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

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Abstract

High pressure combined with elevated temperatures can produce low acid, commercially sterile and shelf-stable foods. Depending on the temperature and pressure levels applied, bacterial endospores pass through different pathways, which can lead to a pressure-induced germination or inactivation. Regardless of the pathway, Bacillus endospores first release pyridine-2,6-dicarboxylic acid (DPA), which contributes to the low amount of free water in the spore core and is consequently responsible for the spore’s high resistance against wet and dry heat. This is therefore the rate-limiting step in the high pressure sterilization process. To evaluate the impact of a broad pressure, temperature and time domain on the DPA release, Bacillus subtilis spores were pressure treated between 0.1 and 900 MPa at between 30 and 80 °C under isothermal isobaric conditions during dwell time. DPA quantification was assessed using HPLC, and samples were taken both immediately and 2 h after the pressure treatment. To obtain a release kinetic for some pressure-temperature conditions, samples were collected between 1 s and 60 min after decompression. A multiresponse kinetic model was then used to derive a model covering all kinetic data. The isorate lines modeled for the DPA release in the chosen pressure-temperature landscape enabled the determination of three distinct zones. (I) For pressures <600 MPa and temperatures >50 °C, a 90% DPA release was achievable in less than 5 min and no difference in the amount of DPA was found immediately 2 h after pressurization. This may indicate irreversible damage to the inner spore membrane or membrane proteins. (II) Above 600 MPa the synergism between pressure and temperature diminished, and the treatment temperature alone dominated DPA release. (III) Pressures >600 MPa and temperatures <50 °C resulted in a retarded release of DPA, with strong increased differences in the amount of DPA released after 2 h, which implies a pressure-induced physiological like germination with cortex degradation, which continues after pressure release. Furthermore, at 600 MPa and 40 °C, a linear relationship was found for the DPA release rate constants ln(kDPA) between 1 s and 30 min.

1. Introduction

Thermal retorting is the process commonly used for producing shelf-stable low acid food products. For a complete inactivation of all endospores, however, high levels of heat have to be applied. To reduce this undesirable amount of over-processing, an isostatic high pressure treatment combined with elevated starting temperatures (Tinit >70 °C) has been identi-
due to some pressure-resistant spore populations that cannot be germinated by pressure. Consequently, higher treatment temperatures are needed. If the food is preheated to moderate treatment temperatures (T > 60 °C), the inactivation of spores proceeds at least as a two-step process (Heinz and Knorr, 1996; Margosch et al., 2004b; Mathys et al., 2007; Paidhungat et al., 2002; Reineke et al., 2012; Wuytack et al., 1998). In this process the endospores are first induced to release DPA by high pressure, and the germinated spores are subsequently killed by the combination of pressure and heat, owing to their increased overall sensitivity to stress (Paidhungat et al., 2002; Reineke et al., 2011a, 2012; Sale et al., 1970; Wuytack et al., 1998).

Whereas pressure induced germination can be strongly accelerated by increasing the treatment temperature (Gould, 1970; Reineke et al., 2012), a temperature decrease to room temperature strongly retards or even inhibits the germination process (Nakayama et al., 1996; Sale et al., 1970).

In addition to the treatment temperature, the applied pressure level has a significant impact on the germination pathway of all Bacillus and Geobacillus spore strains (Paredes-Sabja et al., 2011). At pressures between 150 MPa and 400 MPa, a nutrient-like physiological germination is induced without the presence of nutrients (Gould, 1970), through triggering the spore’s nutrient receptors (Setlow, 2003). The activation of the nutrient receptors causes the release of pyridine-2,6-di-carboxylic acid (dicapilloinic acid [DPA]), by an assumed opening of the spore’s dicapilloinic acid channels. The existence of such DPA-channel proteins is unconfirmed, but it is likely that the SpoVAD protein is involved in DPAA2 movement into and out of the spore (Li et al., 2012). The release of DPA is accompanied by activation of the spore’s cortex lytic enzymes, which are responsible for degradation of the cortex (Paidhungat et al., 2001).

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

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

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

2. Materials and methods

2.1. Sporulation and spore preparation

The strain B. subtilis PS832 (obtained from P. Setlow; University of Connecticut, Health Center) that was used in this study was sporulated according to a method described by Nicholson and Setlow (1990). To induce sporulation a liquid preparatory culture obtained from a single colony was grown in 50 mL LB-Lennox-broth (Molekula, Manchester, U.K.) at 37 °C under continuous shaking (120 rpm). After 24 h, 200 μL of the cell suspension was surface plated on 2× SG medium agar plates and incubated at 37 °C. After sporulation the spores were harvested and the suspension was cleaned by repeated centrifugation (3-fold at 5000 g). It was then washed with cold distilled water (4 °C) and sonicated for 1 min. The cleaned spore suspension contained approximately 1.0×10^11 spores/mL (≥95% phase bright) with nearly no spore agglomerates, as verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.).

2.2. Isostatic high pressure treatment

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

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

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

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

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

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

The pressure-treated samples were filtered 2 h after the treatment, and additionally an aliquot was immediately made for DPA analysis. After decompression for all samples pressurized in the U111 system. In an independent test series, samples were treated at 200 MPa and 600 MPa at 40 °C, and after 1 min, 5 min, 10 min, 20 min, and 1 h. Between sampling they were stored at 4 °C and subjected to continuous shaking at 150 rpm (HLC Cooling-Thermo-Mixer MKR13, Ditabis, Pforzheim, Germany). Pressure-induced DPA release was calculated as a constant ratio of spores for the staining. The concentrations of the fluorescent dyes for staining were 15 μM SYTO16 (Invitrogen, Carlsbad, CA, USA) and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA), according to the method of Mathys et al. (2007). Both fluorescent dyes are able to stain DNA, but the membrane impermeant SYTO16 acts as an indicator for spore germination, whereas the membrane impermeant PI indicates membrane permeability. The treated spore suspensions were diluted with ACES buffer solution (0.05 M, pH 7) to achieve a flow rate of about 1000 events/s, as well as a constant ratio of spores for the staining. The concentrations of the fluorescent dyes for staining were 15 μM PI and 0.5 μM SYTO16 in the diluted spore suspension. Afterwards, the samples were stored in the dark at ambient temperature for 15 min. The analyses were carried out using a BD FACSCalibur flow cytometer (BD Bioscience, San José, CA, USA) equipped with a 15 mW, 488 nm air-cooled argon ion laser. BD CellQuest Pro (Version 5.2, BD Biosciences) was used as operation and acquisition software. To achieve a clear determination of the different spore populations, and to discriminate non-spore particles, the amplification of the forward scatter (FSC) was set to E01 (10-fold) and the FSC threshold was set to 72. The amplification of the side scatter (SSC) was set to 721 V and the SSC threshold was set to 706. All analyses were performed to a minimum in duplicate.

2.4. Flow cytometry analysis

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

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

2.5. Calculation of isokineticity lines

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

Firstly:

$$\frac{d(c_{DPA})}{dt} = -k_{isp}(p, T)[c_{DPA}]$$

describes the release of DPA under isothermal isobaric conditions, with $k_{isp}$ as a rate constant. The amount of released DPA under dynamic pressure–temperature conditions ($\tau_0 = 1$ s dwell time) was excluded. This equation was also utilized to calculate the time-dependent release of DPA after decompression at 200 MPa and 600 MPa, at 40 °C.

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

$dormant \rightarrow germinated \rightarrow inactivated.$

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

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

$$\frac{d[germinated]}{dt} = k_s(p, T)[dormant] - k_h(p, T)[germinated]$$

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

with $k_s$ and $k_h$ as rate constants.

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

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

$$[dormant] = [dormant]_0 \exp(-k_s t)$$

$$[germinated] = [germinated]_0 \exp(-k_h t) + \frac{k_s [dormant]_0}{k_h - k_s} \left[\exp(k_h t) - \exp(-k_h t)\right]$$

$$[inactivated] = [inactivated]_0 + \frac{[germinated]_0}{k_h - k_s} \left[1 - \exp(-k_h t) + [dormant]_0 \frac{k_h \exp(-k_h t) - k_s \exp(-k_s t)}{k_h - k_s}\right]$$

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

2.6. Reproducibility of results

All experiments were carried out at least in duplicate. The presented data are either the means taken from two replicate experiments or from single representative experiments. Generally, the cell counts of pressure-treated samples were reproducible within 0.5 log₁₀. For kinetic modeling, at least 4 kinetic points for each pressure–temperature combination were used, excluding dynamic pressure temperature conditions during compression and decompression.
3. Results and discussion

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

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

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

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

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

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

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

Table 1

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>Pressure [MPa]</th>
<th>ln(kDPA)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td></td>
<td>600</td>
<td>−3.51</td>
</tr>
</tbody>
</table>

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

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

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

A comparison of the isorate lines in Fig. 1 with those in Fig. 2B shows an equal characteristic between 300 and 600 MPa. This finding demonstrates that in addition to data for heat sensitive and inactivated spores (Reineke et al., 2012), the release rates for DPA can also be compared between different HP systems, if they are derived under isothermal-isobaric conditions during pressure dwell time. Furthermore, it can be confirmed that, above a threshold pressure of 600 MPa, the release of DPA from the spore core is nearly independent of the pressure and can be accelerated by a factor of 10, if the temperature is increased from 50 °C to 70 °C. These findings are in agreement with published DPA release kinetics for pressure treatments of \( B. \ subtilis \) at 600 MPa and 40 °C and 800 MPa at 70 °C. Wuytack et al. (1998) reported an 87% DPA release after 1 h at 600 MPa, and a 98% release was measured by Margosch et al. (2004b) after 2 min at 800 MPa.

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

The synergism between pressure and temperature above 50 °C and below 600 MPa could be explained by a pressure-induced crystallization of the inner spore membrane, which is likely to consist of a high proportion of gel phase lipids (Cowan et al., 2004). This phase transition of lipids under pressure makes biological membranes one of the most pressure-sensitive cellular components (Winter and Jeworrek, 2009). Another explanation of the synergism, however, could be that a ceasing of membrane protein function occurs above 200 MPa (Winter and Jeworrek, 2009), which might be attributed to a physiologically unacceptable overall state of the spore’s inner membrane. Hence, the assumed opening of the Ca²⁺-DPA-channel proteins (Paidhungat et al., 2002) or a denaturation of other membrane proteins may also occur.

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

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

![Fig. 2: Iso-rate lines from 2 to 20 min for a 90% DPA release immediately after the pressure treatment.](image1)

![Fig. 3: Iso-rate lines from 2 to 20 min for a 90% DPA release immediately after the pressure treatment for isobaric and isothermal conditions.](image2)

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### Table 2

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

<table>
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<th>Temperature [°C]</th>
<th>Pressure [MPa]</th>
<th>U111 ln(k(DPA)) after 0 s</th>
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<td>−3.73</td>
<td>−2.98</td>
</tr>
<tr>
<td>100</td>
<td>90</td>
<td>−1.38</td>
<td>−1.50</td>
<td>−1.48</td>
<td>−1.59</td>
</tr>
<tr>
<td>105</td>
<td>100</td>
<td>−1.37</td>
<td>−1.50</td>
<td>−1.48</td>
<td>−1.59</td>
</tr>
</tbody>
</table>

* Data from thermal inactivation in thin glass capillaries.

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

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

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

When compared to the 200 MPa treatment, the DPA release at 600 MPa was strongly retarded. Whereas a ~100% DPA release was measured after 10 min at 200 MPa, more than 1 min was needed at 600 MPa. Furthermore, the DPA release after decompression was retarded as well, which enabled (through the use of Eq. (1)) the calculation of a DPA release rate $k_{DPA}$ (Fig. 5). The calculated release rates $\ln k_{DPA}$ clearly show that a compression to 200 MPa or 600 MPa and 40 °C (1 s dwell time) was insufficient to induce DPA release. Furthermore, nearly no further DPA release occurred after decompression for dwell times of ≥10 min at 200 MPa or ≥60 min at 600 MPa.

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

3.4. Spore germination and inactivation

In order to enable correlation between the release of DPA and the loss of heat resistance, as well as the subsequent spore inactivation, 3 log$_{10}$ isorate lines were calculated for each case (Fig. 6). A multiresponse kinetic model was used, which enabled simultaneous calculation of both rate constants ($k_h$ and $k_i$) by using multiple data (Reineke et al., 2012). This increases the precision of the parameters (Van Boekel, 2009). The individual rate constants for the heat sensitive and inactivated spores are presented in Table 2. For low pressures (≤600 MPa) and short pressure dwell times (≤10 min) the whole inactivation process shifts towards a purely thermal inactivation. However, especially under these conditions, a strong synergism between pressure and temperature is depicted in Fig. 6. These data therefore extend previously published kinetics

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

![Fig. 5. Calculated release rate $\ln(k_{DPA}) [1/s]$ for an isothermal isobaric pressure treatment at 40 °C and 200 MPa (▲) or 600 MPa (○).]
(Reineke et al., 2012) to higher temperatures and lower pressures. Moreover, kinetic data from both studies are nearly identical under equal process conditions (e.g. 600 MPa and 3 min or 10 min).

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

In this study, the temperature needed to achieve 3 log10 heat sensitive spores at 800 MPa was 61.2 °C for a 3 min treatment and 56 °C for a 10 min treatment. However, to inactivate 3 log10 of spores at 800 MPa, temperatures of 70.2 °C and 63.7 °C were needed, respectively. It can therefore be seen that an impact of pressure and temperature is present at high treatment pressures, but is not so dominant as for lower pressures and high temperatures. Similar behavior has also been reported for *C. botulinum* (Margosch et al., 2004a), where a transient increase in temperature resulted in a strongly accelerated inactivation. Furthermore, this accelerated inactivation was reflected by an accelerated release of DPA.

For pressure dwell times above 10 min, the shape of the isorate lines for the DPA release and heat sensitive spores differs. Whereas the DPA release for pressures below 300 MPa strongly shifts towards lower treatment temperatures (Fig. 2A), this behavior was not found for the formation of heat-sensitive spores after pressurization (Fig. 6A) or for spore inactivation during the treatment (Fig. 6B). This suggests a different inactivation pathway under these conditions, which probably includes at least a successful pass through germination stage I. However, the temperature difference between a 3 log10 germination within 20 min (43.3 °C) at 300 MPa and a 3 log10 inactivation (52 °C) remained nearly constant, compared to higher process pressures and temperatures.

Wuytack et al. (1998) have stated that spores treated at 100 MPa and 40 °C were able to degrade their small soluble acid proteins (SASPs) and further to rapidly generate ATP, in contrast to spores treated at 600 MPa. When these findings are combined with inactivation data of the same *B. subtilis* spore strain (PS832) (Reineke et al., 2012), in which a 3 log10 inactivation was achieved for pressure dwell times exceeding 40 min, they lead to the assumption that a pressure-induced physiological spore germination is possible for pressures up to 600 MPa and ≤50 °C. To further evaluate this assumption flow cytometry analysis was performed.

### 3.5. Flow cytometry analysis

The results of the flow cytometry (FCM) analysis for each kinetic point are presented in Fig. 7. Due to the diverse behavior of the assumed spore DPA release mechanisms in this study, all stained spores were regarded as no longer in the dormant state. They were plotted against the calculated amount of heat sensitive spores based on Eq. (4). This plot was used because this population includes spores that are PI as well as SYTO16 stained. Fig. 7A shows the results for the FCM analysis at 40 °C and 60 °C. For the 40 °C treatment a good correlation of the residuals was found (adj. R² = 0.964) for all pressure dwell times, as well as an adequate fit (adj. R² = 0.76) at 60 °C and pressure dwell times below 3 min. Furthermore, under these conditions all treated samples showed a positive SYTO16 staining, which indicates spore germination. This shifted to a positive PI staining for longer pressure dwell times, indicating an inactivation. The detection of SYTO16 fluorescence suggests that spores are able to degrade their cortex (Black et al., 2005) under pressure, for pressure treatments up to 600 MPa and moderate treatment temperatures, and even for pressures up to 900 MPa with short pressure dwell times at 60 °C.

For pressure dwell times above 3 min (Fig. 7A) or at higher treatment temperatures (80 °C, Fig. 7B), the inactivation mechanism seems to change. As shown in Fig. 2, a rapid release of DPA was detected under these conditions. However, a defined SYTO16 or PI staining of the spores was no longer possible. Whereas spores treated at 60 °C and dwell times ≥3 min showed an intermediate fluorescent signal between a positive SYTO16 or PI staining (described as the unknown state; Mathys et al., 2007), or were even not stainable, all spores showed a positive PI staining after a thermal treatment at 80 °C for 20 min. This indicates that a rupture of the inner spore membrane occurred after an additional mild thermal heat treatment.

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

Interestingly, no staining with SYTO16 or PI was possible for spores that were pressure treated for more than 1 min at 80 °C under all tested pressure conditions (200 MPa–900 MPa). Furthermore, these spores also showed no positive PI staining after a mild thermal treatment (20 min at 80 °C), even though under some treatment conditions a full spore inactivation (>5.7 log10) was achieved during the pressure treatment. There could be various explanations for these diverse staining behaviors, and only a few authors have used FCM analysis for

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**Fig. 6.** Isorates lines from 1.5 to 20 min for 3 log10 heat sensitive (A) and inactivated (B) *Bacillus subtilis* spores under isothermal isobaric conditions during pressure dwell times. Arrows depict the time needed for a thermal 3 log10 inactivation. Dotted lines represent the adiabatic heat of compression for water. The dashed line represents the freezing line between water and the different pressure-dependent ice modifications.
pressure-treated spores or for spores in general (Baier et al., 2011; Black et al., 2005, 2006; Laflamme et al., 2005; Mathys et al., 2007).

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

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

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

4. Conclusions

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

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

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.12.010.

Acknowledgments

The authors thank Peter Setlow from the University of Connecticut-Health Center for his helpful comments. Further we acknowledge Irene Hemmerich for her assistance during the HPLC analysis.

This work was partially financially supported by a grant from the Federal Agency for Agriculture and Food (BLE-2816302307).

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