Food sterilisation under high pressure- Fundamentals, new insights and challenges

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Abstract. Consumers all over the world demand high quality and convenient products with natural flavour and taste, and greatly appreciate the fresh appearance of minimally processed food. Sterilization under pressure has been shown to be valuable for global production of high quality products. However, the mechanism of inactivation of bacterial spores by heat and pressure is still a topic 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 G*eobacillus. 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. After particle analysis it could be shown that the lag phase often found in thermal spore inactivation can sufficiently be described by first-order inactivation kinetics when the agglomeration size is considered. The physiological response of *B. licheniformis* spores to pressure and thermal inactivation was investigated using multiparameter flow cytometry. For pressure treated spores, four distinct sub-populations were detected. For these sub-populations, 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's inner membrane. With an improved understanding of the spore inactivation mechanism it will be possible to assess the benefits and sustainability of emerging technologies e.g. pressure assisted thermal sterilization with regards microbiological safety and stability.

1. Introduction

The use of high pressure technology in food processing has steadily increased during the past 10 years and at present, 112 industrial installations exist worldwide with volumes from 35 to 420 l and an annual production volume of more than 120,000 tons (Tonello, C., personal communication). Pressure-assisted heating as emerging technology can homogeneously heat up and cool down products, and it allows the accurate control of the treatment intensity required for sterilization (Heinz & Knorr, 2002). Although it is widely accepted that pressure assisted thermal sterilization is environmentally friendly and can retain the fresh-like characteristics of foods better than heat treatment (Toepfl, Mathys, Heinz & Knorr, 2006), it has not yet been successfully introduced into the food industry—often may be due to the less known inactivation mechanism of bacterial spores. Consequently, for microbiological safety and control of this technology, new methodologies for the detailed investigation of heat and/ or pressure sterilization are required.

During the first phase of such basic inactivation studies buffer systems to obtain constant pH-values and medium properties are in common use. However, the dissociation equilibrium in water and buffer solutions varies with pressure and temperature (Marshall & Franck, 1981; Hamann, 1982; Kitamura & Itoh, 1987). Experimental methods are limited up to 450 MPa (Stippl, Delgado & Becker, 2004) and not suitable for the investigation of combined thermal and pressure dependencies. Due to the shift of dissociation equilibria during heating and/ or compression the results of inactivation experiments are prone to error if not designed correctly.

The effectiveness of thermal and pressure treatments is often verified by challenge tests using foods that have been spiked with highly concentrated microbe suspensions. Due to preparation, storage and handling of those suspensions the clumping and the formation of aggregates can hardly be avoided (Aiba & Toda, 1966; Bueltermann, 1997; Mathys, Heinz, Schwartz & Knorr, 2007). This phenomenon is well known, but its importance for the quantitative assessment of survivors in inactivation experiments has rarely been addressed. Its importance becomes clearer by the following. Agglomerates always produce one colony per each plate. Consequently agglomerates of unknown numbers inside are always counted as one spore until all spores in the agglomerate are inactivated. Beyond this agglomeration and disintegration can change the colony forming units per milliliter.

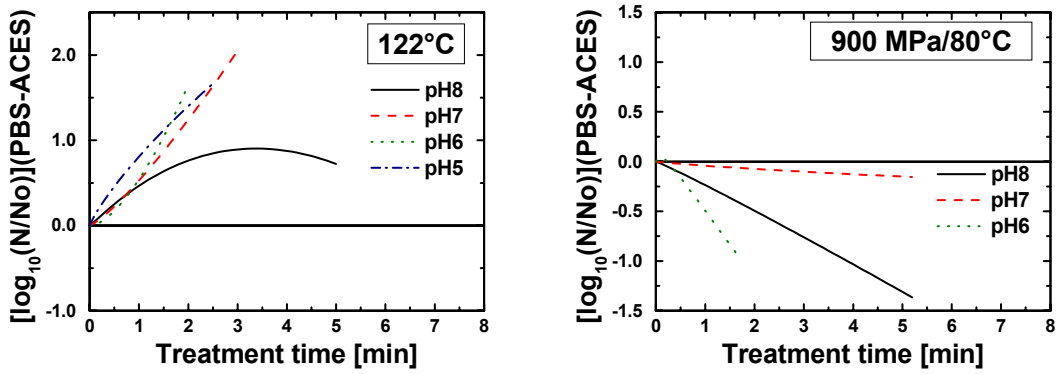
It is firmly held (Clouston & Wills, 1969; Knorr, 1999; Mathys, Chapman, Bull, Heinz & Knorr, 2007), however, that spore inactivation, either by high pressure or combined high pressure and heat, is partly attributed to germination of a proportion of the spore population during the high pressure process. Recently, others have demonstrated the application of flow cytometry to the detection of spore germination by high pressure. Black, Koziol-Dube, Guan, Wei, Setlow, Cortezzo et al. (2005) and Black, Wei, Atluri, Cortezzo, Koziol-Dube, Hoover et al. (2007) reported the use of the green fluorescent nucleic acid dye SYTO 16 as an indicator of germination in pressure treated spores of *Bacillus subtilis*. The first use of the functional dyes 3,3´-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] was reported for the flow cytometric assessment of membrane potential activation in the inner membrane of spores germinated by nutrients (Laflamme, Ho, Veillette, de Latrémoille, Verreault, Mériaux et al., 2005). Thus great potential remains for research into the application of flow cytometry for the assessment of spore physiological response to various processes, including high pressure processing. Changes in the physiological state of bacterial spores by high pressure and thermal processes alone and in combination, should be determined in order to improve the understanding of spore inactivation mechanisms by both pressure and temperature. Further, the rapid, high throughput nature of flow cytometric data acquisition offers substantial benefits in collection of large data sets, which can in turn be used to predict and model bacterial spore inactivation.

An understanding of the mechanism(s) of inactivation, and the use of larger data sets, will aid in the food safety assessment of pressure assisted thermal sterilization in particular, and also assist in the commercialization of these processes facilitating adoption by industry.

2. Results and Discussion

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 ([$\log_{10}$ (N/No)](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. 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).

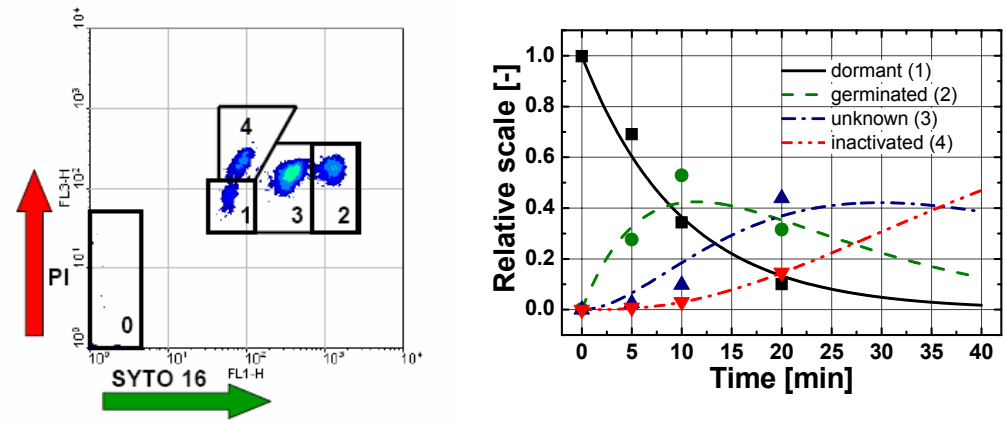


a) **b)**

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

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.



a) **b)**

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 (Mathys, Chapman et al., 2007).

2b) Heterogeneous population distribution in a predicted three-step-model (N1→N2→N3→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) (Mathys, Chapman et al., 2007).

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 (Stocks, 2004).

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 ($N_1 \rightarrow N_2$), an unknown step ($N_2 \rightarrow N_3$) and finally the inactivation step ($N_3 \rightarrow N_4$). 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

$$dN_1/dt = -k_1 N_1 = -v_1 \quad (1)$$

$$dN_2/dt = k_1 N_1 - k_2 N_2 = v_1 - v_2 \quad (2)$$

$$dN_3/dt = k_2 N_2 - k_3 N_3 = v_2 - v_3 \quad (3)$$

$$dN_4/dt = k_3 N_3 = v_3 \quad (4)$$

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 (Mathys, Chapman et al., 2007). Variations between the model and experimental data occurred because of partial overlap of some sub-populations.

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 (Mönch, Heinz, Guttmann & Knorr, 1999). 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 (1611) both arrangements have an average density of $\pi(\sqrt{18})^{-1}$. 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 $\pi(\sqrt{12})^{-1}$ is the unique densest lattice sphere packing in two dimensions (Lagrange, 1773). 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 (Mathys, Heinz et al., 2007), 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).

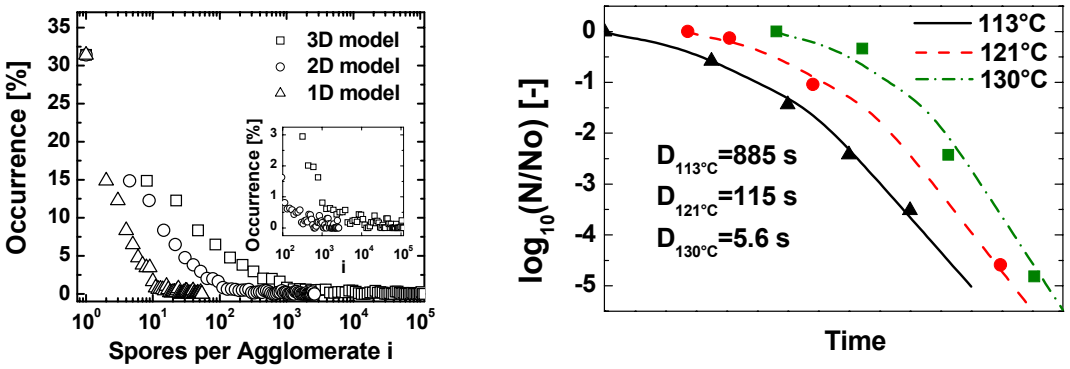


Fig. 3a): Agglomeration size distribution of a *G.stearothermophilus* spore suspension with different geometrical assumptions for the agglomerates and an inset, which shows agglomerations sizes between 10^2-10^5 spores per agglomerate (Mathys, Heinz et al., 2007).
3b): Experimental plots for ▲113°C, ●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 (Mathys, Heinz et al., 2007).

3. Conclusion

The mechanistic background of the inactivation of bacterial spores is still matter of discussion (Heinz et al., 2002). 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.
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.

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