Kinetics of the high pressure inactivation of bacteriophage P008 in milk

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Summary

High pressure treatment has the potential to inactivate microorganisms and maintain valuable enzymes and vitamins. In the field of dairy technology the inactivation of not only pathogenic and spoiling microorganisms but also of bacteriophages is important in terms of process safety. Therefore, kinetics of the high pressure inactivation of the lactococcal bacteriophage P008 in milk were investigated. Pressure was varied from 300 to 600 MPa at temperatures between 15 and 70 °C. Remaining active bacteriophages were detected by the plaque assay. Inactivation depended on both pressure and temperature but at pressures below 450 MPa and temperatures above 50 °C pressure and heat acted antagonistically, i.e. high pressure seemed to protect phages from heat inactivation. Similar findings have been made for the pressure treatment of proteins. For an effective pressure inactivation of phages either pressures higher than 450 MPa or low temperatures should be applied.

Introduction

Bacterial fermentations require aseptic conditions to guarantee the highest process safety. This applies not only to dairy technology but also to modern food biotechnology where strains of *Lactococcus lactis* are employed as “microreactors” because these unlike *Escherichia coli* have received the GRAS (generally recognized as safe) status.

To ensure a high hygienic standard of the nutrient medium heat treatment, microfiltration and also high pressure treatment may be applied. Heat treatment is effective in terms of sterilization but valuable nutrients such as vitamins and biofunctional components may be destructed. Microfiltration with a cut-off of about 1 μm is efficient in maintaining the nutrients and removing microorganisms including spores but bacteriophages pass the membrane (bacteriophages: 0.05-0.5 μm), which put the fermentation at risk. High pressure is known to inactivate pathogens while vitamins and enzymes mostly persist (Knorr 1993). Several studies suggest that phages can be pressure-inactivated. The first investigations by Solomon (1966) dealt with the *E. coli* phage T4 and hydrostatic pressures up to 400 MPa. He

described the kinetics by 1st order and found that the reaction rate rised sharply if pressures were higher than 350 MPa. Brauch *et al.* (1990) reported on the pressure inactivation (300 MPa) of the three *E. coli* phages ϕx, λ and T4, which inactivation curves exhibited a tailing (reaction order > 1) in all cases. Groß and Ludwig (1992) showed on phage T4 that an inactivation at 400 MPa was faster at low temperatures (5-20 °C) than at higher temperatures of up to 60 °C. Herdegen (1998) worked on the pressure-induced inactivation of *Listeria*-phages. At room temperature 300 MPa/15 min were necessary to reduce the phage titre by 7 log. Chen as well as Müller-Merbach observed the tailing effect of inactivation of both, *E. coli* phage (Chen *et al.* 2004) and lactococcal phage (Müller-Merbach *et al.* 2005b) and approximated the kinetics by nonlinear regression. Moroni *et al.* (2002) researched on lactococcal phages under dynamic high pressure which involves a different inactivation mechanism due to sudden pressure drop.

The kinetics of high pressure inactivation of the wide-spread lactococcal bacteriophage P008 (Neve 1996) suspended in milk were to be investigated in this study. The formal kinetics were evaluated by nonlinear regression.

Experimental Method

Phages and host

The phage P008 and its mesophilic host *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* F7/2 were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and were propagated in the lab. Both bacteria and phages grew well at 30 °C in M17-broth (BD Diagnostic Systems, Heidelberg, Germany) to which 10 ml of 1 mol l^{-1} $CaCl_2$-solution per l broth had been added. To propagate the phage, its host culture was grown in the broth at 30 °C for 4 to 8 hours so that it had reached exponential growth and had made the broth turbid; a few drops of phage suspension were then added and the culture was further incubated until it cleared up. Bacteria were removed *via* a 10 min centrifugation at 3074 x g at room temperature. The phage lysates, having an initial titre of > 10^8 pfu ml^{-1}, were kept at 4 °C.

High pressure treatment

The phage lysate of P008 was treated with pressures from 300 to 600 MPa at temperatures between 15 and 70 °C. The high pressure treatments were carried out on a laboratory high pressure equipment of the University of Hohenheim, Stuttgart (Resato High Pressure

Technology, Roden, Netherlands, 6 high pressure units with an inner volume 32 ml, pressure transmitting medium: glycol) and on a comparable high pressure equipment at the Federal Research Centre for Nutrition and Food, Karlsruhe (Dunse, Frankfurt am Main, 5 high pressure units with an inner volume of 20 ml, pressure transmitting medium: mixture of glycol and water in equal volumes). For both plants, the pressure can be controlled separately for each pressure unit and can be applied up to 600 MPa.

Phage lysate (1 ml) and 4 ml of milk (3.5 % fat) as dilution medium were put in a plastic-tube (Greiner Labortechnik, Germany) with an inner volume of 5 ml and closed with a rubber plug (VWR, Germany) so that no headspace was left; tubes were then placed in the thermostated pressure chambers. After 3 to 5 minutes the sample reached the outer temperature (15, 25, 50, 60 and 70°C). Then, hydrostatic pressure was applied (300, 450, 550 and 600 MPa) and the build-up rate was fixed at 200 MPa min^{-1}. Due to the slow pressure build-up phase, the temperature of the samples did not rise markedly (< 10 °C). When the target pressure was reached, the first sample was depressurised at 200 MPa min^{-1} and taken out of the pressure chamber and cooled down in icy water in order to stop the reaction. Its phage titre, N_0, represented the reference for the high pressure inactivation (t = 0). The treatment of the samples representing N_0 is comprised of the heating-up (3-5 min), the pressure build-up (1.5-3 min) and the pressure release (1.5-3 min). Pressure holding times were 0, 5, 10, 30, 60 and 120 min.

Phage count

The phage titre, or the concentration of active phages, was determined by the double layer method or “plaque assay” (Adams 1959) using Calcium-enriched M17 growth medium (Terzaghi and Sandine 1975).

Nonlinear regression

Since kinetics of phage inactivation deviate from 1st order (Groß and Ludwig 1992, Chen *et al*. 2004, Müller-Merbach *et al.* 2005a) they were approximated by nonlinear regression. The regression as well as the generation of the pressure-temperature- (p,T-)diagram were performed with the software Sigma Plot 8.0 (SPSS Inc., Chicago, USA). For high pressure induced inactivation at constant temperature the regression eq. 1 (Hinrichs and Rademacher 2005, Müller-Merbach *et al.* 2005b) was applied which is based on the Eyring-law. The resultant parameters are the reaction order, n, the rate constant, $k_{p_{ref}}$ at the reference pressure and the activation volume, $\Delta V^{\#}$, which indicates the pressure dependency of the reaction. At a constant temperature and within a relatively small pressure range from 300 to

600 MPa the activation volume can be regarded as being constant. According to the principle of Brown and Le Chatelier, the activation volume has to be negative for reactions which are favoured by pressure because they run under reduction in volume. Reactions with positive activation volume are not favoured by pressure.

$$log\ N_t = log\left[N_0 \left(1 + (n-1) \cdot k_{p_{ref}} \cdot exp\left(-\frac{\Delta V^{\#}}{RT} \cdot (p - p_{ref}) \right) \cdot N_0^{n-1} \cdot t \right)^{\frac{1}{1-n}} \right]$$

for $n \neq 1$, T = const (1)

N_0: reference phage titre in pfu ml^{-1}, i. e. after temperature and pressure build-up and release; $k_{p_{ref}}$: rate constant at reference pressure in s^{-1}; $\Delta V^{\#}$: activation volume in ml mol^{-1}; T: absolute temperature in K; reference pressure p_{ref} = 450 MPa

Results and Discussion

High pressure inactivation of phage P008 at different temperatures

Phage P008 was pressurised up to 600 MPa. Figure 1 shows the reduction in phage titre (logarithmic scale) during the first 30 min at temperatures of 15, 25, 50 and 60 °C. The initial phage titre was between 10^8 and 10^{10} pfu ml^{-1}. The highest phage titre reduction was achieved at 600 MPa/60 °C but did not exceed 3 log inactivation. A pressure of 450 MPa did not markedly affect the phage titre at any of the temperatures shown. At 70 °C (not shown) the inactivation progressed faster so that phages could not be detected after 30 min holding time.

Bacteriophage P008 showed high resistance to pressure. For an effective inactivation pressures higher than 450 MPa have to be applied. Therewith, the phage is more pressure-stable than yeast cells (Hoover *et al.* 1989), fungi, fungal spores and the vegetative forms of bacteria (Cheftel 1995, Arroyo *et al.* 1997), whereas bacterial spores of the genera *Bacillus* and *Clostridium* can survive higher pressures (Takahashi *et al.* 1993).

The “non-log-linear” course (not linear on logarithmic scale) of the graphs (tailing) indicates a non-1st-order reaction to be analysed by nonlinear regression.

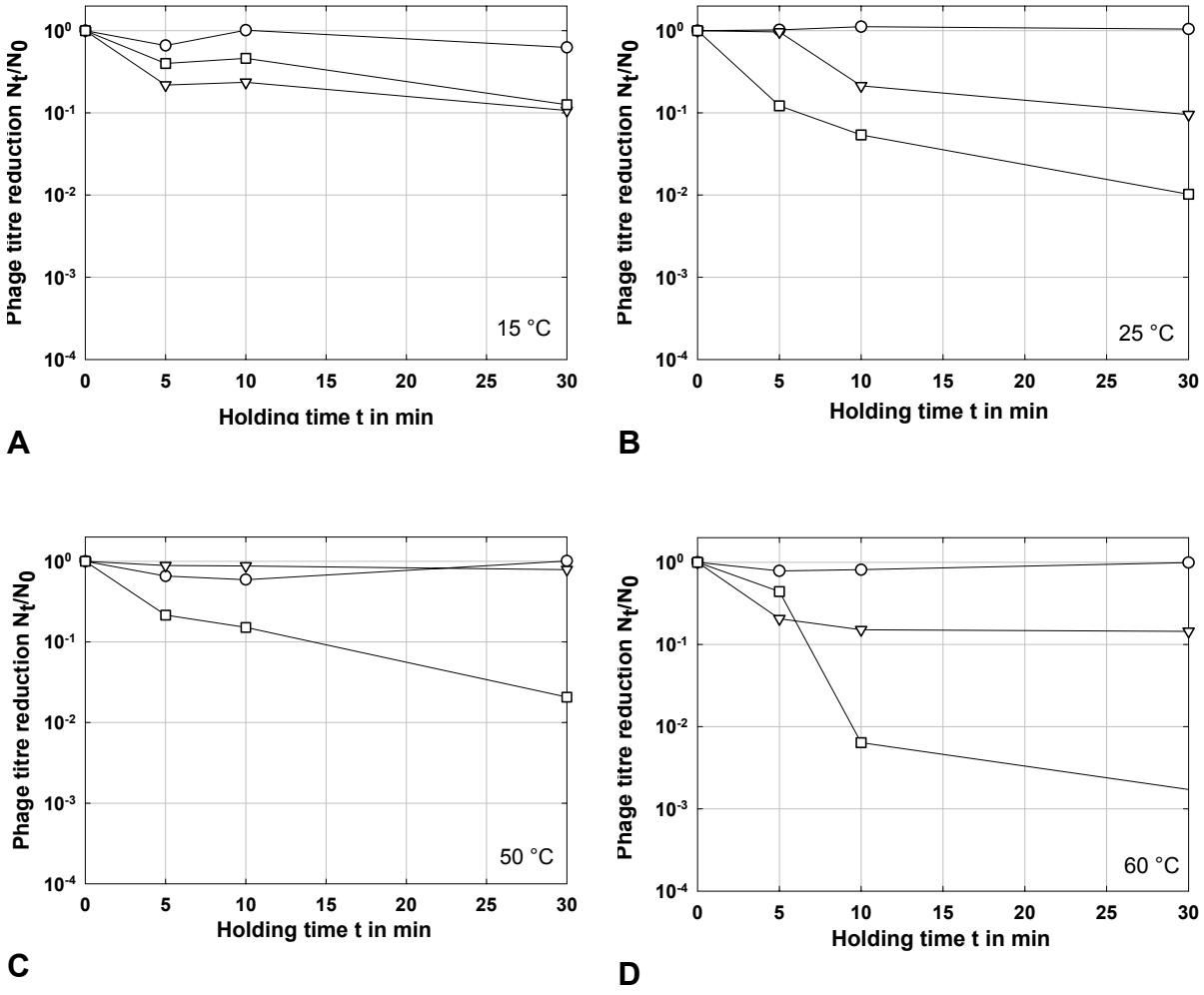

Figure 1: High pressure inactivation of phage P008 in milk at 15 °C (A), 25 °C (B), 50 °C (C) and 60 °C (D) as a function of holding time (0-30 min) for different pressures (○ 450 MPa, ▽ 550 MPa, □ 600 MPa)

Formal kinetics of phage inactivation by high pressure

The data were evaluated using nonlinear regression according to eq. 1 for each temperature. Table 1 gives the resultant formal kinetic parameters. The order of reaction ranged between 1.2 and 1.5. The activation volume was highest and the rate constant lowest for 50 °C. The correlation coefficient r^2 was 0.82 for the data at 15 °C and above 0.97 for all other temperatures.

Table 1: Formal kinetic data of the high pressure inactivation of phage P008 in milk at constant temperature according to eq. 1

Temperature ϑ in °C	Activation energy $\Delta V^{\#}$ in ml mol^{-1}	Rate constant $k_{p_{ref}}$ in s^{-1}	Order of reaction n	Correlation coefficient r^2	Samples
15	-48 ± 12	(1.1 ± 5.9) · 10^{-8}	1.41 ± 0.20	0.821	18
25	-74 ± 7	(1.8 ± 3.0) · 10^{-8}	1.48 ± 0.08	0.971	24
50	-177 ± 7	(2.0 ± 2.9) · 10^{-9}	1.26 ± 0.07	0.987	24
60	-120 ± 14	(7.3 ± 6.6) · 10^{-7}	1.23 ± 0.04	0.985	24
(70	-127 ± 68	(1.3 ± 2.6) · 10^{-3}	1.25 ± 0.31	0.997	7)*

* too little data available for evaluation due to the fast inactivation

To verify the regression according to eq. 1, the calculated data were plotted against the experimental data of the phage titre N_t. The closer the points are to the bisecting line, the better the calculated data correlate with the experimental data.

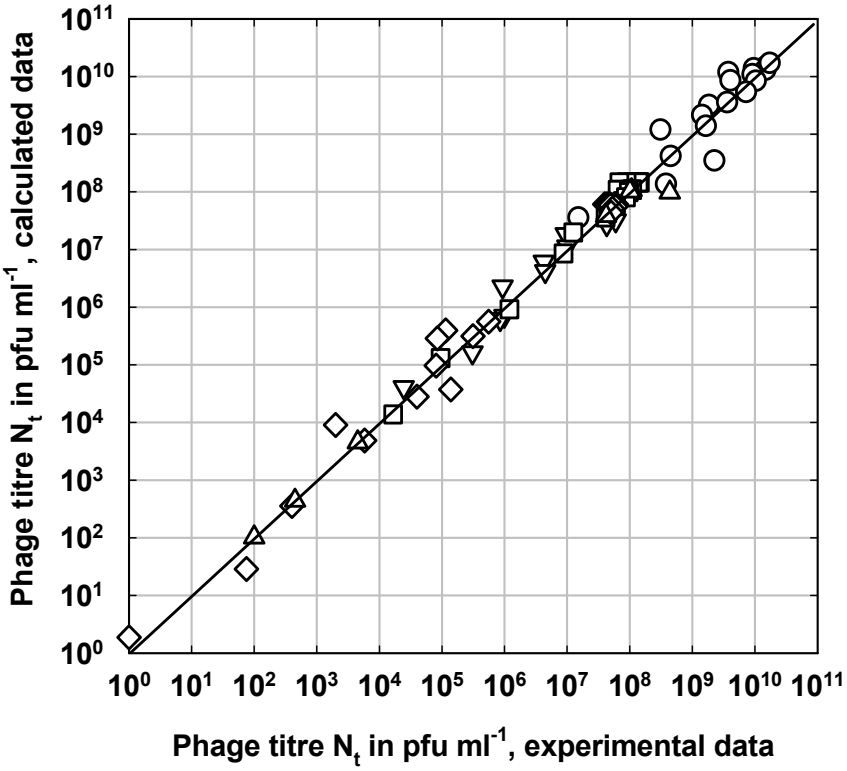

Figure 3: Correlation of the experimental N_t-values and calculated N_t-values of the high pressure inactivation of phage P008 in milk at 15 °C (○), 25 °C (▽), 50 °C (□), 60 °C (◇) and 70 °C (△)

Coaction of heat and pressure

A p,T-diagram (figure 3) for the combined pressure-induced and thermal inactivation of phage P008 was generated. It is based on the Eyring-law (eq. 2) and the formal kinetic data (table 1) as well as the Arrhenius-law (eq. 3) and the data (not shown) for the thermal inactivation at ambient pressure. It indicates the dependency of the rate constant $k_{p,T}$ from temperature and pressure. Each line represents an equal reaction rate induced by the coaction of heat and pressure.

$$k_{p,T} = k_{p_{ref}} \cdot exp\left(-\frac{\Delta V^{\#}}{RT} \cdot \left(p - p_{ref}\right) \right)_T \tag{2}$$

$$k_{p,T} = k_{T_{ref}} \cdot exp\left(-\frac{E_A}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right)_p \tag{3}$$

The reaction rate increases following the arrows, thus, with increasing pressure, with increasing temperature or both. In the left upper section the temperature dependence is very low as the lines are nearly parallel to the x-axis. In the right upper section pressure and temperature act synergistically, i.e. in the same direction. To increase $k_{p,T}$ either the pressure can be raised or the temperature. In the right bottom section pressure and temperature act antagonistically, i.e. if the pressure is raised the reaction at constant temperature becomes slower. In this part the activation volume becomes positive. Note that at 50 °C the rate constant equals 10^{-8} s^{-1} for ambient pressure as well as for approximately 450 MPa, and the pressures in between are less effective.

Also for phage inactivation (Groß and Ludwig 1992) and for the inactivation of virus (turnip yellow mosaic virus, Goldbeck *et al*. 1991) it was shown that temperature stability was increased under hydrostatic pressure. According to Goldbeck this could be due to the formation of hydrogen bonds. The formation of hydrogen bonds is promoted by pressure (Masson 1992) and could support a stabilisation by protein–protein- or RNA–protein-interactions.

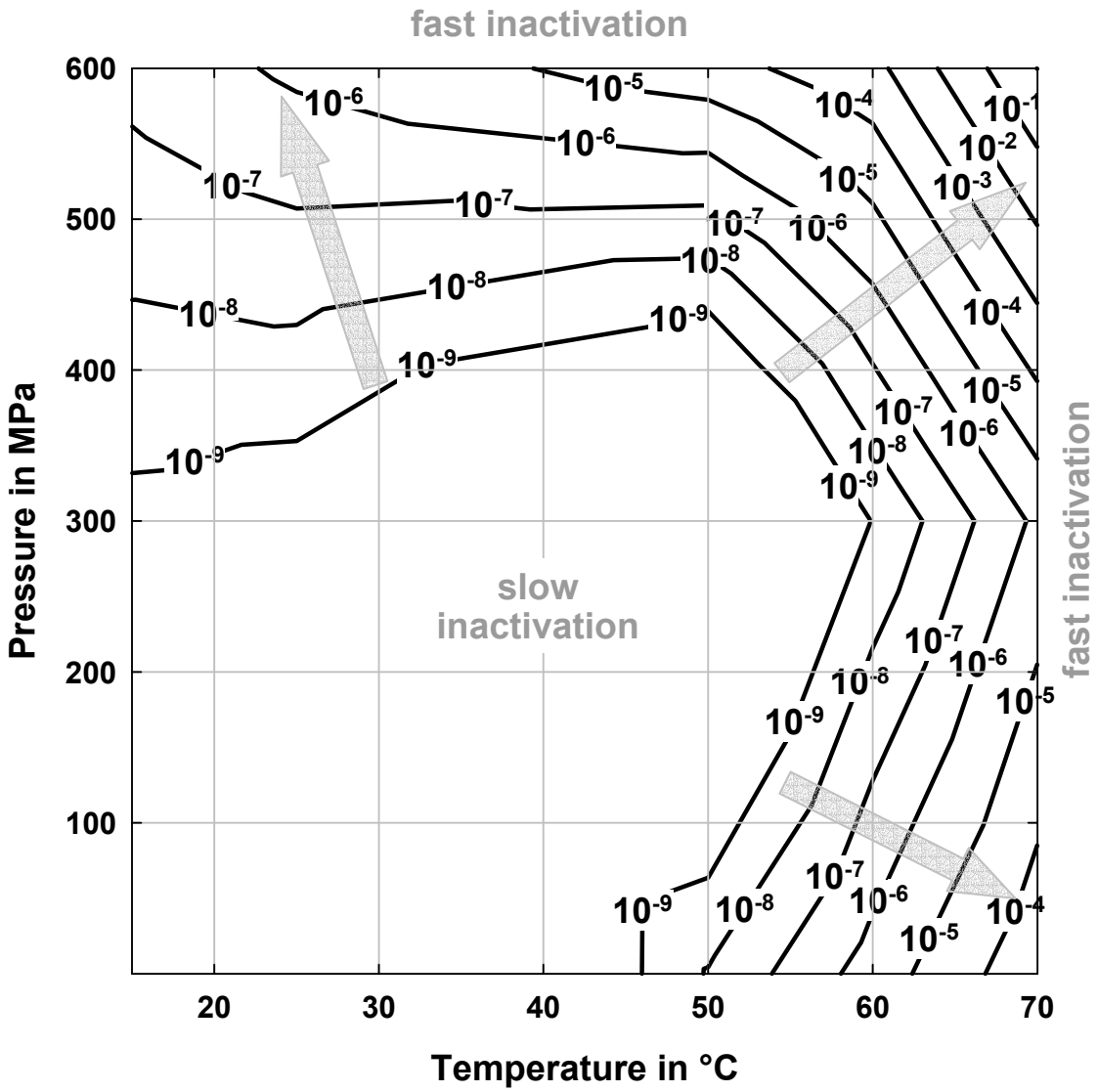

Figure 4: Pressure-temperature-diagram indicating lines of equal rate constants $k_{p,T}$ of high pressure inactivation of phage P008 in milk

The characteristic elliptic shape of the p,T-diagram with its synergistic and antagonistic parts are often described for protein denaturation and enzyme inactivation (Heremans 1993, Cheftel 1995, Ludikhuyze *et al.* 2000). What is detected as denaturation (or inactivation) can be the result of different molecular reaction mechanisms according to the prevailing conditions of pressure and temperature. For further explanations and interpretations refer to Suzuki (1960), Gross and Jaenicke (1994) and Balny *et al.* (2002).

Conclusion

The resultant kinetics of the high pressure inactivation of phage P008 at different temperatures were non-1st-order and exhibited a tailing. The kinetics were approximated by a nonlinear regression model which is based on the Eyring-law and the rate law of n^{th} order. The reaction order n, the rate constant $k_{p,T}$, and the activation volume $\Delta V^{\#}$ were calculated. From the kinetic data a p,T-diagram was established. The experimental data as well as the p,T-diagram showed that pressures below approximately 450 MPa and temperatures above 50 °C acted antagonistically., i.e. for the effective pressure-induced inactivation of bacteriophages pressure should be above 450 MPa or if lower it should be combined with low temperature. In further studies the high pressure inactivation of phages at temperatures below 0 °C should be investigated to verify the expectation that also in the very low temperature region phage inactivation follows protein denaturation in terms of temperature-pressure-coaction.

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