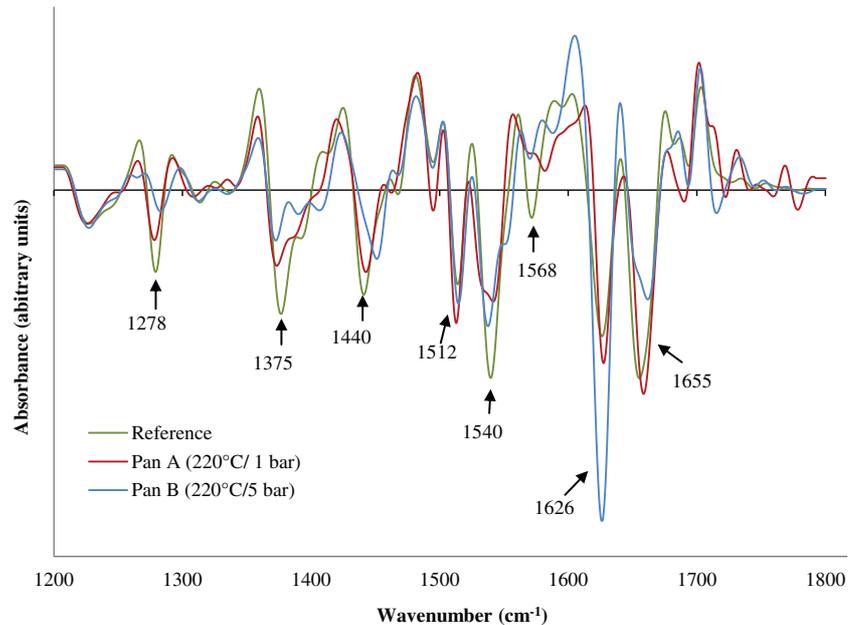
One important finding in this work was that spores equilibrated at a very low water activity of 0.13 still had a water content of 8.1 to 11.5 % (w/w) (Table 3) considering that all the weight loss was the result of water evaporation and depending on the strain. This value is significantly higher than those found for the sorption isotherm obtained for *Bacillus subtilis* ATCC 31324 spores [19]. It was observed that the weight loss was always superior to the total water content estimated by the water activity measured in the spores. This paradox can be explained taking into account that the water activity only measures the water in equilibrium with the surroundings and

not the water in the core, which is slowly affected by the changes in the atmosphere but does not contribute to the water activity measured. This can be explained by the low water permeability of the inner membrane which is consistent with the high degree of lipid immobilization in this membrane [43].

Two Different Evaporation Events Were Observed in Pan B and Could be Related to Water Located in Different Parts of the Spore (Core and Non-Core)

Considering these results, presented in Fig. 3, we believe that the water left the spore at two different moments, once at around 150 °C and then at around 200 °C. One hypothesis is that the external water, or noncore water (from the cortex and coat), of the spore evaporates easily when the pan seal breaks

Fig. 6 Changes in composition of *Bacillus subtilis* 168 during heat treatment performed by DSC. Derivative (2nd) FTIR spectra of untreated spores (Reference) and treated spores (Pan A and Pan B)



at 150 °C. The second evaporation, which corresponds to a theoretical vapor pressure of 16 bar, event may be related to the water that is inside the spore core and because of this it needs to surpass the pressure exerted by the spore cortex and the internal membrane in order to evaporate. These results confirm that water is compartmentalized in the spore and that a significant fraction of the total water is located in the core. Moreover, these results suggest that water is not equally distributed within the spores in the two strains. The strain ATCC 31324 presented a higher percentage of water in the outer layers than strain 168 strain, whereas strain 168 had more water in the core than strain ATCC 31324. Other studies did not report the same results probably because they used completely hermetic pans and stopped the treatment at lower temperatures. In addition, dried spores with precise water activities were used in this study, differing from studies that used “moist” or “dry” spores [15, 28, 44].

Spore Inactivation and Changes in Their Structure After Heat Treatment

The results presented in Figs. 4 and 5 show that it is possible to maximize the inactivation of dried spores if they are heated without their internal water evaporating (Fig. 5). Inactivation with an equivalent heat treatment was found to be superior in Pan B than in Pan A. For instance, spores that had lost almost all water in Pan A (at 220 °C) were still able to germinate when put in favorable conditions.

As already shown by Beaman and Gerhardt [11], these results confirm that spores with a higher core water content, in this case strain 168, are less heat resistant. Moreover, we clearly show that heat resistance is related to the initial water

activity of the spores as well as the preservation of this water content throughout the heat treatment. Consequently, when high temperatures are reached with no previous evaporation, the spore is more sensitive to heat.

In order to understand why more hydrated spores are less resistant to heat treatment as observed in this study, we can examine the FTIR spectra of the spores. DNA is usually the target of spore inactivation in dry heat treatment [1] but interestingly many of the observed changes in Pan B were also found in autoclaved spores [33]. The loss of intensity or absence of peaks related to DPA (1,568, 1,450, 1,378 and 1,279 cm^{-1}) in Pan B suggests that there was a rupture of the cortex and the inner membrane allowing DPA leakage from the core. The peaks related to the structure of proteins were also affected by the heat treatment in Pan B, which could indicate that a key germination protein or enzyme was denatured and lost its activity. In contrast, in Pan A no major modification was observed in the protein structure suggesting that the dehydration protected the proteins from heat.

The inactivation of one or more key spore proteins is believed to be important in spore heat killing [45]. When the heat surpasses a threshold level, changes in the structure of vital growth proteins become irreversible causing spore inactivation [46]. It is known that in low moisture states the temperature of protein denaturation increases [47] making it more difficult to inactivate these key proteins. In this context, the high spore heat resistance at low water content can be explained by the low mobility and changes in the conformation of proteins and enzymes. Indeed, most spore proteins are rotationally immobilized, which may contribute to heat resistance by preventing heat-denatured proteins from aggregating irreversibly [16]. Consequently, when the original water

content of the spore is maintained, damage to proteins is increased during heat treatment and such damage is certainly related to the difference in spore inactivation found in our experiments.

Conclusion

The results obtained through the heating of dried spores under pressure indicate that there are two water vaporization events in the spore. The first one occurs at around 100 °C at atmospheric pressure and is believed to correspond to vaporization of water from the outer layers of the spore that is easily exchanged with the environment. The second occurs at a higher temperature of around 200 °C that could correspond to water from the spore core.

This work presents new insight on the inactivation of dried spores and the mechanism involved and proposes the basis for the optimization of dried spores' inactivation without moisture addition to the product. The use of a method that prevents drying during heat treatment has shown that water conservation in the spores is directly related to spore destruction. This destruction was found to be highly related to protein denaturation/aggregation and DPA release, which are maximized in moist atmospheres. In addition, when spores are progressively dried (including loss of core water) at atmospheric pressure they show a very high resistance to heat. These findings can be used for the optimization of decontamination procedures for dried foods and powders, which remains a major issue for the food industry.

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COMPENDIUM DE THÈSE EN FRANÇAIS

Introduction

Les spores bactériennes sont produites dans la nature comme moyen de survie face à des perturbations environnementales drastiques et dans cette forme elles peuvent résister à des conditions que normalement sont létales pour les cellules végétatives (Nicholson et al. 2000).

De nombreux microorganismes peuvent former des spores comme mécanisme de survie, notamment les bactéries Gram-positives comme *Alicyclobacillus*, *Clostridium*, *Sporolactobacillus*, and *Sporosarcina* ainsi que des bactéries gram négatives comme *Desulfotomaculum*. Les espèces sporulantes les plus importantes sont *Bacillus* (*B. cereus*, *B. anthracis*) et *Clostridium* (*C. perfringens*, *C. botulinum*, *C. Difficile*) qui peuvent provoquer des maladies chez l'humain et certaines sont même considérées comme des armes biologiques (Nicholson 2004).

Les spores sont résistantes à de nombreuses perturbations environnementales telles que la chaleur (humide et sèche), la pression, les rayonnements Ultra-Violets (UV), les produits chimiques ainsi que la déshydratation (Nicholson et al. 2000). Par conséquent, les spores peuvent survivre au processus de transformation des aliments. Ainsi le développement la germination des spores durant leurs conservations peut conduire à la détérioration des aliments et à des intoxications alimentaires.

Cependant, les bactéries sporulantes peuvent montrer un aspect bénéfique. En effet, leur utilisation comme indicateurs de stérilisation (*Geobacillus stéarothermophilus*), insecticide naturel (*Bacillus thuringiensis*), ou producteurs d'enzymes, d'antibiotiques (*B. subtilis*) et probiotique (*B. subtilis*) commence à être reconnue et développée dans le monde industriel (Höfte and Whiteley 1989; Albert et al. 1998; Schallmey et al. 2004; Cutting 2011). De nouvelles applications telles que l'utilisation des spores en tant que véhicules de vaccination sont actuellement en cours de développement (Oggioni et al. 2003).

Les aliments à faible humidité ont toujours été considérés comme présentant un faible risque de provoquer des intoxications alimentaires, parce qu'ils ne sont pas propices la croissance de microorganismes pathogènes. Cependant, au cours des dernières décennies, nombreux cas de TIAC dans différents pays ont été associés à la consommation de produits alimentaires de faible humidité. Les pathogènes les plus fréquents liés à ces TIAC étaient *Salmonella* et *Bacillus* spp. Ces incidents soulignent la nécessité d'assurer des pratiques d'hygiène adaptées et un traitement d'inactivation adéquat dans la production d'aliments à faible humidité. En plus, l'industrie alimentaire a été obligée

de modifier ses pratiques afin de mieux lutter contre le risque de contamination des aliments à faible teneur en humidité ([FAO/WHO 2012](#); [Van Doren et al. 2013](#)). Entre 2007 et 2010, aux Etats Unis, trois épisodes importants de salmonellose ont été attribués à la consommation des épices et condiments (Sotir et al. 2009; CDC, 2010; Higa, 2011). En effet, la dose infectieuse de *Salmonella* est faible, 100 à 1000 bactéries sont suffisantes pour provoquer l'infection chez certaines personnes. Le FDA a rapporté que la contamination des épices par *Salmonella* était la cause de 95% des rappels alimentaires aux États-Unis associés à des épices sur la période 1980-2000 (Vij et al., 2006).

Les spores du genre *Bacillus* sont présentes en concentrations très élevées dans les aliments secs ($> 10^2$ - 10^5 spores/g) dans des ingrédients tels que les épices, la poudre de cacao, le lait en poudre et les céréales ([Chen et al. 2004](#); [Lima et al. 2011](#); [Witkowska et al. 2011](#)). Cependant, la décontamination des aliments secs est difficile puisque la faible teneur en eau entraîne des changements dans la conformation des protéines en les rendant plus résistant aux traitements. Bien que les spores soient dormantes dans les produits secs, leur survie représente un problème lorsque ces produits sont ajoutés à des aliments humides et puisqu'ils sont plus favorables à la germination et la multiplication bactérienne.

Les facteurs impliqués dans la résistance des spores ont été partiellement expliquée par leur structure et par la présence de composants internes spécifiques. En effet les spores présentent une structure multicouche très différente de celle des cellules en croissance (Nicholson et al. 2000) et ces différentes couches jouent un rôle dans leur résistance. Le manteau protéique offre une protection contre les produits chimiques et les enzymes lytiques et agit aussi comme une barrière de perméabilité (Driks, 1999). Le cortex, fait de peptidoglycanes, est essentiel au maintien de l'intégrité de la membrane interne. La membrane interne présente une faible perméabilité aux petites molécules, peut-être même à l'eau, ce qui est l'un des facteurs impliqués dans la résistance des spores à certains produits chimiques, en particulier des ceux qui endommagent l'ADN. Enfin, la partie la plus interne est le protoplaste qui contient le matériel génétique et la plus part des enzymes et possède une faible teneur en eau (25 à 50 % poids humide selon l'espèce). Le protoplaste contient également deux substances uniques qui sont l'acide dipicolinique (Ca-DPA) (25% de base de poids sec) et les petites protéines solubles dans l'acide (SASP) ([Setlow et al. 2006](#)). On croit que la résistance remarquable de spores aux différents stress est une conséquence directe de la faible teneur en eau dans le protoplasme de la spore qui en milieu aqueux contient seulement une fraction de l'eau présent dans les cellules végétatives (Black et Gerhardt 1962b).

Afin de répondre aux exigences de sécurité et aux attentes des consommateurs, l'industrie agroalimentaire consacre des ressources considérables au développement de méthodes capables

d'éliminer les microorganismes sans affecter négativement la qualité des aliments. La production d'aliments salubres et propres exige le contrôle des matières premières entrant dans la chaîne alimentaire, la réduction de la croissance microbienne et la diminution ou l'élimination des microorganismes par des traitements adéquats. Par conséquent, l'utilisation d'un traitement de décontamination est essentiel pour limiter la flore microbienne à des niveaux inférieurs à 4 log₁₀ CFU / g et pour garantir l'absence de pathogènes tels que *Salmonella* dans le produit final.

Les traitements couramment utilisés pour décontaminer les poudres alimentaires sont la vapeur, le chauffage ohmique, l'irradiation, les agents chimiques ou des traitements par effet Joule. La fumigation avec l'oxyde d'éthylène, autrefois standard, a été interdite dans plusieurs régions du monde, comme l'Union Européenne ([Schweiggert et al. 2007](#)). En outre, les fournisseurs de produits alimentaires séchés préfèrent éviter l'utilisation d'irradiation en raison de la barrière psychologique ainsi que les obligations d'étiquetage ([Laroche et al. 2005](#)). En général, quel que soit le traitement de décontamination choisi, un compromis doit être trouvé entre l'efficacité maximale du processus et la qualité de produit.

En plus, un certain nombre d'études réalisées dans le laboratoire PMB ont abordé le problème de la décontamination des poudres alimentaires. Par exemple un procédé où des très hautes températures (entre 200 et 600°C) sont appliqués aux produits pulvérulents pendant des temps très courts (0,1 à 30 secondes), suivi par un refroidissement instantané avec un gaz réfrigérant (-80°C) a été développé et ensuite breveté par le laboratoire ([Gervais et al. 2002](#); [Fine and Gervais 2005](#)). Nouvelles technologies comme les lumières UV ([Fine and Gervais 2004](#)) et la haute pression gazeuse ([Espinasse et al. 2008](#); [Colas de la Noue et al. 2012](#)) ont été utilisés dans l'inactivation des microorganismes secs (levures et spores). Ces procédés offrent des perspectives pour le développement des nouveaux procédés de décontamination des produits déshydratés dans lequel les microorganismes présentent une plus grande résistance aux traitements conventionnels.

L'irradiation est la seule méthode vraiment efficace pour l'inactivation des spores et même si elle s'est avérée un moyen efficace, écologiquement propre et avec une faible consommation d'énergie, il est rarement utilisé en raison de sa faible acceptation par le consommateur. Malheureusement, tous les autres procédés actuels présentent une capacité limitée pour la décontamination des spores sèches (habituellement 2 log).

Dans ce contexte, l'objectif de cette étude était d'étudier le rôle d'eau, sa distribution et son implication dans la résistance des spores sèches. Cette compréhension permettra l'optimisation de la décontamination des aliments secs.

Ce mémoire de thèse débute par une introduction sur le sujet suivi par une étude bibliographique qui établit l'état des connaissances sur les spores bactériennes. Les points majeurs sur les spores comme leur formation, structure et résistance sont présentés ainsi que les méthodes plus couramment utilisés pour décontaminer des poudres alimentaires. Ensuite le chapitre Matériel et Méthodes présente les souches, les étapes de productions de spores et les traitements et analyses réalisés. Le chapitre Résultats et Discussion est divisé en deux parties: la fondamentale, où la technique de AED est utilisé d'une façon originale pour réaliser un traitement thermique sur des spores et simultanément récupérer des données associées aux transitions thermiques, à la teneur en eau et à l'inactivation des spores; la deuxième partie présente l'application des résultats de la première partie pour le développement d'une système pour optimiser la destruction des spores sèches.

Conclusion and Aim of the Thesis

Cette étude bibliographique a donné une vue d'ensemble des propriétés de spores telles que leur structure et leur résistance à différents types de stress ainsi que des différents procédures de décontamination utilisés dans l'industrie pour traiter les aliments secs.

En réponse à des conditions défavorables, ces bactéries ont donc la capacité de former des spores métaboliquement inactives et qui peuvent survivre sous cette forme plusieurs millions d'années. Ces spores représentent une préoccupation majeure des industries alimentaires, car elles sont responsables de détérioration d'aliments et de toxi-infections alimentaires en raison de leur haute résistance aux procédés de conservation des aliments. Certaines souches, comme *Clostridium botulinum*, peuvent causer des maladies mortelles du système nerveux, certaines souches de *Bacillus cereus* peuvent provoquer des formes graves de diarrhée et des vomissements et *Bacillus anthracis* qui peut être utilisé comme une arme de bioterrorisme.

Plusieurs facteurs sont impliqués dans la résistance des spores bactériennes, y compris une faible teneur en eau du protoplaste, un faible pH interne, une forte concentration de DPA, la saturation de l'ADN des spores avec des protéines de petites protéines solubles en acide (SASP) ainsi que la faible mobilité des composants du protoplasme.

A ce jour, bien qu'il y ait eu des avancées majeures dans la compréhension de la résistance extrême des spores bactériennes, quelques points clés de cette résistance ainsi que les mécanismes d'inactivation aux différents stress ne sont pas complètement élucidés.

Il est connu que la chaleur humide tue les spores par l'inactivation des protéines essentielles, cependant l'identité de cette protéine reste inconnu ainsi que le moment exact de la mort de spores.

L'inactivation des spores par haute pression hydrostatique a reçu beaucoup d'attention ces dernières années et il est maintenant connu que les spores sont détruites que quand l'haute pression est couplé à un traitement thermique doux. Bien que les mécanismes aient été élucidés, l'effet de matrices alimentaires reste mal connu. Ces deux méthodes sont normalement utilisées pour le traitement de liquides ou des produits à haute teneur en eau.

Cependant, l'inactivation de spores déshydratées reste une préoccupation majeure et un grand défi pour l'industrie alimentaire en raison de sa résistance augmenté aux différents traitements par rapport aux spores hydratées. Les rayonnements UV et l'ionisation inactivent les spores par l'endommagement des molécules d'ADN mais l'influence de différents facteurs tels que la teneur en eau, ne sont pas complètement compris. Enfin, l'inactivation des spores par la chaleur sèche semble être due à des lésions accumulées dans l'ADN des spores, par conséquent, les systèmes de réparation de l'ADN et les SASPs jouent un rôle majeur dans la résistance des spores à la chaleur sèche. Toutefois, de nombreux aspects de la résistance des spores en milieu sec n'ont pas été examinées à fond, par exemple, le rôle de l'activité de l'eau et la teneur en eau.

Un nombre insuffisant de recherches ont été effectuées sur le lien entre l'activité de l'eau et la résistance des spores sèches à la chaleur puisque la plupart des études précédentes ne contrôlait pas cette propriété, c'est à dire, les spores ont été déshydratés en utilisant différentes méthodes, puis traités à la chaleur sans mesurer ou contrôler leur teneur en eau. Un autre problème souvent rencontré est l'écart entre la recherche fondamentale sur l'inactivation des spores et une application effective des connaissances acquises. De plus, il y a actuellement un intérêt accru pour la décontamination de produits alimentaires secs, comme des herbes, des épices, des céréales, le lait en poudre et le cacao après de nombreux cas d'intoxication alimentaire liés à la consommation de produits alimentaires de faible humidité.

Dans ce contexte, l'objectif de cette thèse est l'étude du rôle du teneur en eau et de sa distribution sur l'inactivation par la chaleur de spores de *Bacillus* déshydratées.

Ce manuscrit est divisé dans deux parties: une étude fondamentale et le développement d'un nouveau procédé de décontamination.

Dans la première partie, nous avons utilisé l'Analyse Enthalpique Différentielle avec une approche originale: les capsules en aluminium ont été utilisées comme des mini-réacteurs pour l'application d'un traitement thermique dans des conditions bien contrôlés de montée en température et, simultanément, récupérer des informations sur les transitions thermiques et la perte de poids. Nous

étions surtout intéressés par l'évaporation de l'eau des spores donc capsules non-hermétiques avec différentes résistances ont été choisies afin de moduler la température d'évaporation.

La deuxième partie de l'étude a visé la mise au point d'un nouveau procédé de décontamination à l'aide des résultats obtenus lors de l'étude avec l'AED. L'effet de l'activité de l'eau, de la température et de la pression sur l'inactivation des spores en utilisant ce procédé a été étudié.

3. MATERIEL ET MÉTHODES

3.1. Matériel biologique

3.1.1 Production des spores

Deux souches de *Bacillus* ont été utilisés de façon à permettre la comparaison des résultats obtenus: *B. subtilis* ATCC 31324 (DSMZ 704) obtenus auprès de Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Allemagne) et *B. subtilis* 168 du BGSC (Bacillus Genetic Stock Center, USA). La souche mutante de *B. subtilis* sans la plus part des SASPs a été obtenus auprès de la BGSC.

Les spores de *B. subtilis* ont été produites dans un milieu complexe, dans un réacteur à 37 ° C, pH 8,0, avec un débit d'air de 4 l / min et sous agitation (450 rpm) (Nguyen Thi Minh et al. 2008) et le protocole de la préparation est présentée dans le Tableau 3 (p. 73). Tout au long de la production, le nombre d'UFC après un traitement de 80°C et 10 min a été déterminé et dès que la sporulation concerne 95% de la population, la production est arrêtée. Les spores sont ensuite récoltées par centrifugation, puis lavées trois fois avec de l'eau distillée froide et enfin les spores ont été atomisées et stockées à 4°C. La concentration de spores est estimée d'environ 10¹¹ UFC/g.

La souche mutante $\alpha\beta^-$ et *B. subtilis* 168 ont été produites selon la méthode de Nicholson et Setlow (Nicholson and Setlow, 1990) et ont été ensuite lyophilisés. Les spores lyophilisées ont été utilisés seulement dans le test de présence de mutations avec l'acide nalidixique.

Les poudres de spores on été équilibrées à différentes aw comprises entre 0,13 et 0,50 dans des boîtes hermetiques contenant des solutions de sel saturées à 25°C. Les solutions de sel utilisées pour obtenir différentes aw sont présentées dans le Tableau 4. L'aw a été suivie par l'osmomètre Decagon-AQUALAB CX-2 (Pullman, USA)

3.1.3 Mesure de la viabilité

Les spores traitées à la chaleur ont été dilués avec de l'eau physiologique (NaCl à 0,9% p / v) et 100 µl de chaque dilution sont étalées sur milieu BCP (Biokar diagnostics) et incubées une nuit à 37 °C. Le dénombrement est réalisé sur les boîtes de Petri, les boîtes comptées sont celles ayant un nombre d'unité formant colonie (UFC) compris entre 30 et 300. Toutes les expériences ont été effectuées au moins en triplicate et les barres dans les graphiques représentent l'écart type. L'inactivation des spores a été exprimé en utilisant un facteur de réduction logarithmique $\log N_t/N_0$, où N_0 est le nombre de spores avant le traitement et N_t le nombre de spores après le traitement.

3.2 Differential Scanning Calorimetry

Les analyses thermiques ont été réalisées dans un calorimètre Q-20 DSC (TA Instruments, USA) équipés d'un système de refroidissement par azote liquide. L'indium (température de fusion $T_m=156,6^\circ\text{C}$ et enthalpie de fusion $28,54 \text{ J.g}^{-1}$) est utilisé pour calibrer l'appareil. Les analyses sont réalisées à des températures comprises entre 25°C et 220°C à une vitesse de chauffage de $10^\circ\text{C}/\text{min}$ sous flux d'azote ($50 \text{ ml}/\text{min}$). Une capsule vide est utilisée comme référence.

L'idée derrière cette expérience est que les spores agissent comme des capsules fermées avec une certaine résistance à la pression et lorsque il y a une accumulation de pression à l'intérieur de la spore, l'eau contenue dans le protoplasme peut être vaporisé.

Trois types d'expériences ont été réalisés à l'aide du calorimètre. Premièrement, les caractéristiques des capsules a été évaluée et ensuite les thermogrammes, la perte de poids et l'inactivation des spores ont été étudiés, comme présenté dans la Figure 13 (p. 77).

1. La température d'évaporation de l'eau a été évaluée en utilisant une masse de 1 - 3 mg de l'eau distillée. Deux capsules en aluminium (TA Instruments, USA) avec différentes résistances à la pression ont été utilisés: Tzero™ avec un couvercle non hermétique (901683.901/901671.901) et Tzero™ avec un couvercle hermétique (901683.901/901684.901) et qui seront appelés Pan A et Pan B, respectivement.

2. Les spores équilibrées à différentes a_w ont été chauffées dans les deux capsules (A et B) pour obtenir leurs thermogrammes. Ensuite les capsules ont été refroidies rapidement à la température initiale et immédiatement réchauffés pour déterminer la réversibilité des transitions. Les échantillons ont été pesés à nouveau après le traitement AED pour vérifier la perte de poids au cours du chauffage.

3. L'inactivation des spores a été évaluée après un traitement thermique dans le calorimètre. Environ 5 mg de spores ont été pesés dans les capsules (A et B), scellés et subis au traitement thermique.

3.3 Fourier Transform Infrared Spectroscopy

IRTF a été utilisée pour évaluer les changements structuraux et biochimiques dans les spores de *B. subtilis* 168 traitées thermiquement avec l'AED. Les expériences ont été réalisées à l'aide d'un spectromètre infrarouge à transformée de Fourier BRUKER IFS Vector 22 (Bruker, Allemagne). La méthode ATR (réflectance totale atténuée) a été utilisée avec un cristal ZnSe (sélénure de zinc) ([De Lamo-Castellví and Rodríguez-Saona 2011](#)).

Les spores sèches ont été diluées dans l'eau distillée et lavées quatre fois. Une goutte de 10 µl d'échantillon (10^8 CFU/ml) est déposée sur le cristal puis séchée sous un flux d'azote pendant 5 à 10 min pour obtenir un film homogène. Les spectres sont obtenus par l'acquisition de 10 scans à une résolution de 8 cm^{-1} et à une température de 25 °C . Avant chaque mesure, un background est réalisé afin de soustraire le bruit de fond. Le logiciel OPUS (Bruker, Allemagne) est utilisé pour obtenir la dérivée seconde des spectres et obtenir la position des pics.

3.4 Treatment at high temperatures and low pressures

Le méthode d'inactivation des spores en utilisant de basses pressions et des températures élevées permet de résoudre l'un des problèmes rencontrés lors de la décontamination des poudres: le séchage simultané de l'échantillon. Cette méthode permet de chauffer l'échantillon sans perte d'eau, de façon à maintenir l'activité de l'eau initiale, ce qui n'est pas possible avec d'autres méthodes utilisées pour les produits déshydratés.

Le réacteur développé consistait en une petite chambre hermétique en acier inoxydable (1,7 ml de volume) qui peut être mise sous pression avec du gaz (Figure 15). Ici, l'azote a été utilisé pour ajouter la pression et une fois que la pression a été réglée, la valve du réacteur était fermée en maintenant la pression appliquée tel que présenté sur la Figure 16.

Spores séchées équilibrées à différentes a_w ont été placées dans des carrés d'aluminium qui ont été pliés et mis à l'intérieur du réacteur. Le réacteur a été ensuite mis sous pression entre 2 et 7 bars, fermé et chauffé dans un bain d'huile à des températures (mais toujours au-dessus de 100 °C).

Dans le cas de traitements sans ajout de pression, la vanne a été laissée ouverte pour permettre l'évaporation de l'eau où fermée pour retenir de l'eau évaporée. Après le traitement, les réacteurs ont été refroidis dans un bain glacé, puis la vanne est ouverte pour libérer la pression.

La pression indiquée est toujours la pression absolue ce qui signifie que la pression d'azote est ajouté à la pression atmosphérique pour donner la pression totale du système. Les différentes conditions de pression appliquées et le codage utilisé dans les sections des résultats sont présentés dans le Tableau 6.

3.4.1 Experimental design

Un plan expérimental de Doehlert (Doehlert 1970) a été utilisé pour étudier l'effet des variables de traitement sur l'inactivation des spores de *Bacillus* secs. La température, la pression et l'activité de l'eau ont été choisis en tant que variables d'entrée indépendantes, même si elles ne sont pas indépendantes puisque la pression augmente à l'intérieur du réacteur lors d'un chauffage. Cette conception a été conçu uniquement pour donner une vision globale du système et des conclusions statistiques doivent être examinées avec soin. Inactivation des spores a été utilisée en tant que variable de sortie en fonction, comme indiqué dans le tableau 7.

Le plan d'expérience de Doehlert (Doehlert 1970) a été utilisé pour étudier l'effet des variables de traitement dans l'inactivation des spores de *Bacillus* sèches. La pression, la température et l'activité de l'eau ont été choisis comme variables indépendantes, même si elles ne sont pas indépendantes puisque la pression augmente à l'intérieur du réacteur lors du chauffage. Cette conception a été conçu uniquement pour donner une vision globale du système et des conclusions statistiques doivent être examinée avec attention. L'inactivation des spores a été utilisée en tant que variable de sortie, comme indiqué dans le Tableau 7.

4.1.3 Discussion

Les transitions observées sont liées à la vaporisation d'eau

D'autres transitions thermiques, en dehors de la vaporisation de l'eau, qui pourraient normalement se produisent lorsque les spores sont chauffées sont liés à la dénaturation des protéines, des altérations des structures cellulaires complexes et l'agrégation de macromolécules induite par la dénaturation ([Belliveau et al. 1992](#)). Cependant, Snyder et al ([2005a](#); [2005b](#)) ont utilisé l'analyse thermogravimétrique couplée à la spectrométrie de masse ou *Pyrolysis Bioaerosol Detection* et ont montré que les composants de spores commencent à subir une décomposition thermique seulement au-dessus de 250 °C et le même a été trouvé en ce qui concerne l'acide dipicolinique et protéines. De plus, dans notre étude les transitions étudiées se sont produites à la même température de vaporisation de l'eau pure et ils étaient fortement corrélés avec la quantité d'eau présente dans l'échantillon et en plus elles étaient aussi irréversibles. Par conséquent, la perte de poids observée

dans cette étude ne peut pas être liée à la décomposition des composants de spores et peut être attribuée à l'évaporation de l'eau à partir de la spore.

Une fraction de l'eau de spores localisée dans le protoplasme semble être isolé par la faible perméabilité à l'eau de la membrane interne

Une conclusion importante de ce travail était que les spores équilibrées à une très faible activité de l'eau (0,13) avaient encore une teneur en eau de 8,1 à 11,5% (p / p) (Figures 26 et 27) considérant que toute la perte de poids est due l'eau évaporation et en fonction de la souche. Cette valeur est significativement plus élevés que ceux trouvés pour l'isotherme de sorption obtenues pour *Bacillus subtilis* ATCC 31324 spores ([Nguyen Thi Minh et al. 2010](#)). Il a été observé que la perte de poids était toujours supérieure à la teneur totale en eau estimée par l'activité de l'eau mesurée dans les spores. Une hypothèse pour expliquer ce paradoxe tient compte du fait que l'activité de l'eau ne mesure que l'eau en équilibre avec l'environnement, et non de l'eau dans le protoplasme, qui est lentement affecté par les changements dans l'atmosphère mais ne contribue pas à l'activité de l'eau mesurée. Ceci peut être expliqué par la faible perméabilité à l'eau de la membrane interne qui est compatible avec le degré élevé d'immobilisation des lipides dans cette membrane ([Cowan et al. 2004](#)).

Deux événements d'évaporations différentes ont été observés dans Pan B et pourraient être liés à l'eau dans les différentes parties de la spore

En considérant les résultats présentés sur la figure 38, nous pensons que l'eau a quitté la spore à deux moments différents, une fois à environ 150 °C et ensuite à environ 200 °C. Une hypothèse est que l'eau externe, ou l'eau de l'extérieur (du cortex et manteau), de la spore s'évapore facilement lors de la rupture d'étanchéité de la capsule à 150 °C. Le deuxième événement d'évaporation peut être lié à l'eau qui est à l'intérieur du protoplasme de spores et de ce fait il doit dépasser la pression exercée par le cortex et la membrane interne de la spore afin d'évaporer. Ces résultats confirment que l'eau est en fait compartimentée dans la spore et qu'une fraction significative de la quantité totale d'eau est situé dans le protoplasme (voir les figures 39 et 40).

Inactivation des spores et changements dans leur structure après traitement thermique

Afin de comprendre pourquoi les spores plus hydratées sont moins résistantes à un traitement thermique à sec nous pouvons examiner les spectres FTIR des spores. A partir des spectres infra-rouge, il est possible d'obtenir des informations sur le type de dommage subi par les spores pendant

le traitement thermique. La cible de l'inactivation des spores dans le traitement à la chaleur sèche est généralement l'ADN ([Nicholson et al. 2000](#)), ce qui est intéressant cependant, c'est que la plupart des changements observés dans Pan B ont également été trouvés dans les spores autoclavées ([Subramanian et al. 2006](#)). La perte d'intensité ou l'absence de pics liés au DPA (1568, 1450, 1378 et 1279 cm⁻¹) dans Pan B suggère qu'il y a eu une rupture du cortex et de la membrane interne qui a permis une sortie de DPA du protoplasme. Ces résultats sont cohérents avec notre hypothèse de vaporisation de l'eau à partir du protoplasme à des températures plus élevées puisque la vaporisation ne peut avoir lieu que si une pression spécifique est dépassée et provoque donc le collapse de la membrane interne et du cortex.

Les pics liés à la structure des protéines ont été également affectés par le traitement thermique dans Pan B, ce qui pourrait indiquer que quelque protéine clé de germination ou enzyme a été dénaturée et a perdu son activité. En revanche, dans Pan A aucune modification majeure a été observée dans la structure des protéines ce qui suggère que la déshydratation a protégé les protéines de la chaleur.

L'inactivation d'une ou de plusieurs protéines clés des spores est considérée comme étant importante dans la destruction thermique de spores ([Coleman et al. 2010](#)). Lorsque la chaleur dépasse un niveau de seuil, des changements dans la structure des protéines de croissance vitales deviennent irréversibles entraînant l'inactivation des spores ([Grinshpun et al. 2010](#)). Il est connu que dans des états de faible humidité, la température de dénaturation des protéines augmente ([Sochava 1997](#)), ce qui rend plus difficile l'inactivation de ces protéines clés. Dans ce contexte, la forte résistance de spores à faible teneur en eau à la chaleur peut être expliquée par la faible mobilité et par des changements dans la conformation des protéines et des enzymes. En effet, la plupart des protéines de spores sont immobilisées de manière rotationnelle, ce qui peut contribuer à la résistance à la chaleur en empêchant l'agrégation irréversible des protéines dénaturées par la chaleur ([Sunde et al. 2009](#)).

Par conséquent, lorsque la teneur en eau initiale de la spore est maintenue, des dommages aux protéines sont augmentés au cours du traitement thermique et un tel dommage est certainement lié à la différence de l'inactivation des spores trouvé dans nos expériences. Un autre facteur qui peut influencer sur cette faible inactivation des spores est la diminution locale de la température provoquée par l'évaporation continue de l'eau, i.e., le refroidissement par évaporation qui se produit étant donné que l'évaporation est une réaction endothermique.

Tous ces résultats nous ont permis de proposer un scénario concernant l'évolution de la teneur en eau dans la spore et la mortalité correspondante.

Ces résultats montrent que les spores de *Bacillus subtilis* contiennent deux fractions d'eau différentes : un facilement disponible, situé dans le manteau et le cortex et un second situé dans le protoplasme de spores, dans lequel les échanges avec l'extérieur sont limités par la membrane interne (Figure 39). Cette fraction n'est pas prise en compte dans les mesures de l'activité de l'eau, où le temps d'équilibre n'est pas très long.

Dans Pan B, qui support jusqu'à 5 bar (150 °C), les spores restent hydratées jusqu'à cette température et par conséquent, elles sont très sensibles à la chaleur (Figure 39). Cette sensibilité est liée à la teneur en eau initiale de la spore, et elle augmente avec des teneurs en eau élevées. Entre 150 et 200 °C, la capsule reste ouverte et les fractions d'eau disponibles s'évaporent progressivement, cependant une fraction importante de l'eau interne reste dans la spore et ne sort que comme vapeur à environ 200 °C. Après 200 °C les spores deviennent extrêmement sèches et, si elles ont survécus au traitement, elles deviendront très résistantes à la chaleur.

Contrairement, dans Pan A, à pression atmosphérique, il y a une longue phase de séchage au cours de laquelle, même l'eau interne s'évapore progressivement comme montré dans le diagramme de la figure 40. Au-dessous de 100 °C, l'eau présente dans les spores va s'évaporer lentement en raison de la différence de gradient de concentration d'eau ainsi que le haut débit d'azote dans le four du calorimètre. Entre 100 °C et 130 °C, nous pouvons supposer que l'eau se vaporise, en particulier la fraction située dans le protoplasme de la spore. En conséquence, la spore devient extrêmement sèche et donc très résistante à la chaleur comme le montre la Figure 42.

En ce qui concerne l'inactivation des spores dans Pan B (figure 41), les spores restent hydratées jusqu'à 150 °C et, par conséquent, elles sont très sensibles à la chaleur, en particulier lorsque les teneurs en eau initiales sont élevées. Entre 150 et 200 °C, la capsule est ouverte, donc les fractions d'eau disponibles vont s'évaporer progressivement mais une fraction importante de l'eau interne restera dans la spore et ne sortira qu'à environ 200 °C sous forme de vapeur. Après 200 °C, la spore devient extrêmement sèche et très résistant à la chaleur, si elle a survécu le traitement.

Cependant, dans Pan A, lorsque les spores atteignent des températures létales (> 110 °C), elles ont déjà perdues la plupart de leur eau initiale, ce qui les rend plus résistantes à la chaleur. Par conséquent, il n'y a presque pas de diminution de la population de spores avant 160 °C.

Cette analyse a montré que les spores sèches résistent beaucoup mieux que les spores hydratées au même traitement thermique et, plus important, compte tenu de la présence d'eau interne fortement lié à la spore. Une hypothèse est que certaines macromolécules (protéines, ADN) sont beaucoup plus sensibles à la dénaturation en phase aqueuse, ce qui a été confirmé par les résultats de FTIR.

4.2.6 Discussion

Aujourd'hui, les exigences commerciales et légales relatives à la sécurité, la qualité et le stockage des produits alimentaires sont concentrées sur le développement et l'amélioration des méthodes de décontamination. Cependant la destruction des spores bactériennes est assez difficile et la plupart des techniques couramment utilisées pour traiter les aliments secs résultent en une très faible inactivation des spores.

Les techniques de décontamination les plus souvent utilisés pour les produits secs sont la vapeur, l'irradiation et la fumigation. Cependant, la vapeur ajoute de l'humidité et entraîne des changements de couleur, la fumigation a été interdite dans la plupart des pays en raison de sa toxicité et l'irradiation est fortement rejeté par les consommateurs. En outre, ces techniques de décontamination provoquent une perte de qualité et présentent des taux d'inactivation parfois faibles. En conséquence, le développement de technologies innovantes pour la production d'ingrédients alimentaires secs sûrs et de bonne qualité s'est fait nécessaire.

Même si les épices sont des ingrédients non périssables, une fois qu'ils sont ajoutés à des produits alimentaires riches en eau, la flore naturelle ou contaminant peut rapidement se développer et par conséquent, mettre en danger la santé des consommateurs. C'est une question importante, en particulier lorsque les épices sont ajoutées aux aliments prêts-à-consommer qui ne sont pas soumis à d'autres traitements thermiques (Van Doren et al. 2013).

Différentes techniques de décontamination de produits alimentaires secs, y compris les épices, ont été étudiées. Staack et al. (2008) ont étudié l'effet de la chaleur infrarouge sur l'inactivation de *B. cereus* dans la poudre de paprika et ont montré que la réduction de spores était dépendante de l'*aw*. Cependant les spores n'ont été significativement inactivées que à très haute *aw* (> 0,80) et la réduction maximale était inférieure à 2 log. Cette faible inactivation a été attribuée principalement à un séchage des spores en cours de traitement. L'utilisation de lumière pulsée a également montré une très faible réduction microbienne sur le traitement des épices, même pour les cellules végétatives de *Bacillus* (~ 1 log) (Nicorescu et al. 2013). D'autres techniques comme les micro-ondes, la haute pression et le CO₂ à haute pression ont peu d'effet sur inactivation microbienne dans les milieux de faible humidité. Par conséquent, leur utilisation sera conditionnée à une augmentation précédente de la teneur en humidité de l'aliment à traiter, ce qui conduirait à la même problème constaté avec l'utilisation de la vapeur d'eau.

La technique développée dans ce travail présente des avantages en ce qui concerne d'autres procédures comme par exemple :

- Une forte destruction microbienne même à faible aw: l'inactivation dépend de l'aw initiale, mais même dans les faibles niveaux d'aw (<0,30) il y a eu une réduction considérable de spores ; en plus à des niveaux de aw couramment trouvés dans les poudres alimentaires (0,30 à 0,50), nous avons observé une inactivation supérieure à 5 log dans les traitements de courte durée (3 à 4 minutes).
- Aucune augmentation de la teneur en eau des spores: malgré son efficacité, le traitement à la vapeur ajoute de l'eau au produit comme résultat de la condensation de la vapeur ainsi, une étape ultérieure de séchage doit être effectuée afin d'éviter la formation de moisissures et la détérioration du produit. Notre technique fonctionne en maintenant la teneur en eau initiale afin d'augmenter la destruction des spores.
- Aucun séchage simultané du produit: la chaleur sèche normale et des traitements thermiques infrarouges conduisent à l'évaporation de l'eau initialement présente dans le produit, ce qui diminue l'inactivation des spores. Dans notre cas, l'utilisation d'une contre-pression a augmenté la température de vaporisation de l'eau dans le système et ainsi elle a empêché le séchage des spores.

Le dernier point est considéré comme étant la clé de l'inactivation des spores en milieu sec, comme indiqué à la fois dans l'étude de DSC ainsi que dans les résultats provenant du réacteur. Le traitement doit être effectué sous pression afin d'éviter le séchage, mais comme la pression interne augmente avec l'augmentation de la température, la pression initiale peut être inférieure au niveau nécessaire pour éviter la vaporisation de l'eau. Par exemple, à 150 °C la P_{vap} est de 5 bars, mais, comme l'a montré l'analyse thermodynamique (tableau 11), un P_i de 4 bars est suffisante pour éviter le séchage puisque à 150 °C la P_f sera de 5,68 bar, soit plus élevé que la P_{vap}. Pour les traitements à des températures différentes les mêmes considérations doivent être prises.

Cependant, l'étude actuelle présente quelques limitations. Tout d'abord, les traitements ont été faits en utilisant que des spores sèches et pas des produits alimentaires réels. Par conséquent, des essais doivent être effectués en utilisant différentes matrices alimentaires inoculées avec des spores de *Bacillus* pour vérifier les niveaux d'inactivation obtenus ainsi que les possibles changements dans les paramètres de qualité des aliments (couleur, arôme, composés volatils, et les fonctionnalités, etc.).

Une autre limitation est l'utilisation d'une seule espèce de spore bactérienne, même si deux souches ont été utilisées. *B. subtilis* est normalement utilisé comme un modèle pour d'autres spores pathogènes, mais il y a des différences de résistance entre les espèces et même entre des souches de

la même espèce. En outre, l'utilisation de différents mutants de *B. subtilis* pourrait fournir une connaissance plus approfondie des mécanismes impliqués dans leur destruction dans ce réacteur.

Enfin, le système a été développé dans une échelle de laboratoire, et par conséquent il n'est pas optimisé. Afin de traiter les matrices alimentaires, un réacteur de volume plus important doit être construit avec un système d'agitation pour assurer un chauffage homogène et éviter la surchauffe produit plus proche des parois.

CONCLUSION ET PERSPECTIVES

L'objectif de cette étude était la compréhension des facteurs et des mécanismes impliqués dans la résistance à la chaleur des spores déshydratées. La thèse a été divisée en deux parties principales: d'abord, une étude fondamentale sur l'effet de la teneur et la distribution d'eau dans la spore sur sa résistance à la chaleur suivi par une seconde partie qui décrit l'application de ces résultats. L'originalité de ce travail réside sur la méthode choisie pour analyser les spores sèches (Analyse Enthalpique Différentielle) et dans le développement d'une application à partir des résultats fondamentaux. Cette conclusion réaffirmera les principales résultats et souligner des directions futures et des perspectives en ce qui concerne le système de traitement thermique développé.

AED

Bien que la technique d'AED a déjà été utilisé pour analyser des spores, ces études ont surtout portés sur la transition vitreuse, à savoir, une application classique de la calorimétrie. Dans cette étude, la capsule d'AED a été pensé comme un réacteur qui permet la réalisation d'un traitement thermique dans des conditions extrêmement contrôlées ainsi que la récupération de différentes données sur l'état des spores pendant et après le traitement, en particulier la relation entre les résultats de viabilité avec les thermogrammes et la perte de poids. De plus, l'utilisation de deux bacs avec des résistances à la pression différentes a rendu possible la modulation de la température d'évaporation de l'eau des spores, ce qui a créé par conséquent, deux traitements thermiques très différents.

Le premier résultat important est la remarquable teneur en eau trouvé dans des spores extrêmement sèches ($a_w = 0,13$) et aussi que la plupart de l'eau se trouve probablement dans le protoplasme. Dans le cas où une capsule résistante à la pression a été utilisée (capsule B), deux événements différents liés à l'évaporation de l'eau ont été observés et elles ont été associées à l'eau externe et interne. Il est intéressant de noter que le second événement représente une fraction de l'eau qui s'est évaporée à environ 200 °C, ce qui est l'équivalent d'une pression de vapeur de 16 bars.

D'après les estimations, 30% de l'eau présente dans des spores complètement hydratées se trouve dans le protoplasme et 70% dans les couches plus externes (Kong et al. 2012).

Cependant, lorsque sont séchées, l'eau de couches plus externes s'évapore avant tout changement dans l'eau du protoplasme (Maeda and Koga 1981; Ishihara et al. 1994; Kong et al. 2013) due la faible perméabilité de la membrane interne (Cowan et al. 2004). Par conséquent, comme le transfert de l'eau est très faible le temps d'équilibre est extrêmement long et donc les spores auront une proportion d'eau plus importante dans le protoplasme que dans les couches externes. En plus, nous avons observé que à faible activité de l'eau (<0,30) la quantité d'eau évaporé dans l'AED est plus élevé que la valeur donnée par l'isotherme de sorption de la même souche (Nguyen Thi Minh et al. 2010). Ce résultat suggère que les couches extérieures et le protoplasme ont des activités de l'eau distinctes, et que la valeur mesurée est en fait l'activité de l'eau externe. Pour conclure, même étant un important paramètre opérationnel important, l'activité de l'eau ne constitue pas la meilleure estimation de la teneur en eau de spores.

Cette étude a aussi mise en évidence l'effet cruciale de l'état hydrique de la spore sur sa résistance à la chaleur. Les spores équilibrées à différentes activités de l'eau ont présenté de profils d'inactivation différents, celles plus hydratées ont été inactivées davantage pour un même traitement thermique. En outre, les deux capsules ont produit deux traitements thermiques différentes, puisque dans la capsule A une longue phase de séchage à faible température a été observé tandis que dans la capsule B cette phase de séchage était courte et elle a commencé à très hautes températures. Donc les spores qui ont été traitées à la chaleur et séchées simultanément (Capsule A) étaient beaucoup plus résistant à la chaleur que ceux qui ont maintenu leur teneur en eau initiale jusqu'à des températures élevées ont été atteintes (Pan B).

La différence d'inactivation a été mise en évidence par l'analyse des spectres de FTIR des spores traitées. Ceux qui ont séché (Capsule A) ont gardé la structure protéique presque intacte, en particulier lorsque les spores très sèches ont été utilisées. Par contre, les spores qui ont gardé leur teneur en eau initiale (Capsule B) présentaient des modifications majeures sur la structure des protéines et ont perdu tout leur DPA, ce qui suggère que la conformation des spores a été largement affectée par ce traitement.

Ces résultats confirment que simplement en gardant leur teneur en eau initiale lors de l'application de la chaleur sèche, il est possible de changer complètement les mécanismes de l'inactivation des spores. Par conséquent, la faible inactivation associée à la modification de la protéine minimal confirme que de la faible teneur en eau des spores permet de les protéger

de la chaleur en réduisant la dénaturation des protéines de ces dernières. En conclusion, l'inactivation faible associée à la modification minimale des protéines confirme que de faibles teneurs en eau peuvent protéger la spore de la chaleur, en réduisant au minimum la dénaturation des protéines.

Chaleur sèche versus chaleur humide

Jusqu'à présent, il était bien connu que la chaleur humide est plus efficace que la chaleur sèche dans la destruction des spores bactériennes. Toutefois, compte tenu de tous ces résultats, nous pensons que cette hypothèse doit être rediscutée et nous apportons un point de vue différent concernant l'inactivation des spores bactériennes. Nous proposons que c'est en fait le niveau d'hydratation de la spore qui détermine sa destruction par la chaleur à des températures supérieures à 100 ° C. Il est donc raisonnable de supposer que le traitement par la chaleur humide entraîne l'hydratation des spores, ce qui contribue à leur destruction, tandis que le traitement conventionnel de chaleur sèche provoque la déshydratation des spores ce qui les rend moins sensible au traitement.

Pour vérifier ces hypothèses, deux expérimentations différentes pourraient être proposées. Dans la première, les spores très sèches (<10% d'eau en poids sec) sont soumis à un traitement très court avec de la chaleur humide. Puisque le temps nécessaire pour hydrater les spores est suffisamment long, nous pouvons supposer qu'ils resteront "sèches" pendant le traitement, et comme il a été montré, leur résistance augmente remarquablement lorsque les teneurs en eau sont très faibles. Par conséquent, on s'attend à ce traitement devrait résulter dans un taux de mortalité très faible. Dans ce cas, il serait démontré que le traitement par la chaleur humide par lui-même ne sera pas capable de détruire les spores sèches.

La deuxième expérience serait le contraire: les spores totalement hydratés doivent être traités à la chaleur sèche à des températures élevées pendant de temps très courts. Comme les spores hydratées sont plus sensibles à des traitements thermiques, et comme dit précédemment, le temps nécessaire pour équilibrer des spores à faibles teneurs en eau est assez long. Donc le temps de traitement doit être plus long que le temps nécessaire pour augmenter la température des spores. En conclusion, tant que le traitement a une courte durée et n'affecte pas la teneur en eau de spores, ils seront très sensibles à la chaleur et des taux de mortalité très élevés sont attendus. Ainsi, dans ce cas, il serait démontré que la chaleur sèche peut inactiver les spores humides.

Réacteur

D'après les résultats intéressants obtenus avec l'étude AED, nous avons développé une Preuve de Concept afin de vérifier les résultats. Par conséquent, pour éviter l'évaporation de l'eau à partir des spores, une contre-pression a été utilisée avec l'ajout d'azote gazeux. Le nouveau système s'est avéré très efficace dans l'inactivation des spores de *Bacillus* sèches, puisque une forte inactivation a été atteinte avec des temps de traitement très courts (de 5 à 6 log, en quelques minutes).

Les traitements à la vapeur d'eau sont un moyen simple et efficace pour décontaminer les aliments; c'est pourquoi la plupart des industriels utilisent, à l'heure actuelle, de la vapeur saturée comme fluide décontaminant. Toutefois la vapeur présente des problèmes comme par exemple la condensation d'eau sur le produit qui doit être éliminée pour éviter la formation de moisissures et la détérioration ultérieure. En outre, d'autres traitements disponibles en milieu aqueux ne sont pas efficaces dans des faibles activités de l'eau tels que l'haute pression et les champs électriques pulsés. Donc, de nouvelles procédures pour la décontamination des aliments secs qui soient capables de détruire les spores sont nécessaires et notre système est un bon candidat.

Le système décrit ici peut être utilisé pour le traitement des aliments à faible teneur en eau, il a l'avantage de ne pas ajouter de l'humidité du produit et il en résulte des taux élevés de destruction de spores (<5 log). La prochaine étape serait de réaliser un système à plus grande échelle afin d'effectuer un test sur des produits alimentaires réels et d'évaluer à la fois la décontamination microbiologique et les changements dans les paramètres de qualité.

Ce projet pilote pourrait ensuite être utilisé pour traiter les produits comme les épices par exemple. L'activité de l'eau normalement trouvée dans les épices est comprise entre 0,30 et 0,50 et, comme il a été montré, une inactivation supérieure à 5 logs peut être atteinte avec un traitement de 3 minutes à ces activités de l'eau. Par conséquent, si les épices sont déjà autour de cette activité de l'eau, aucune équilibration d'humidité n'est nécessaire au préalable, cependant si le produit à traiter présente une faible a_w , une étape d'équilibration serait devoir être ajoutée.

Dans ce cas, les épices seraient stockées dans une atmosphère avec une humidité relative de 50% pendant quelques semaines, puis ils seraient traités. Certes, les différentes conditions de temps, de température et de pression doivent être testés pour optimiser cette nouvelle procédure de décontamination.

Ainsi, nous travaillons actuellement sur le dépôt d'un brevet de ce système miniature qui sera déposé d'ici la fin de l'année 2013.

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