Modelling combined effects of temperature and pH on the heat resistance of spores of Bacillus cereus

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Abstract

The development of relationships between the pH of a heating medium and the thermal resistance of contaminant microorganisms is important and of a public health significance. A number of mathematical models have been presented in recent years, including that of Mafart and Leguérinel (1998) . However, in this This model , the effect of possible interactions between temperature and pH on D-values was not assessed. The consequences of ignoring interaction terms in models has been assessed and a comparison with Mafart's model that includes an interaction term showed that interaction terms can be neglected and that Mafart's model can be used in thermal process calculations. It appears possible to adopt a standard value of z_{pH} , for example the 3.6 value and the conventional concept of biological destruction value L(T) (ratio of the sterilization value and the exposure time at a fixed heating temperature) may then be extended to L(T,pH) (the same ratio at a fixed temperature with a fixed pH of the heating menstruum).

Keywords:
Temperature, pH, Heat resistance, Spores, *Bacillus cereus*.

Introduction

Interactions between heating temperature and pH are frequently observed. In most cases, increasing temperature reduces the effect of pH. This type of interaction can be seen in the data of Xezones and Hutchings (1965) for *Clostridium botulinum*. This same behaviour is observed for *Bacillus stearothermophilus* (Rodrigo et al., 1993; Fernandez et al., 1994; Lopez et al., 1996). Rodrigo et al.(1993) observed that pH did not have any effect on the D_T values of spores of *Clostridium sporogenes* when they were heated at high temperature. These results do not agree with those of Cameron et al.(1980) from which it can be shown that increasing temperature enhances the effect of pH. Acidifying the heating medium generally results in a decreased effect of temperature: an increase of the z value is observed when the pH of the medium decreases. This behaviour was showed for *Bacillus stearothermophilus* (Fernandez et al., 1994; Lopez et al., 1996), *Bacillus subtilis* (Condon and Sala, 1992), *Bacillus licheniformis* (Palop et al., 1992) and *Clostridium sporogenes* (Silla Santos et al.1992). However, Cameron et al.(1980) pointed out an opposite effect for *Clostridium sporogenes*(a decrease of the z value with decreasing pH), that disagrees with results of Silla Santos et al.(1992) .

The mathematical relationship between the pH of the heating medium and the thermal resistance of microorganisms has been poorly documented. Jordan and Jacobs (1948) found a linear relationship between pH and the logarithm of the D_T values for *E. coli*. Davey et al.(1978) developed the first model for predicting the combined effects of both process temperature and pH on thermal resistance of bacteria. This empirical model , which was developed from data of Xezones and Hutching (1965) for inactivation of *Clostridium botulinum*, shows a satisfactory goodness of fit and is relatively parsimonious (4 parameters for 2 factors). However it can be shown that Davey's model is still over parameterized (see appendix 1). The four empirical coefficients of the model have no biological significance. As an alternative, Mafart and Leguérinel (1998) proposed a new model with only three parameters, each having a physicochemical significance:

$$\log D_{(T,pH)} = \log D_{(T^*,pH^*)} - \frac{T - T^*}{z_T} - \frac{(pH - pH^*)^2}{z_{pH}^2} \qquad (1)$$

Where T* is the reference temperature (for example 121.1°C), pH* is the pH of maximal heat resistance of spores (generally pH 7), z_T is the conventionnal thermal z-value, z_{pH} is the distance of pH from pH* which leads to a ten fold reduction of D value. Lastly, $D_{(T^*, pH^*)}$ is the D value at T* and pH*.

This model was derived from the same data used by Davey et al., 1978 (i. e. Xezones and Hutching, 1965) and from two other sets of data related to *Clostridium sporogenes* (Cameron et al., 1980) and to *Bacillus stearothermophilus* (Lopez et al.,1996).

Neither Davey's nor Mafart's models took into account interactions between temperature and pH on thermal resistance of spores. This paper aims to check from our own data related to *Bacillus cereus* that the lack of term accounting for interactions in the Mafart's model can be justified.

The standard z-value used to compute most heating process is 10°C because it nearly corresponds to the value for *Clostridium botulinum* spores and is closed to z_T-value of most other types of bacterial spores. Another aim of this paper is to check that the z_{pH} value of *Bacillus cereus* is in the same range than that of others studied types of spores. If this paper, confirmed by further works, shows that most of z_{pH} values are close to that of *Clostridium botulinum*, it would be possible to adopt a standard z_{pH} for calculations of heat treatements.

Materials and methods

Microorganism and spore production

The strain of *Bacillus cereus* (CNRZ 110) was obtained from the Institut National de Recherche Agronomie (France). Spores were kept in distilled water at 4°C.

Cells were precultivated at 37°C during 24 h in Brain Heart Infusion (Difco). The preculture was used to inoculate nutritive agar plates (Biokar Diagnostics BK021) added with $MnSO_4$ 40mg l^{-1} and $CaCl_2$ 100 mgl^{-1} on the surface aera. Plates were incubated at 37°C for 5 days. Spores were then collected by scraping the surface of the agar and suspended in sterile distilled water and washed three times by centrifugation (10000xg for 15 min) (Bioblock Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml distilled water and 5 ml ethanol. The obtained suspension was then kept at 4°C during 12 hours in order to eliminate vegetative non sporulated bacteria, and washed again three times by centrifugation. The final suspension (about 10^{10} spores ml^{-1}) was at last distributed in sterile Eppendorfs microtubes and kept at 4°C.

Thermal treatment of spore suspension

$D_{(T,pH)}$ values in citrate-phosphate buffers adjusted respectively to 4.1, 4.5, 5.5, 6.5 and 6.9 were determined for temperatures of 86.6°C, 89°C, 95°C, 101°C and 103.4°C with one replicate at each temperature and pH combination. The whole set of data involves a complete 3x3 factorial design from 89°C to 101°C and from pH4.5 to pH 6.5 and in order to extend the range of validity of the model, the four following combinations were added: pH 5.5 at 86.6°C and 103.4°C, pH 4.1 and 6.9 at 95°C.

First, 30µl of spore suspension was diluted in 3 ml buffer. Capillary tubes of 25 µl (vitrex) were filled with 10µl of sample and submited to a thermal treatment in a thermostated oil bath. After heating, the tubes were cooled in water/ice bath, washed in a 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 sterile tryptone salt broth (Biokar Diagnostics) by rincing with 1 ml tryptone salt broth contained in a syringe equipped with a needle.

Viable spore count

The viable spores were counted by duplicate plating in nutritive agar (10g tryptone, 5g meat extract, 5g sodium chloride, 15 g agar for 1000ml water)(Biokar Diagnostic) and incubating at 30°C for 48h.

Data analysis

D_T values were based on the reciprocal of slopes obtained when the log number of survivors was plotted against time. Multiple linear regressions used to fit the model were carried out with the STAT-ITCF software (Institut Technique du Fourrage France).

The goodness of fit of the model was evaluated by using the per cent variance accounted for (Snedecor and Cochran, 1969) which is given by:

$$R^2 = 1 - \frac{(1 - r^2)(n - 1)}{(n - N - 1)}$$

where n is the number of observations, N the number of terms and r^2 is the multiple regression coefficient.

Results

Raw data of the experimental design are shown in Table 1. As the thermal resistance of *Bacillus cereus* is relatively low, we adopted the standard temperature T*=100°C instead of 121.1°C. On the other hand, a preliminary experiment showed that the pH of maximal thermal resistance related to the studied strain of *Bacillus cereus* was close to 7.5 (data not shown). The model was then fitted with pH*=7.5. Fitted parameters according to equation 1 were:

$$D_{(T^*, pH^*)} = 60 \text{ seconds}$$

$$z_T = 9.15°C$$

$$z_{pH} = 3.70$$

with a per cent variation accounted for $R^2 = 97.7\%$.

Families of graphs of $D_{(T, pH)}$ as a function of temperature at constant values of pH and as a funtion of pH at constant values of temperature are shown in Figures 1 and 2 respectively. Decreasing pH reduced the effect of temperature: at pH 6.5, the z_T value was 7.97°C whilst it becomes 9.27°C and 10.3°C at pH5.5 and 4.5 respectively. At 95°C and 101°C, the z_{pH} value remains constant and equal to 3.87. On the other hand, a decrease of the z_{pH} value is observed at 89°C (z_{pH}=3.06). It is then clear that the heat resistance of spores depends not only on temperature and pH of the medium, but also on interactions between these two factors. In order to take into account interactions, the model (1) was modified by adding an interaction term :

$$\log D_{(T, pH)} = \log D_{(T^*, pH^*)} + C_1(T - T^*) + C_2(pH - pH^*)^2 + C_3(T - T^*)(pH - pH^*)^2 \quad (2)$$

Where C_1, C_2 and C_3 are empirical parameters without known physiological significance. Fitted parameters according to this new model were:

$$D_{(T^*, pH^*)} = 83 \text{ seconds}$$

$$C_1 = -0.2900°C^{-1}$$

$$C_2 = -0.0281$$

$$C_3 = 0.1807°C\text{-}1$$

with a per cent variation accounted for R2 = 98.5%.

The new interaction term was highly significant (P<0.01%)

The comparaison between goodness of fit of both models (equations 1 and 2) are illustrated by Figures 3 and 4.

4. Discussion

Variation of the thermal resistance of *Bacillus cereus* spores as a function of temperature and pH is similar to those for many bacterial spores. It is confirmed that the pH of the heating medium has a prominent effect on the $D_{(T,pH)}$ values. For example, at 95°C a decrease of D-value of 5.7 times can be observed when the pH decrease from 6.9 to 4.1. As for of most bacterial spores, an increase of the temperature results in a decrease in the effect of the pH while a decrease of the pH medium results in a decrease of the effect of the temperature. This behaviour means that some interactions on $D_{(T,pH)}$-values exist between temperature and pH.This is confirmed by the fact that the interactions term of equation (2) is highly significant. However, adding one more parameters to our model in order to account for interactions results in a poor improvement of its goodness of fit : (with a R^2-value of 0.985 instead of 0.977). Moreover, in Equation 2, only one parameter ($D_{(T*,pH*)}$) keeps a biological significance: because of the occurence of the interaction term, it is not possible to reparameterize C_1 and C_2 into z_T and z_{pH} respectively. Fernandez et al.(1996) proposed two models for combined effects of temperature and pH on the thermal resistance of *Bacillus stearothermophilus* and *Clostridium sporogenes*: one a polynomial quadratic model including an interaction term (6 parameters), the other a simple linear model without interaction terms (3 parameters). From their data it may be concluded that the slight improvement of goodness of fit obtained by the quadratic model with respect to the linear model does not justify the choice of the 6 parameters model rather than that of 3 parameters model. The linear model of Fernandez et al. is of the form:

$$\log K = a + bT + cpH \qquad (3)$$

where K is the death rate of spores and a, b and c empirical coefficients. It can be easly shown (see Appendix 2) that equation(3) can be reparameterized to give:

$$\log D = \log D^* - \frac{1}{z_T}\left(T - T^*\right) - \frac{1}{z_{pH}}\left(pH - pH^*\right) \qquad (4)$$

Disregarding pH terms, both models (1) and (4) are reduced to Bigelow's equation. However equation (1) where the pH term is squared differs from the

simple linear model (equation (4)) and is the only one to take the sigmoïdal pattern of D values curves versus pH into account.

The z_{pH} value (3.7) related to *Bacillus cereus* have the same magnitude that the observed values for *Clostridium botulinum* (3.56-3.61), *Clostridium sporogenes* (3.33-4.29) and *Bacillus stearothermophilus* (2.94-3.97) (Mafart and Leguérinel, 1998). Like conventional z_T values of spores close to 10°C and keep generally the range of 8°C to 12°C, it appears that z_{pH} values are, in most situations, included in a 3 to 4 range. If this observation is confirmed, it may be possible to adopt a standard value of z_{pH} (for example the 3.6 value of *Clostridium botulinum*) in order to include the pH in thermal process calculations. The sterilization value could be defined as a heat treatment equivalent to an exposure timeof 1 min at 121.1°C and at pH 7. The conventional concept of biological destruction value L(T) could then be extended to L(T,pH) with

$$L(T,pH) = 10^{\frac{T-T^*}{z_T}+\left(\frac{pH-pH^*}{z_{pH}}\right)^2} \qquad (5)$$

This function corresponds to the exposure time, at a T temperature and at a given pH, which would be necessary in order to obtain one sterilization unit.

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Appendix 1: Reparameterization of Davey's model

Davey et al.(1978) described variation of death rates versus temperature and pH by the following equation:

$$LnK = C_0 + \frac{C_1}{T} + C_2\,pH + C_3\,pH^2 \quad (1)$$

where K is the death rate constant, T is the absolute temperature and C_0, C_1, C_2 and C_3 are empirical coefficients. Let pH* be the pH value of maximal thermal resistance of spores (in most cases pH*=7)

In the situation of maximal thermal resistance,

$$\frac{d(LnK)}{dpH} = C_2 + 2C_3\,pH = 0 \quad (2)$$

$$pH^* = \frac{-C_2}{2C_3} \quad (3)$$

so that Davey's model can be rewritten with only three parameters:

$$LnK = C_0 + \frac{C_1}{T} + C_2\,pH\left(1 - \frac{1}{2}\frac{pH}{pH^*}\right) \quad (4)$$

For example, in the case of the heat treatment of *Clostridium botulinum* spores in spaghetti tomato sauce, Davey's coefficient values were C_2=-2.3967; C_3=0.1965. In macaroni creole, C_2=- 2.6170 and C_3= 0.1871. Corresponding pH* values are then 7.07 and 6.99 respectively.

Appendix 2: Reparameterization of Fernandez's linear model

The following model was proposed:

$$\log K = a + bT + cpH \quad (1)$$

with $K = \frac{Ln10}{D}$

so equation (1) is equivalent to

$$\log D = \log(Ln10) - a - bT - cpH \quad (2)$$

or

$$\log D = \log D^{*} - \frac{1}{z_T}\left(T - T^{*}\right) - \frac{1}{z_{pH}}\left(pH - pH^{*}\right) \quad (3)$$

By comparison between equation (2) and (3) new parameters D*, z_T and z_{pH} can be identified versus Fernandez's parameters:

$$\log D^{*} = \log(Ln10) - a - bT^{*} - cpH^{*}$$

$$z_T = \frac{1}{b} \quad (5)$$

$$z_{pH} = \frac{-1}{c} \quad (6)$$

For example, for *Bacillus stearothermophilus* heated in mushroom extract acidified with glucono-δ-lactone, a=-12.0495; b=0.1156; c=0.2990. With T*=121.1°C and pH*=7, new parameters are

D*=3.20 minutes

z_T=8.65°C

z_{pH}=3.34

Table 1

pH/T	86.6°C	89°C	95°C	101°C	103.4°C
4.1			43.2 46.5		
4.5		158.2 156.4	51.9 45.4	10.9 10.6	
5.5	706 692	394.8 411.0	92.1 92.0	21.8 21.4	10.6 10.1
6.5		1185 1189	180.8 197.8	38.2 36.1	
6.9			257.3 256.6		

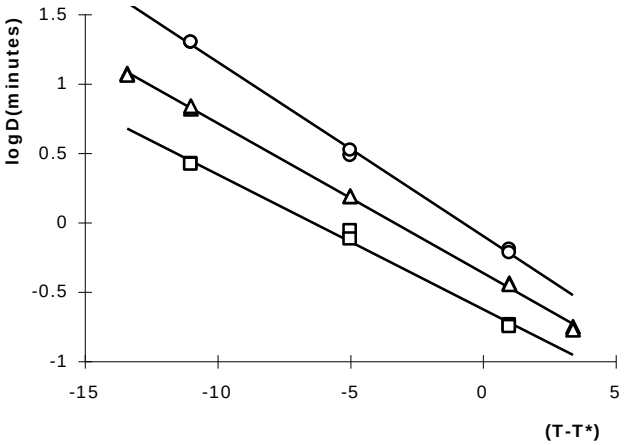


figure 1

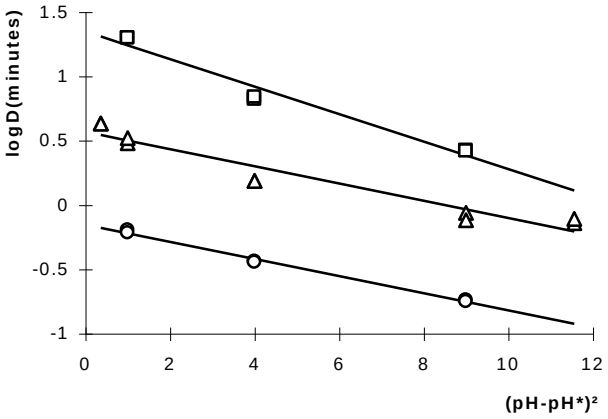


figure2

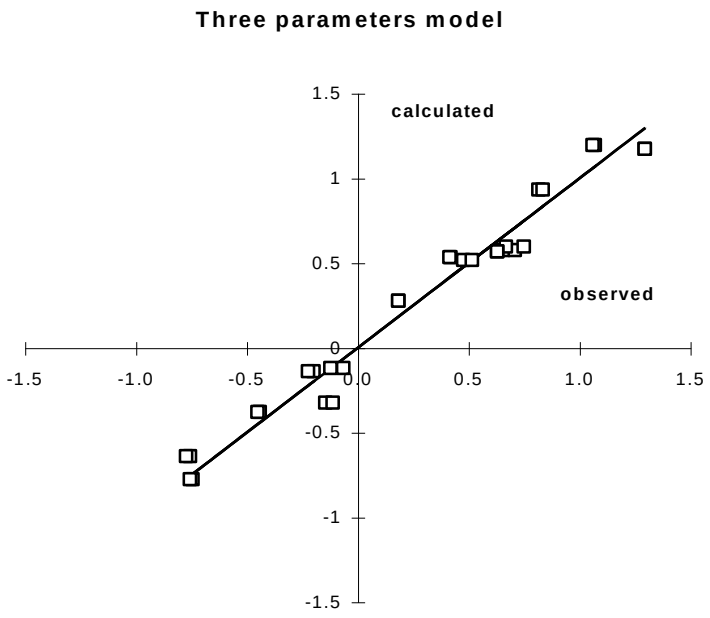

Three parameters model
calculated
observed
1.5
1
0.5
0
-0.5
-1
-1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5

Figure 3

Erreur! Objet incorporé incorrect.

Figure 4

Legends of figures

Figure 1 :
Log D value of *Bacillus cereus* spores versus (T-T*).
Key : □, log D at pH 6.5 ; Δ, log D at pH 5.5 ; ○, log D at 4.5.

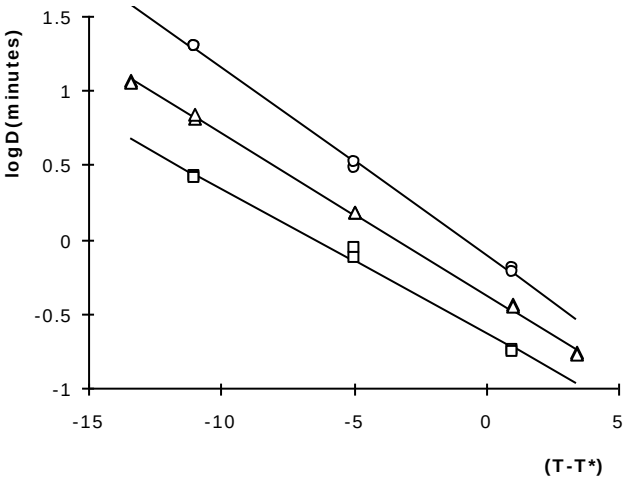
Figure 2 :
Log D value of *Bacillus cereus* spores versus (pH-pH*)
Key : □, log D at 89°C ; Δ, log D at 95°C ; ○, log D at 101°C.

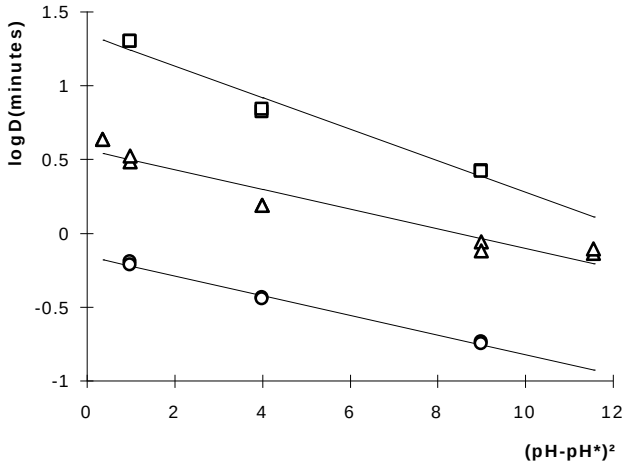
Figure 3 :
Observed log D value compared to log D value calculated according to the three parameters model

Figure 4 :
Observed log D value compared to log D value calculated according to the four parameters model

Legends of Table

Table 1
D values (minutes) of experimental design.


1.5
1
0.5
0
-0.5
-1
logD(minutes)
-15
-10
-5
0
5
(T-T*)


1.5
1
0.5
0
-0.5
-1
logD(minutes)
0
2
4
6
8
10
12
(pH-pH*)²