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1 Modelling combined effects of temperature and pH on the
2 heat resistance of spores of *Bacillus cereus*.

3
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7

8 Abstract

9

10 The development of relationships between the pH of a heating medium and the
11 thermal resistance of contaminant microorganisms is important and of a public
12 health significance. A number of mathematical models have been presented in
13 recent years, including that of Mafart and Leguérinel (1998) . However, in this
14 This model , the effect of possible interactions between temperature and pH on
15 D-values was not assessed. The consequences of ignoring interaction terms in
16 models has been assessed and a comparison with Mafart's model that includes
17 an interaction term showed that interaction terms can be neglected and that
18 Mafart's model can be used in thermal process calculations. It appears possible
19 to adopt a standard value of z_{pH} , for example the 3.6 value and the conventional
20 concept of biological destruction value $L(T)$ (ratio of the sterilization value and
21 the exposure time at a fixed heating temperature) may then be extended to
22 $L(T,pH)$ (the same ratio at a fixed temperature with a fixed pH of the heating
23 menstruum).

24

25 Keywords:

26 Temperature, pH, Heat resistance, Spores, *Bacillus cereus*.

27

28

29 Introduction

30

31 Interactions between heating temperature and pH are frequently observed. In
32 most cases, increasing temperature reduces the effect of pH. This type of
33 interaction can be seen in the data of Xezones and Hutchings (1965) for
34 *Clostridium botulinum*. This same behaviour is observed for *Bacillus*
35 *stearothermophilus* (Rodrigo et al., 1993; Fernandez et al., 1994; Lopez et al.,
36 1996). Rodrigo et al.(1993) observed that pH did not have any effect on the D_T
37 values of spores of *Clostridium sporogenes* when they were heated at high
38 temperature. These results do not agree with those of Cameron et al.(1980) from
39 which it can be shown that increasing temperature enhances the effect of pH.
40 Acidifying the heating medium generally results in a decreased effect of
41 temperature: an increase of the z value is observed when the pH of the medium
42 decreases. This behaviour was showed for *Bacillus stearothermophilus*
43 (Fernandez et al., 1994; Lopez et al., 1996), *Bacillus subtilis* (Condon and Sala,
44 1992), *Bacillus licheniformis* (Palop et al., 1992) and *Clostridium sporogenes*
45 (Silla Santos et al.1992). However, Cameron et al.(1980) pointed out an
46 opposite effect for *Clostridium sporogenes*(a decrease of the z value with
47 decreasing pH), that disagrees with results of Silla Santos et al.(1992) .

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

61

$$62 \log D_{(T,pH)} = \log D_{(T^*,pH^*)} - \frac{T - T^*}{z_T} - \frac{(pH - pH^*)^2}{z_{pH}^2} \quad (1)$$

63

64

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

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

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

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

84

85 **Materials and methods**

86 **Microorganism and spore production**

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

90 Cells were precultivated at 37°C during 24 h in Brain Heart Infusion
91 (Difco). The preculture was used to inoculate nutritive agar plates (Biokar
92 Diagnostics BK021) added with $MnSO_4$ 40mg l⁻¹ and $CaCl_2$ 100 mg l⁻¹ on the
93 surface aera. Plates were incubated at 37°C for 5 days. Spores were then
94 collected by scraping the surface of the agar and suspended in sterile distilled
95 water and washed three times by centrifugation (10000xg for 15 min) (Bioblock
96 Scientific, model Sigma 3K30). The pellet was then resuspended in 5 ml
97 distilled water and 5 ml ethanol. The obtained suspension was then kept at 4°C
98 during 12 hours in order to eliminate vegetative non sporulated bacteria, and
99 washed again three times by centrifugation. The final suspension (about 10¹⁰
100 spores ml⁻¹) was at last distributed in sterile Eppendorfs microtubes and kept at
101 4°C.

102

103 Thermal treatment of spore suspension

104 $D_{(T,pH)}$ values in citrate-phosphate buffers adjusted respectively to 4.1,
105 4.5, 5.5, 6.5 and 6.9 were determined for temperatures of 86.6°C, 89°C, 95°C,
106 101°C and 103.4°C with one replicate at each temperature and pH combination.
107 The whole set of data involves a complete 3x3 factorial design from 89°C to
108 101°C and from pH4.5 to pH 6.5 and in order to extend the range of validity of
109 the model, the four following combinations were added: pH 5.5 at 86.6°C and
110 103.4°C, pH 4.1 and 6.9 at 95°C.

111 First, 30µl of spore suspension was diluted in 3 ml buffer. Capillary tubes of 25
112 µl (vitrex) were filled with 10µl of sample and submitted to a thermal treatment
113 in a thermostated oil bath. After heating, the tubes were cooled in water/ice
114 bath, washed in a solution of soap and rinsed with sterile distilled water.
115 Finally, ends were flamed with ethanol. The capillary tubes were broken at both
116 ends and their contents poured into a tube containing 9 ml sterile tryptone salt
117 broth (Biokar Diagnostics) by rining with 1 ml tryptone salt broth contained in
118 a syringe equipped with a needle.

119

120 Viable spore count

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

124

125 Data analysis

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

130

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

133

134
$$R^2 = 1 - \frac{(1 - r^2)(n - 1)}{(n - N - 1)}$$

135

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

138

139

140

141 Results

142

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

149

$$150 \quad D_{(T^*, \text{pH}^*)} = 60 \text{ seconds}$$

$$151 \quad z_T = 9.15^\circ\text{C}$$

$$152 \quad z_{\text{pH}} = 3.70$$

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

154 Families of graphs of $D_{(T, \text{pH})}$ as a function of temperature at constant values of
 155 pH and as a function of pH at constant values of temperature are shown in
 156 Figures 1 and 2 respectively. Decreasing pH reduced the effect of temperature:
 157 at pH 6.5, the z_T value was 7.97°C whilst it becomes 9.27°C and 10.3°C at
 158 pH 5.5 and 4.5 respectively. At 95°C and 101°C , the z_{pH} value remains constant
 159 and equal to 3.87. On the other hand, a decrease of the z_{pH} value is observed at
 160 89°C ($z_{\text{pH}}=3.06$). It is then clear that the heat resistance of spores depends not
 161 only on temperature and pH of the medium, but also on interactions between
 162 these two factors. In order to take into account interactions, the model (1) was
 163 modified by adding an interaction term :

164

$$165 \quad \log D_{(T, \text{pH})} = \log D_{(T^*, \text{pH}^*)} + C_1(T - T^*) + C_2(\text{pH} - \text{pH}^*)^2 + C_3(T - T^*)(\text{pH} - \text{pH}^*)^2$$

166

$$167 \quad (2)$$

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

$$170 \quad D_{(T^*, \text{pH}^*)} = 83 \text{ seconds}$$

$$171 \quad C_1 = -0.2900^\circ\text{C}^{-1}$$

$$172 \quad C_2 = -0.0281$$

$$173 \quad C_3 = 0.1807^\circ\text{C}^{-1}$$

174 with a per cent variation accounted for $R^2 = 98.5\%$.

175

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

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

179

180 4. Discussion

181

182 Variation of the thermal resistance of *Bacillus cereus* spores as a function of
183 temperature and pH is similar to those for many bacterial spores. It is confirmed
184 that the pH of the heating medium has a prominent effect on the $D_{(T,pH)}$ values.

185 For example, at 95°C a decrease of D-value of 5.7 times can be observed when
186 the pH decrease from 6.9 to 4.1. As for of most bacterial spores, an increase of
187 the temperature results in a decrease in the effect of the pH while a decrease of
188 the pH medium results in a decrease of the effect of the temperature. This
189 behaviour means that some interactions on $D_{(T,pH)}$ -values exist between
190 temperature and pH. This is confirmed by the fact that the interactions term of
191 equation (2) is highly significant. However, adding one more parameters to our
192 model in order to account for interactions results in a poor improvement of its
193 goodness of fit : (with a R²-value of 0.985 instead of 0.977). Moreover, in
194 Equation 2, only one parameter ($D_{(T^*,pH^*)}$) keeps a biological significance:
195 because of the occurrence of the interaction term, it is not possible to
196 reparameterize C₁ and C₂ into z_T and z_{pH} respectively. Fernandez et al.(1996)
197 proposed two models for combined effects of temperature and pH on the
198 thermal resistance of *Bacillus stearothermophilus* and *Clostridium sporogenes*:
199 one a polynomial quadratic model including an interaction term (6 parameters),
200 the other a simple linear model without interaction terms (3 parameters). From
201 their data it may be concluded that the slight improvement of goodness of fit
202 obtained by the quadratic model with respect to the linear model does not justify
203 the choice of the 6 parameters model rather than that of 3 parameters model.
204 The linear model of Fernandez et al. is of the form:

$$205 \quad \log K = a + bT + cpH \quad (3)$$

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

$$209 \quad \log D = \log D^* - \frac{1}{z_T} \left(T - T^* \right) - \frac{1}{z_{pH}} \left(pH - pH^* \right) \quad (4)$$

210

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

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

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

$$227 \quad L(T, pH) = 10^{\frac{T - T^*}{z_T} + \left(\frac{pH - pH^*}{z_{pH}} \right)^2} \quad (5)$$

228
229 This function corresponds to the exposure time, at a T temperature and at a
230 given pH, which would be necessary in order to obtain one sterilization unit.

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286 Appendix 1: Reparameterization of Davey's model

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

$$289 \quad \text{Ln}K = C_0 + \frac{C_1}{T} + C_2 \text{pH} + C_3 \text{pH}^2 \quad (1)$$

290 where K is the death rate constant, T is the absolute temperature and C₀,
291 C₁, C₂ and C₃ are empirical coefficients. Let pH* be the pH value of maximal
292 thermal resistance of spores (in most cases pH*=7)

293 In the situation of maximal thermal resistance,

$$294 \quad \frac{d(\text{Ln}K)}{d\text{pH}} = C_2 + 2C_3 \text{pH} = 0 \quad (2)$$

295

$$296 \quad \text{pH}^* = \frac{-C_2}{2C_3} \quad (3)$$

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

$$298 \quad \text{Ln}K = C_0 + \frac{C_1}{T} + C_2 \text{pH} \left(1 - \frac{1}{2} \frac{\text{pH}}{\text{pH}^*} \right) \quad (4)$$

299 For example, in the case of the heat treatment of *Clostridium botulinum*
300 spores in spaghetti tomato sauce, Davey's coefficient values were C₂=-2.3967;
301 C₃=0.1965. In macaroni creole, C₂=- 2.6170 and C₃= 0.1871. Corresponding
302 pH* values are then 7.07 and 6.99 respectively.

303

304 Appendix 2: Reparameterization of Fernandez's linear model

305 The following model was proposed:

306 $\log K = a + bT + cpH$ (1)

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

308 so equation (1) is equivalent to

309 $\log D = \log(Ln10) - a - bT - cpH$ (2)

310 or

311 $\log D = \log D^* - \frac{1}{z_T}(T - T^*) - \frac{1}{z_{pH}}(pH - pH^*)$ (3)

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

314 $\log D^* = \log(Ln10) - a - bT^* - cpH^*$

315 $z_T = \frac{1}{b}$ (5)

316 $z_{pH} = \frac{-1}{c}$ (6)

317 For example, for *Bacillus stearothermophilus* heated in mushroom extract
318 acidified with glucono- δ -lactone, $a=-12.0495$; $b=0.1156$; $c=0.2990$. With
319 $T^*=121.1^\circ\text{C}$ and $pH^*=7$, new parameters are

320 $D^*=3.20$ minutes

321 $z_T=8.65^\circ\text{C}$

322 $z_{pH}=3.34$

323

324

325

326

Table 1

327

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		

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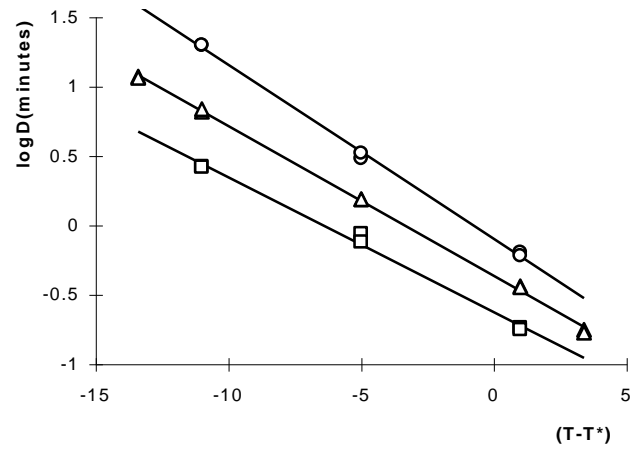
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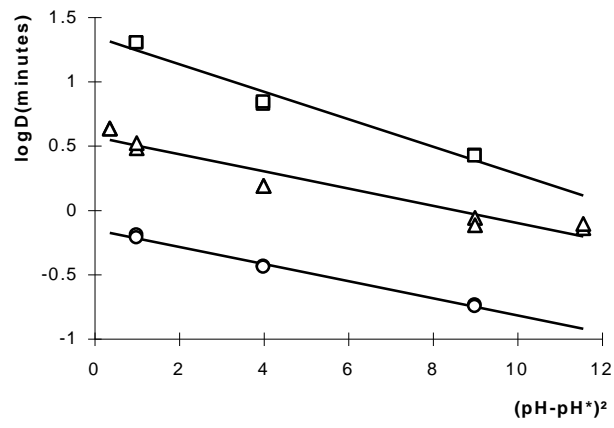
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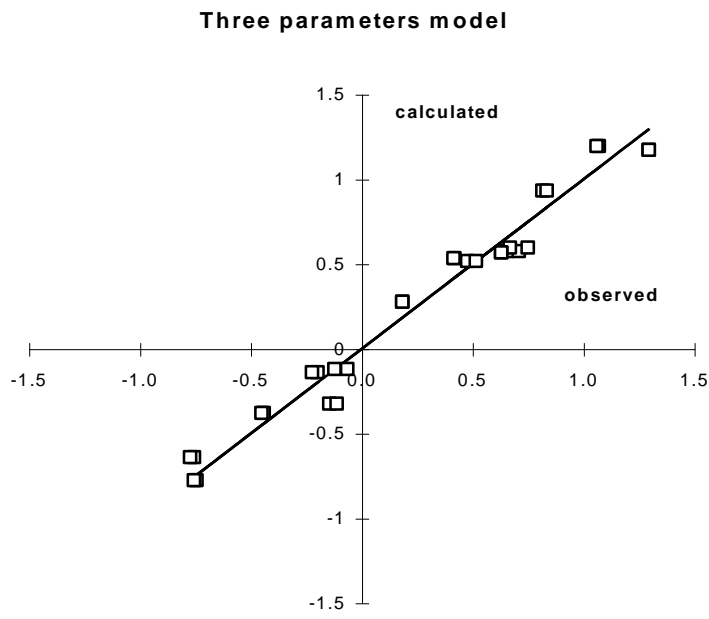
figure 1

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figure2



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Figure 3

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Erreur! Objet incorporé incorrect.

360

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362

363

364 Figure 4

365

366

367

368 Legends of figures

369

370 Figure 1 :

371 Log D value of *Bacillus cereus* spores versus (T-T*).

372 Key : □, log D at pH 6.5 ; Δ, log D at pH 5.5 ; ○, log D at 4.5.

373

374 Figure 2 :

375 Log D value of *Bacillus cereus* spores versus (pH-pH*)

376 Key : □, log D at 89°C ; Δ, log D at 95°C ; ○, log D at 101°C.

377

378 Figure 3 :

379 Observed log D value compared to log D value calculated according to the three
380 parameters model

381

382 Figure 4 :

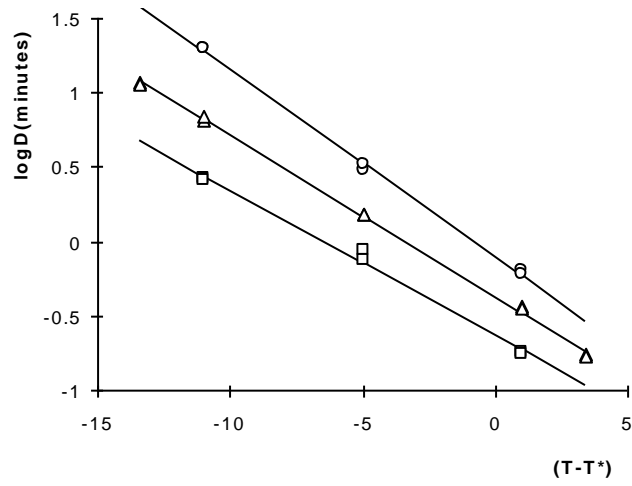
383 Observed log D value compared to log D value calculated according to the four
384 parameters model

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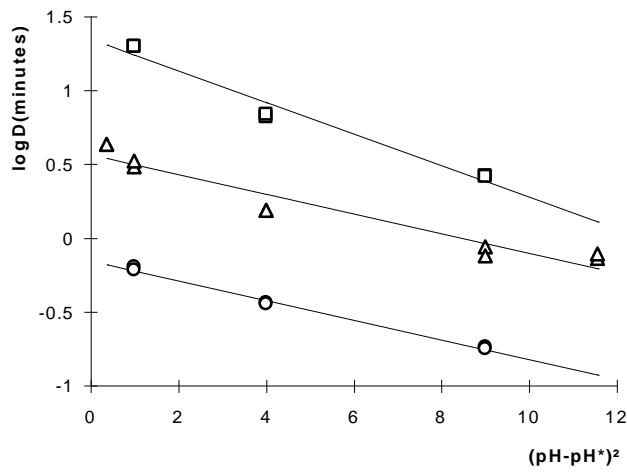
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387 Legends of Table
388
389 Table 1
390 D values (minutes) of experimental design.
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