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QUANTIFYING THE COMBINED EFFECTS OF THE HEATING TIME, THE TEMPERATURE AND THE RECOVERY MEDIUM PH ON THE REGROWTH LAG TIME OF *BACILLUS CEREUS* SPORES AFTER A HEAT TREATMENT

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Abstract

The purpose of this study was to quantify the lag time of re-growth of heated spores of *Bacillus cereus* as a function of the conditions of the heat treatment: temperature, duration and pH of the recovery medium. For a given heating temperature, curves plotting lag times versus time of heating show more or less complex patterns. However, under a heating time corresponding to a decrease of 2 decimal logarithms of the surviving populations of spores, a linear relationship between the lag time of growth and the time of the previous heat treatment can be observed. The slope of this linear relationship followed itself a Bigelow type linear relationship, the slope of which yielded a ζ- value very close to the observed conventional z-value. It was then concluded that the slope of the regrowth lag time versus the heating time followed a linear relationship with the sterilisation value reached in the course of the previous heat treatment. A sharp effect of the pH of the medium which could be described by a simple "secondary" model was observed. As expected, the observed intercept of the linear

relationship between lag time and heating time (lag without previous heating) was dependent on only the pH of the medium and not on the heating temperature.

Keywords: Bacillus cereus, heat treatment, lag time, recovery.

1. Introduction

Bacillus cereus is a pathogen that produces two heat resistant toxins which are likely to cause more or less severe infections (Granum, 1994; Johnson,1984). The level of population which can generate infections is in the magnitude of 10^5-10^6 spores per gram (Granum and Lund, 1997). This bacteria belongs also to the spoilage flora of dairy products because of the action of its lecithinase on the globule membrane of milk fat (Jean Stone, 1952).

The heat resistance of *Bacillus cereus* spores is extremely variable, with a D-value (time of decimal reduction) ranging from 5 to 108 minutes at 85°C and from 0.3 to 27 minutes at 100°C respectively (Bergère and Cerf, 1992; Picoche *et al.*, 1993). Because of their relatively low heat resistance, most of strains are able to survive and grow after a pasteurisation .

It is well recognised that the lag time of growth of bacteria is dependent on the "history" of the bacterial population, especially its previous thermal conditions. As an example, Kaufmann *et al.*, (1959) observed a lag time of growth of 25 hours for the strain of *Micrococcus* MS102 after a heat treatment at 82°C instead of 19 hours after a heating at 76°C. A few studies have been published on modelling the influence of environmental recovery conditions on the viability of injured spores (Mafart and Leguérinel, 1997; Baker and Griffiths, 1993). However, the mathematical description of the growth after heat treatment as a function of the duration and the intensity of the thermal stress was very scarcely studied.

Mafart (1995) proposed a model describing the part of the lag corresponding to the activation and the germination of spores. Bréand *et al.* (1997, 1999) studied the impact of a previous

thermal stress on the growth of *Listeria monocytogenes* and *Eschericia coli*. They observed a three-phases behaviour of the lag time *versus* the duration of the heating exposure. The lag first increased until a maximum before decreasing at a steady level. Similar observations regarding *Bacillus cereus* were reported by Laurent *et al.* (1999), with a decreasing phase which was less clear than that observed by Bréand *et al.*

The aim of our work was to get a quantitative approach of the effect the heating time, heating temperature and pH of the recovery medium on the lag time of growth of *Bacillus cereus.*

2. Material and methods

2.1. Microorganism and spore production

The strain of *B. cereus* (Bce1), isolated from a dairy food line process, was supplied by Danone (France). Spores were kept in distilled water at 4°C. Cells were precultivated at 37°C for 24 h in Brain Heart Infusion (Difco). The preculture was used to inoculate nutrient agar plates (Biokar Diagnostics BK021) added with $MnSO_4$ 40 $mgl^{-}1$ and $CaCl_2$ 100 mg^{-1} on the surface area. Plates were incubated at 37°C for 1 month. Spores were then collected by scraping the surface of the agar and suspended in sterile Phosphate buffer M/15 at pH 7 and washed three times by centrifugation (10 000 x g for 15 min) (Bioblok Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml distilled water and 5 ml ethanol. The suspension obtained was kept at 4°C for 12 h in order to eliminate vegetative nonsporulated bacteria and washed again three time by centrifugation. Lastly, the final suspension (about 10^{10} spores ml^{-1}) was distributed in sterile Eppendorf micro-tubes and kept at 4°C.

2.2. Thermal treatment of spore suspension

First, 30 µl of spore suspension was diluted in 3 ml heating medium (Biokar nutritive broth, 20g / L) . Capillary tubes of 100 µl (vitrex) were filled with 100 µl of sample and submitted to a thermal treatment in a thermostated bath. After heating, the tubes were cooled in water/ice bath, washed in solution of soap and rinsed with sterile distilled water. Finally, ends were flamed with ethanol. The capillary tubes were broken at both ends and their contents poured into a tube containing 9 ml of sterile tryptone salt broth (Biokar Diagnostics) for an appropriated series of decimal dilutions, by rinsing with 1 ml broth contained in a needle-equipped syringe.

When the effect of the duration (up to 50 minutes) and the temperature (85, 90 and 95°C) of the heat treatment on the lag was investigated, the pH of the heating medium was fixed at 7. The investigated ranges of times and temperature correspond to those which are classically used in food process.

2.3. Growth conditions and measurements

Liquid tryptone salt broth was adjusted to the aimed pH by citric acid and distributed by fractions of 10 ml in special flasks topped by a calibrated tube allowing measurements of optical density with a spectrophotometer (600 nm). Each flask was inoculated with 1 ml from the last decimal dilution tube and incubated at 25°C under shaking (150 rotations per minute). A calibration of the spectrophotometer (optical density *versus* cellular concentration) was previously carried out: spores were firstly submitted to a thermal activation (90°C for 1 min at pH 7). The growth was then followed both by the optical density of the culture and the Most Probable Number technique. Because spores and vegetative nonsporulated cells don’t yield the same optical density, the calibration curve was fitted only after the growth time

corresponding to a sufficient cellular concentration (3.10^6 cells per ml) that allowed the concentration of spores to be neglected. The following equation was obtained:

$$\text{Ln OD} = 0.793 \text{ Ln N} - 15.583 \qquad (R^2 = 0.991)$$

. The effect of the pH of the medium, ranging from 5.5 to 7, was investigated by fixing the temperature of the heat treatment at 90°C.

2.4. Lag time estimation

The lag time was defined as the intersection point of the straight line describing the exponential growth expressed as logarithm of the bacterial concentration versus time, with the horizontal line at the logarithm of the initial bacterial concentration. It was checked that the size of the inoculum had no effect on the lag time (data not shown).

2.5. Parameters estimation

The estimations of the parameters of the models are obtained by the least squares method *via* the Matlab© function *`nlcurvefit'*. The criterion of Beale (Beale, 1960) provides us a tool allowing construction, by numerical simulation (technic near to that of Lobry et al. (Lobry, 1991)), of a hypervolume of confidence to the risk 5% which is conventionally retained . Projection on each axis of the space of the parameters gives us an interval of confidence of this parameter.

3. Results

3.1. General pattern of curves

The figure 1 shows the effect of the time of heating at 90°C and different pH, on the lag time of growth after the thermal treatment. As expected, it can be seen that roughly, the lag increases with the time of heating exposure, but patterns of curves are more or less complex. Under pH 6, a three- phase behaviour of the lag versus treatment time which recalls that observed by Bréand *et al.*,1997 can be observed: an increasing phase followed by a short steady or decreasing phase and, lastly, an increasing phase again, with a higher slope than that of the first one. The minimum lag times were observed at pH 6.5.

When duplicates or triplicates were carried out, the repetability of data was relatively fair for short times of heat treatments, but, in proportion as the exposure time increased, curves became more and more divergent (see fig. 2). On the other hand, the observed three-phase patterns , when they existed, were less clear than those observed by Bréand *et al.*, and were always suggested by a single point of curves. For these reasons, it was decided to fit only the first linear phases of curves which was limited to the interval ranging from the initial time to the time corresponding to the decimal destruction ratio n = 2 ($n = \log N_0/N$ herein N_0 and N represents the initial number of spore and the number of surviving bacteria after the heating respectively). It was then assumed that in this restricted range, the lag of growth versus the previous heating time followed a linear relationship:

$$\lambda = \lambda_0 + mt \qquad (1)$$

3.2. Secondary modelling of the initial linear phase

Data corresponding to the previously limited range (0 < n <2) were fitted according to a simple linear regression. The logarithm of the slope (log m) was then plotted versus temperature at pH 7 (fig 3) and versus pH at a heating temperature of 90°C (fig 4).A Bigelow type linear relationship between log m and the heating temperature could be observed. Regarding the effect of pH on the slope m, the pattern of the curve recalled that which was previously observed by plotting logarithm of D-values of spores versus pH (Mafart and Leguérinel, 1998). Assuming *a priori* that interactions between the heating temperature and the pH of the medium could be neglected, we tried to fit the slope of Eq 1 according to the following four parameters equation:

$$\log m = \log m^* + \frac{T - T^*}{\zeta_T} + \left(\frac{pH - pH_{opt}}{\zeta_{pH}} \right)^2 \qquad (2)$$

herein T* represents the reference temperature, while pH_{opt} is a parameter to be estimated which corresponds to the pH at which the m-value is minimum. Parameters ζ_T and ζ_{pH} play the same rules than the conventional z-value and the z_{pH} value which is included in the previous Mafart model respectively. Lastly, m* represents the slope of Eq 1 at the reference temperature and the optimal pH. Parameters estimates are indicated in table 1.

Despite the difficulty of getting accuracy data related to lag times, the adjusted R^2 (0.889) indicates a fair goodness of fit of the model.

Figures 5 and 6 represent the plotting of the logarithm of the intercept λ_0 of the linear Eq 1 versus the heating temperature and pH respectively. As λ_0 corresponds to the lag time of growth without any heat treatment, the absence of effect of the heating temperature on this

parameter was expected. A variance analysis (not shown) detected a slight but significant effect of pH on the value of λ_0. However, the lack of accuracy of results (see fig 6) did not allow any fitting of the data to a relevant model. Rough data allow only to get approximate magnitudes of λ_0 ranging from around 2.5 h at pH 6.5 to 6.3 h at pH 5.5.

4. Discussion

The overall lag time of growth of spores includes both the time of germination of spores and the time of first division of outgrown cells. Moreover, the overall observed lag time decreases as the maximum germination ratio of the population of spores increases (Mafart, 1995). It is then clear that the measured lag is narrowly linked to the conditions of activation and germination of spores, as well as to the lethal effect of heating: the resulting counteracting factors could then explain the relative complexity of the patterns of curves (figure 1) . Powell and Hunter (1955) observed, at temperatures ranging from 50°C to 80°C, that the ratio of germination of *Bacillus* spores decreased when temperatures increased. In the case of *Bacillus megaterium* , Levinson and Hyatt (1970) found an effect of temperature, with an optimal value, both on the rate of activation of spores and on the maximum ratio of germination. Above 68°C, they observed a decrease of the ratio of germination and an increase of the lag time of germination. This last observation is in agreement with our data which point out an increase of the lag with heating temperature.

The low value of ζ_{pH} (0.98) indicates a high sensitivity of the lag time toward the pH of the heating medium: a difference of about 1 pH unit with the optimum pH, inside the investigated range, causes a ten-fold increase of the lag.

The z-value of the strain was previously estimated: it can be seen that this value (z = 8.55°C with a confidence interval of 7.30- 10.29°C, unpublished datum) is close to the ζ_T estimate (ζ_T = 7.06°C with a confidence interval of 4.94- 12.38°C). This comparison allows to consider that, in the case of the studied strain, the assumption of an equality between z and ζ_T values could not be rejected. In these conditions, for a fixed pH of the heating, Eq 2 could be expressed as follows:

$$\log m = \log m^* + \frac{T - T^*}{z}$$

Or (3)

$$m = m^* 10^{\frac{T-T^*}{z}} \quad (4)$$

The combination of Eq 1 and 4 yields:

$$\lambda = \lambda_0 + m^* 10^{\frac{T-T^*}{z}} t = \lambda_0 + m^* F \quad (5)$$

herein F represents the well known sterilisation value (F-value) which is defined as the time (in minutes) of a heat treatment at the constant reference temperature (generally, T* = 121.1°C°), or as any equivalent heat treatment which would cause the same destruction ratio. Then, Eq 5 points out an interesting significance of m* which would correspond to the slope of a linear relationship between the lag time of growth and the sterilisation value submitted by spores before growing.

The high sensitivity of the overall lag time toward the pH can be explained both by the well known effect of this factor on the lag time of outgrown of cells and by its effect on the germination of spores. Blocher and Busta (1985) observed a progressive inhibition of the germination of *Clostridium botulinum* when pH decreased from 7 to 5.5. Regarding *Bacillus cereus*, Vas and Proszt (1957) noted a decrease of the rate and of the maximum ratio of germination when pH was decreasing, regardless of the type of organic acid implemented for controlling pH, except acetic acid which caused a total inhibition of germination at pH as high than 5.5.

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Legend Table

Parameters estimates of equation 2.

Legend Figure

Fig 1 : λ values for different pH (○ pH 7, ● pH 6.5, ►pH 6, ■ pH 5.78 , Δ pH 5.75, pH 5.5) Heating temperature 90°C

Fig 2 a&b : λ values versus heating time at pH 7 (a) and pH 5.5 (b)

Fig 3 : log m values versus heating temperature at pH 7

Fig 4 : log m values versus pH at 90°C

Fig 5 : log λ_0 values versus heating temperature at pH 7

296

297 Fig 6 : log λ_0 values variation versus pH at 90°C

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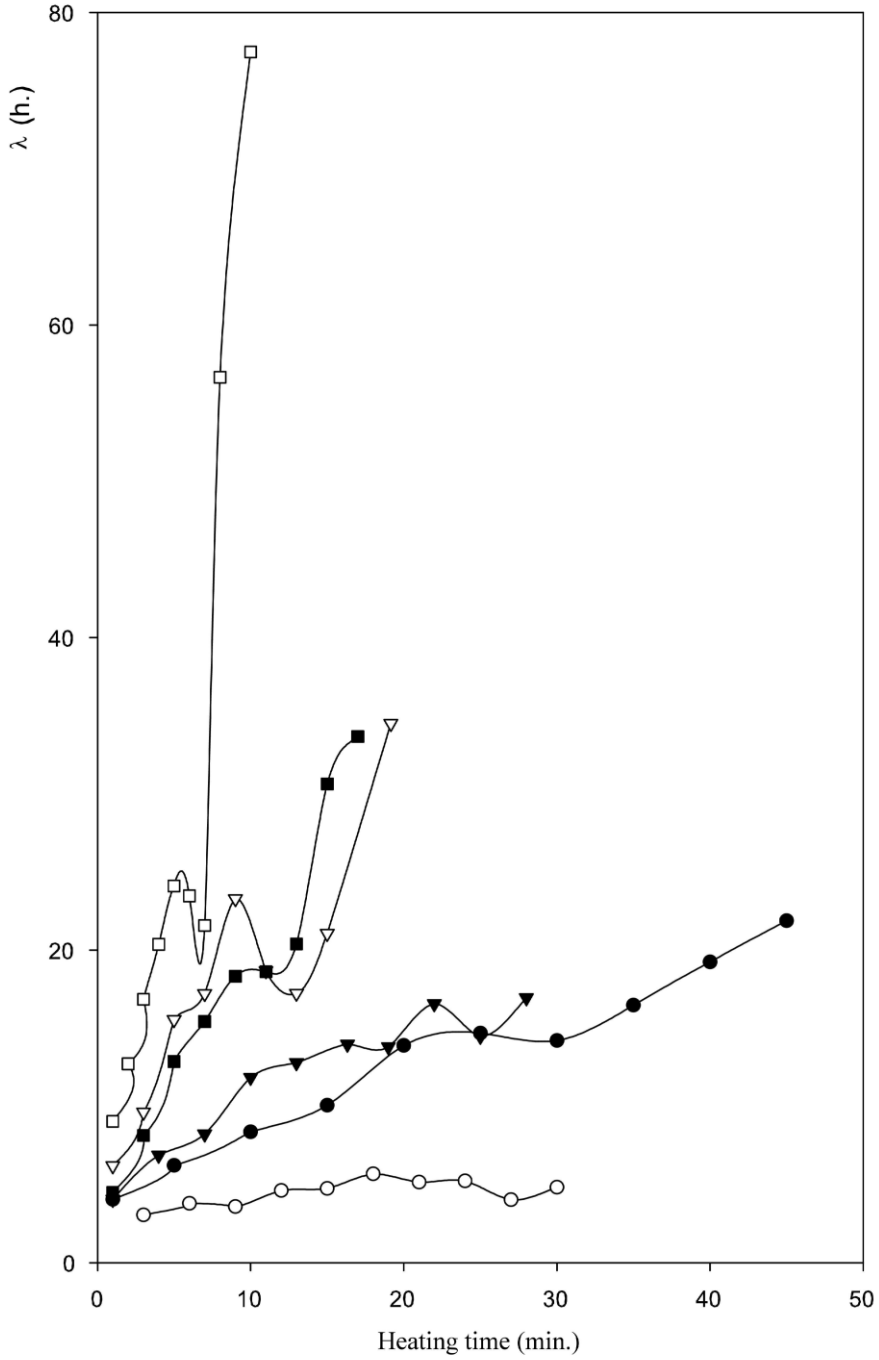
299

300

m^*	ζ_T	ζ_{pH}	pH_{opt}	R^2_{aj}
0.207 h^{-1}	7.060°C	0.977	6.59	0.889
(0.117 – 0.373)	(4.944 – 12.385)	(0.734 – 2.080)	(6.406 – 7.660)	

313



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316 **Fig 1**

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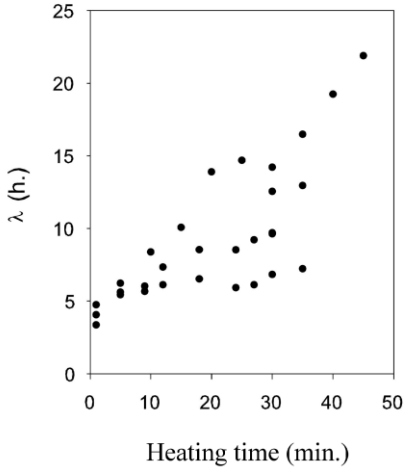
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Heating time (min.)
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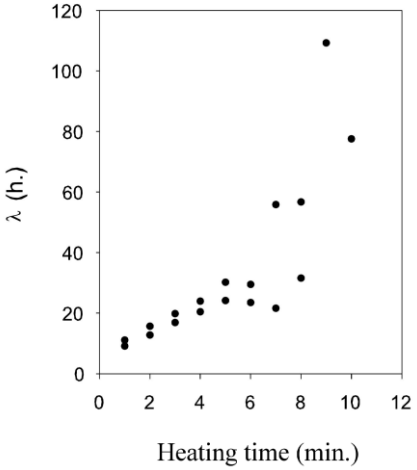
5
10
15
20
25
0
10
20
30
40
50


λ (h.)
Heating time (min.)
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20
40
60
80
100
120
0
2
4
6
8
10
12

Fig 2a

Fig 2b

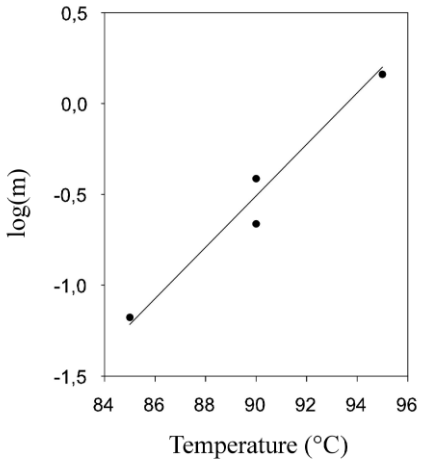

Fig 3

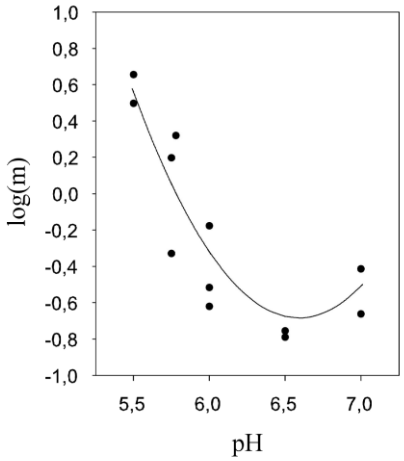

Fig 4

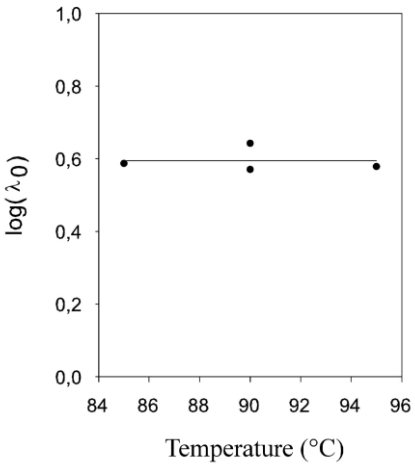

Fig 5

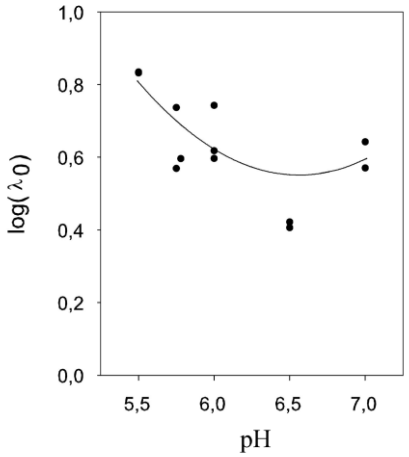

Fig 6