New pressure and temperature effects on bacterial spores

Alexander Mathys¹, Volker Heinz², Dietrich Knorr¹

¹Berlin University of Technology, Department of Food Biotechnology and Food Process Engineering, Koenigin-Luise-Str. 22, D-14195 Berlin, Germany, Tel: +49 30 314-71248, Fax: +49 30 832 76 63

²German Institute of Food Technology, p.o.box: 1165, D-49601, Quackenbrueck, Germany, Tel: +49 5431 183 232, Fax: +49 5431-183114

E-mail for correspondence: alexander.mathys@tu-berlin.de

Abstract. The mechanism of inactivation of bacterial spores by heat and pressure is still a matter of discussion. Obviously, the change of the dissociation equilibrium under pressure and temperature plays a dominant role in inactivation of microorganisms. Heat and pressure inactivation of Geobacillus stearothermophilus spores at different initial pH-values in ACES and phosphate buffer confirmed this view. Thermal inactivation in ACES buffer at 122°C resulted in higher logarithmic reductions. Contrary, after pressure treatment at 900 MPa with 80°C phosphate buffer showed higher inactivation. These results indicated the different dissociation equilibrium shifts in buffer systems by heat and pressure. Due to preparation, storage and handling of highly concentrated spore suspensions the clumping and the formation of aggregates can hardly be avoided. Consequently, the impact of the agglomeration size distribution on the quantitative assessment of G. stearothermophilus spore inactivation was determined by using a three-fold dynamic optical backreflection measurement. Two limiting cases have been discriminated in mathematical modelling: three dimensional, spherical packing for maximum spore count and two dimensional, circular packing for minimum spore count of a particular agglomerate. Thermal inactivation studies have been carried out in thin glass capillaries, where by using numerical simulations the non isothermal conditions were modelled and taken into account. It is shown that the shoulder formation often found in thermal spore inactivation can sufficiently be described by first-order inactivation kinetics when the agglomeration size is considered. In case of high pressure inactivation agglomerations could be strongly changed by high forces at compression and especially decompression phase. The physiological response of Bacillus licheniformis spores to high pressure was investigated using multiparameter flow cytometry. Spores were treated by high pressure at 150 MPa with 37°C, and then dual stained with the fluorescent dyes SYTO 16 and propidium iodide. For pressure treated spores four distinct populations were detected by flow cytometry, and for these we suggest a three step model of inactivation involving a germination step following hydrolysis of the spore cortex, an unknown step, and finally an inactivation step with physical compromise of the spore inner membrane. An understanding of these effects and mechanisms will aid the safety assessment of pressure assisted thermal sterilisation, in turn facilitating the adoption by industry and commercialisation of such processes.

© 2008 IOP Publishing Ltd
1. Impact of dissociation equilibrium shift on spore inactivation

Spore inactivation studies were performed in two buffer systems (phosphate [PBS] and N-(2-Acetamido)-2-aminoethanesulfonic acid [ACES]). For a more obvious presentation of the inactivation difference the log$_{10}$-reductions from each individual buffer solution were subtracted (Fig. 1). By comparing the difference in log$_{10}$-reduction ($\text{[log}_10\text{(N/No)}\text{]}_{\text{PBS-ACES}}$) in phosphate and ACES maximum values up to $2\log_{10}$ after thermal (122°C, Fig. 1a) and $1.5\log_{10}$ after pressure (900 MPa at 80°C, Fig. 1b) treatment could be observed. These data indicated the different dissociation equilibrium shift (pK$_a$-shift) in buffer systems by heat and pressure [1]. At lower initial pH-values a higher spore inactivation was observed, because of increased differences between pH and pK$_a$-value. Direct comparison of different treatments and detailed amounts of pK$_a$-shifts is difficult because of the unknown temperature dependence of the pK$_a$-value at higher temperatures and especially various inactivation mechanisms, which were detected by different non-linear log$_{10}$-reduction (shoulder and tailing).

![Fig. 1](image1.png)

Fig. 1): Difference in log$_{10}$-reduction of *G.stearothermophilus* spores in phosphate (PBS) and ACES ($\text{[log}_10\text{(N/No)}\text{]}_{\text{PBS-ACES}}$) after thermal (122°C, Fig. 1a) and pressure (900 MPa at 80°C, Fig. 1b) treatment at different initial pH-values.

2. Physiological response of spores to high pressure

The physiological response of *Bacillus licheniformis* spores to high pressure and thermal inactivation was investigated using multiparameter flow cytometry. Spores were treated by high pressure at 150 MPa (37°C), and then dual stained with the fluorescent dyes SYTO 16 (permeable DNA dye, indicator of spore germination) and propidium iodide (impermeable nucleic acid dye, indicator of membrane permeabilisation). On the basis of the comparisons among different samples, a population assignment was made (Fig. 2a). Sub-population 1 was assigned as the dormant (culturable) spore sub-population, and sub-population 4 as the inactivated (non-culturable) spore sub-population. Results indicated that sub-population 2 represented the germinated (culturable, but heat sensitive) sub-population, and sub-population 3 a second, unknown heat-sensitive sub-population, of unknown culturability. Sub-population 2 is presumed to be cortex hydrolysed spores with an intact inner membrane. The nature of sub-population 3 is difficult to presume. One possibility is that sub-population 3 represents a population with a hydrolysed cortex and some damage to the inner membrane, thus allowing PI to enter the spores and partially displace SYTO 16 and reduce the observed green staining. SYTO 16 may also be quenched in sub-population 3 by fluorescence resonance energy transfer to PI, as has been reported for other SYTO dyes [2].
Fig. 2a): Sub-population assignment for density plot diagrams of *B. licheniformis* spores in sodium citrate buffer after treatment at 150 MPa with 37°C for 20 min: (0 = noise), 1 = dormant, 2 = germinated, 3 = unknown, 4 = inactivated [3].

2b) Heterogeneous population distribution in a predicted three-step-model (N₁ \(\rightarrow\) N₂ \(\rightarrow\) N₃ \(\rightarrow\) N₄, lines) for *B. licheniformis* spores after pressure treatment at 150 MPa with 37°C in sodium citrate buffer fitted with experimentally determined flow cytometric measurements; dormant (■, black), germinated (●, green), unknown (▲, blue) and inactivated (▼, red) [3].

Using the sub-population assignment described (Fig. 2 a), a three step model of inactivation based on a series of chemical reactions with associated rate constants \(k_i\) (\(i=1, 2, 3\)) is suggested. The whole model includes: a germination step (N₁ \(\rightarrow\) N₂), an unknown step (N₂ \(\rightarrow\) N₃) and finally the inactivation step (N₃ \(\rightarrow\) N₄). The velocity \(v_i\) of each step is related to the rate constants \(k_i\) of the reaction and the concentration of the participating entities \(N_j\) (\(j=1, 2, 3, 4\)), where Eq. 1-4

\[
\begin{align*}
\frac{dN_1}{dt} &= -k_1 N_1 = -v_1 \\
\frac{dN_2}{dt} &= k_1 N_1 - k_2 N_2 = v_1 - v_2 \\
\frac{dN_3}{dt} &= k_2 N_2 - k_3 N_3 = v_2 - v_3 \\
\frac{dN_4}{dt} &= k_3 N_3 = v_3
\end{align*}
\]

represent a differential equation system of this assumptions in which changes in the species concentrations \(N_j\) with time \(t\) proceed according to the velocities \(v_i\) of the reactions that form or remove them. After a multiparameter fit of the whole differential equation system with the sub-population values from each individual population, all three rate constants were obtained. The predictive three step model for the physiological mechanism of inactivation under pressure at 150 MPa showed good regression with the experimental results (Fig. 2b). The modelling enabled the assessment of the continuous population distribution and an extrapolation of the experimental data. Variations between the model and experimental data occurred because of partial overlap of some sub-populations.

3. Impact of spore agglomeration on heat inactivation

The agglomeration size distribution in suspensions of *Geobacillus stearothermophilus* spores was determined by using a three-fold dynamic optical back-reflexion measurement (3D ORM) (Fig. 3). Since 3D ORM accurately yields the maximum length extension of an agglomerate, but provides no information on the packing density. After idealization to the spherical form, the assumed radius of the spores is 1.125 µm based on the X-ray microscopy determinations [4]. The assumption for the agglomerate geometry is divided into three approaches (one, two or three dimensional). The regular
three-dimensional arrangements of sphere (3D) with the highest density are cubic close packing or hexagonal close packing. After Kepler [5] both arrangements have an average density of $\pi/(\sqrt{18})$. In the case of two dimensions (2D) the cross-sectional area can be derived with the measured equivalence diameter. The honeycomb circle packing with a density of $\pi/(\sqrt{12})$ is the unique densest lattice sphere packing in two dimensions [6]. For only one dimension (1D), a chain formation with the product of spore diameter and spore number was supposed. The different geometrical approaches show large differences in number of spores per agglomerate. Thermal inactivation data have been modelled (Fig. 3b) using first-order inactivation kinetics [7], superimposed by the agglomeration size (Fig. 3a). Thermal inactivation studies have been carried out in thin glass capillaries, where by using numerical simulations the non isothermal conditions were modeled and taken into account. It is shown that the shoulder formation often found in thermal spore inactivation can sufficiently be described with the suggested approach (Fig. 3b).

![Fig. 3a): Agglomeration size distribution of a G.stearothermophilus spore suspension with different geometrical assumptions for the agglomerates and an inset, which shows agglomerations sizes between $10^2$-$10^5$ spores per agglomerate [7].](image)

![Fig. 3b): Experimental plots for ▲113°C, ●121°C, ■130°C and predicted data (lines) after thermal inactivation of G.stearothermophilus ATCC 7953 spores. Inactivation curves were modelled with spherical (3D) assumption of the agglomerate geometry [7].](image)

4. Conclusion

The mechanistic background of the inactivation of bacterial spores is still matter of discussion [8]. Different influences and effects were investigated. The change of the dissociation equilibrium under high pressure plays a major role in sensitive reactions e.g. inactivation of microorganisms and/or denaturation of proteins. Heat and pressure inactivation of G. stearothermophilus spores at different initial pH-values in ACES and phosphate buffer confirmed this view. The experimental results indicated the different pK$_a$-shifts in buffer systems by heat and pressure. By using flow cytometry a simple and very fast (within 20 min of processing) method for the rapid assessment of spore physiological state could be developed. This high throughput method offers substantial benefits with regards acquisition of large data sets, which are required to predict and model bacterial spore inactivation by high pressure and heat, and to determine the stochastic nature of such inactivation. After particle measurement and thermal treatment large influence of the agglomeration in spore suspensions on the inactivation could be detected. The high occurrences of middle size agglomerates had the main influence on the curvilinear form in our study. Practical experiments are difficult in this context, because of the necessary defined separation of the agglomerates. Thus it appears that agglomerations in spore suspensions need to be considered by modelling of the thermal inactivation. However, at this time no information about agglomeration of spores under pressure is given. Detail studies to find possible explanations are under way at present.
Applying the above models, spore inactivation can be better anticipated in planning experimental designs. Such extensive empirical data, coupled with an improved understanding of the mechanism(s) of inactivation, will ultimately be required to demonstrate the benefits and risks of high pressure thermal processing to industry.

Acknowledgments
This research was supported by German Ministry of Economics and Technology (BMBF, Grant No. 0330089A), the European Project NovelQ (015710-2 NOVELQ), Food Science Australia and the Commonwealth Scientific and Industrial Research Organisation Food Futures National Research Flagship.

References

[1] Distèche, A. (1959). pH Measurements with a Glass Electrode Withstanding 1500 kg/cm² Hydrostatic Pressure. Review of Scientific Instruments, 30(6), 474-478.
[2] Stocks, S. M. 2004. Mechanism and use of the commercially available viability stain, BacLight. Cytometry Part A 61A:189-195.
[3] Mathys, A., B. Chapman, M. Bull, V. Heinz, and D. Knorr. 2007. Flow cytometric assessment of Bacillus spore response to high pressure and heat. Innovative Food Science & Emerging Technologies. accepted.
[4] Mönch, S., V. Heinz, P. Guttmann, and D. Knorr. 1999. X-ray microscopy in food science. Presented at the International Conference on X-Ray Microscopy-XRM 99, University of California, Berkeley, USA, 1.- 6.08.1999.
[5] Kepler, J. 1611. Ioannis Kepleri S. C. Maiest. Mathematici Strena Seu De Niue Sexangula. Gottfried Tampach, Francofurti ad Moenum.
[6] Lagrange, J. L. 1773. Recherches d’arithmeticque. Nouv. Mem. Acad. Roy. Sc. Belle Letteres. Oeuvres III:265-312.
[7] Mathys, A., V. Heinz, F. H. Schwartz, and D. Knorr. 2007. Impact of agglomeration on the quantitative assessment of Bacillus stearothermophilus heat inactivation. Journal of Food Engineering 81:380-387.
[8] Heinz, V. and D. Knorr. 2002. Effects of high pressure on spores, p. 77-114. In M. E. G. Hendrickx and D. Knorr (ed.), Ultra high pressure treatments of foods. Kluwer Academic/ Plenum Publishers, New York.