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1 **Validation of an overall model describing the effect of**
2 **three environmental factors on the apparent D-value of**
3 *Bacillus cereus* spores

4
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10
11 **ABSTRACT**

12 Several factorial models extending the famous Bigelow model to describe the influence of the
13 heating and recovery pH and a_w conditions on bacterial heat resistance have been developed.
14 These models can be associated in an overall multifactorial model describing the influences of
15 heating and recovery conditions on D values. For *Bacillus cereus* strain ADQP 407 the model
16 parameters characterising the environmental factor influences (pH, Temperature, a_w) were
17 evaluated. Determination of bacterial heat resistance in cream chocolate have been realised to
18 validate these parameter values and to evaluate the level of the influence of food texture or
19 different compounds not taken account of in the model.

20
21 **KEYWORDS:**

22 Heat resistance, pH, a_w , *Bacillus cereus* , secondary model Bigelow model.

23 INTRODUCTION

24 Several environmental conditions influence bacterial heat resistance. In addition to the heating
25 temperature, it has been recognized that pH and water activities of the heating and recovery
26 medium are the main factors that affect the apparent heat resistance of bacteria (Esty and
27 Bigelow, 1920; Esty and Meyer, 1922; Murrel and Scott, 1966; Cook and Gilbert, 1968;
28 Harnulv et al., 1977; Lynch and Potter, 1988; Fernandez et al., 1996; Fernandez et al., 2002).

29 Different multifactorial models which describe the influence of the heating environmental
30 condition have been published (Davey et al., 1978; Reichart, 1994; Cerf et al., 1996;
31 Fernandez et al., 1996) Mafart and Leguérinel (1998) and Gaillard et al. (1999) have
32 developed an extension of the linear Bigelow model to the pH and aw influence Eq. 1.

33
$$\log D = \log D^* - \left(\frac{T - T^*}{z_T} \right) - \left| \frac{pH - pH^*}{z_{pH}} \right| - \left(\frac{a_w - 1}{z_{a_w}} \right) \text{Eq. 1}$$

34 where T^* is the reference temperature (generally, $T^*=121.1^\circ\text{C}$) and pH^* is the reference pH
35 fixed at =7, D^* is the D -value at T^* , pH^* and $a_w = 1$, z_T is the conventional thermal z -value,
36 z_{pH} is the difference of pH from pH^* , which leads to a ten fold reduction of D -value, z_{a_w} is the
37 difference of a_w from $a_w^* = 1$ which leads to a ten fold reduction of D -value. As the Bigelow
38 model, this imbricate model, taking temperature, pH and water activities into account, is used
39 to evaluate the decimal reduction ratio and the sterilization value (F - value) Mafart (2000).

40 However, these models assume that the heat resistance is measured at optimal recovery
41 condition and do not take the influence of the non optimal condition of the recovery media
42 into account. It is well known that the count of survival bacteria after heating treatment is
43 greatly influenced by the characteristic of the recovery medium: temperature, pH, aw and
44 composition. When the recovery condition differs from the optimal condition both a decrease
45 in the number of heated stressed cells capable of producing colonies and a decrease in the
46 estimate decimal reduction time, are observed (Harris, 1963; Katsui et al., 1982; Mallidis and
47 Scholefield, 1986; Feeherry et al., 1987). Recently, according to the same approach as that

48 adopted in the Mafart and Leguerinel model (1998), Couvert et al. (1999) and Coroller et al.
 49 (2001), have developed similar Bigelow models to describe the influence of pH and water
 50 activities respectively, of the recovery medium, on the apparent heat resistance of bacteria
 51 Eq.2-3.

$$52 \quad \log D' = \log D'_{opt} - \left(\frac{pH' - pH'_{opt}}{z'_{pH}} \right)^2 \text{ Eq. 2}$$

$$53 \quad \log D' = \log D'_{opt} - \left(\frac{a'_w - a'_{wopt}}{z'_{a_a}} \right)^2 \text{ Eq. 3}$$

54 Where pH' or aw' are the pH or the water activity of the recovery medium, D' is the apparent
 55 reduction time at pH' or aw' , pH'_{opt} and aw'_{opt} correspond to the maximal D' value and z'_{pH}
 56 and z'_{aw} are the distance from the pH'_{opt} or aw'_{opt} respectively, which leads to a ten fold
 57 reduction of the apparent reduction time D' .

58 Mafart and Leguerinel, Gaillard et al., Couvert et al. and Coroller et al. models can be
 59 associated in an overall nested model which describes the influences of heating and recovery
 60 conditions on the estimated D value of bacteria. This model (eq 4) can be used like the
 61 Bigelow model to estimate the heat resistance and decimal reduction rate of bacterial
 62 population.

$$63 \quad \log D = \log D^* - \left(\frac{T - T^*}{z_T} \right) - \left| \frac{pH - pH^*}{z_{pH}} \right| - \left(\frac{a_w - 1}{z_{a_w}} \right) - \left(\frac{pH' - pH'_{opt}}{z'_{pH}} \right)^2 - \left(\frac{a'_w - a'_{wopt}}{z'_{a_a}} \right)^2 \text{ Eq 4}$$

64 The aim of this paper is to obtain the model's parameters for *Bacillus cereus* spores and
 65 validate these parameter values in the food product of chocolate.

66

67 **Material and methods**

68 *Micro-organism and spore production*

69 The strain of *Bacillus cereus* ADQP407 isolated from shrimp was obtained from the
 70 ADRIA (France). Spores were kept in distilled water at 4°C.

71 Cells were precultivated at 37°C for 24 hrs in Brain Heart Infusion (Difco). The
72 preculture was used to inoculate nutritive agar plates (Biokar Diagnostics BK021) added with
73 MnSO_4 40mg l⁻¹ and CaCl_2 100 mg l⁻¹ on the surface area. Plates were incubated at 37°C for 5
74 days. Spores were then collected by scraping the surface of the agar and suspended in sterile
75 distilled water and washed three times by centrifugation (10000xg for 15 min) (Bioblock
76 Scientific, model Sigma 3K30). The final suspension (about 10¹⁰ spores ml⁻¹) was finally
77 distributed in sterile Eppendorfs microtubes and kept at 4°C.

78

79 *Thermal treatment of spore suspension and recovery conditions.*

80 In basic condition the heating medium was a tryptone salt broth (10gl⁻¹ tryptone
81 Biokar and 10gl⁻¹ sodium chloride) at pH 7 with no sucrose added, the heating temperature
82 was 100°C. The heating medium was sterilized by filtration. The influence of heating
83 temperature was studied ranging from 95°C to 102°C, the heating pH ranging from 4.5 to 7
84 adjusted with HCL and the heating water activities ranging from 1 to 0.92 were adjusted using
85 sucrose. The previous molarities of the different solutes were determined using curves from
86 model UNIFAC-LARSEN (Achard et al. 1992). The a_w values were controlled with an aw-
87 meter (FA-st1 GBX France Scientific Instrument).

88 Firstly, 30µl of spore suspension was diluted in 3 ml adjusted heating medium. Capillary
89 tubes of 200 µl (vitrex) were filled with 100µl of sample, sealed, and submitted to a thermal
90 treatment in a thermostated glycerol bath for different heating times. The heat treatment was
91 stopped by cooling capillary tubes in water / ice bath. Then they were broken at both ends and
92 their contents poured into a tube containing 9 ml sterile tryptone salt broth (Biokar
93 Diagnostics) by rinsing with 1 ml tryptone salt broth.

94 The viable spores were counted by duplicate plating in nutritive agar (10g tryptone, 5g meat
95 extract, 5g sodium chloride, 15 g agar for 1000ml water)(Biokar Diagnostic) and incubated at

96 37°C for 6 days. The recovery medium pH ranging from 5 to 7 was adjusted with sterile
97 solution of HCl after autoclaving. The recovery medium water activity ranging from 1 to 0.95
98 was adjusted with added sucrose. To adjust a_w values, the previous molarities of the different
99 solutes were determined using curves from model UNIFAC-LARSEN (Achard et al. 1992).
100 Nutritive agar and sucrose solutions were sterilized separately by autoclaving to avoid the
101 Maillard reaction. After sterilisation the two solutions were mixed, pH was adjusted to 7 and
102 a_w value was controlled.

103 The validation of the heating sensibility parameters had been realized by heating *Bacillus*
104 *cereus* spores scattered in “chocolate cream” (pH: 6.76 and a_w : 0.968), included in capillary
105 tubes

106

107 *Experimental design and data analysis*

108 For each environmental factor studied a monofactorial experimental design was carried out. D
109 values were determinate by linear regression on the straight portion of curves obtained when
110 the log number of survivors was plotted against heating time. The sensibility parameters of
111 the models “z” were fitted on experimental values using Excel software.

112

113 **Results and discussion**

114 For the strain of *Bacillus cereus* ADQP 407 studied, all survival curves present a log linear
115 relation between the number of colony forming units and heating time. The classical D values
116 were determined by linear regression. One example is presented Fig1. The whole set of data
117 values is presented table 1.

118 The fitting of Bigelow model, related to the heating temperature, on the experimental D
119 values (Fig 2), gives a z_T value equal to 7.1°C, which corresponds to the values currently
120 given for *Bacillus cereus* spores (Bergere and Cerf, 1992).

121 The decrease of heating and recovery medium pH values reduces the apparent bacterial heat
122 resistance. (Fig 3-4). The fitting of parameters (eq 1-2) and associated correlation coefficients
123 were computed Table 2. The decrease in recovery pH medium (z'_{pH} : 2.18) appears to have
124 more influence on the apparent heat resistance than a decrease in heating medium pH (z_{pH}
125 3.45).

126 The dominating influence of recovery pH medium has been observed for other bacterial
127 species (Couvert et al., 1999; Couvert thesis 2002)

128 Regarding the water activity influences, a decrease in a_w value leads to a thermo-protective
129 effect (Fig 5). In the recovery medium heated spores show an apparent maximum heat
130 resistance at an optimum close to 0.985. Under this optimal value, an increase in sucrose
131 concentration reduces the bacterial heat resistance (Fig 6). The a_w decrease of recovery
132 medium (z_{aw} : 0.092) presents a more pronounced effect than the protective effect of heating
133 medium (z_{aw} 0.156).

134 These values correspond to the parameters determined for other *Bacillus cereus* strain with
135 sucrose as the same water depressor (Coroller et al., 2001). For the different models the D^* or
136 D'_{opt} correspond to the D value evaluated to the reference or the optimal conditions
137 respectively, and could not be considered the same. To get one and only D value, the overall
138 equation (Eq 4) was fitted on the whole set of data . The D^* value correspond closely to those
139 determined at heating condition: T^* : 121.1°C, pH^* : 7, a_w^* : 1 and the evaluated optimal
140 recovery conditions pH'_{opt} and $a_w'_{opt}$. The heat resistance parameters; z_T , z_{pH} , z'_{pH} , z_{aw} and z'_{aw}
141 obtained in Table 3, show the parameter values determined from each monofactorial design.

142 Fig 7 illustrates the relationship between experimental and calculated D values

143 In food product the main factors that influence the apparent heat resistance are the
144 temperature, pH and water activities. Moreover different compounds or food textures can
145 influence the bacterial heat resistance. However, previous studies, not published, have shown

146 that these secondary factors had a low influence on the sensibility parameters “z”. The part of
147 these factors is evaluated by the ratio between the experimental heat resistance determined in
148 food and the corresponding calculated values.

149 This comparison is made on the heat treatment only on the one hand and, on the overall
150 apparent heat resistance, heating and recovery on the other hand. Fig 8 shows the comparison
151 of the experimental and calculated *Bacillus cereus* death kinetics determined in chocolate
152 cream (pH 6.76 and aw:0.968). The figure and ratio 1.73, higher than 1, shown that the model
153 taking temperature, pH and water activities into account, underestimates the bacterial heat
154 resistance. However, on the overall apparent heat resistance heating and recovery, the
155 experimental result confirms the calculated forecast concerning the overall apparent heat
156 resistance (Table 4); after heat treatment at 100°C for 35 minutes no growth was observed in
157 cream chocolate incubated at 37°C for 7 days.

158 The confrontation between the validation ratio and the food texture and composition could
159 bring to the fore new factors or compounds that affect apparent heat resistance.

160

161 **Acknowledgement**

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220 Legend of figure

221 Fig 1: log N versus heating time

222 Fig 2: Log D versus heating temperature

223 Fig 3: Log D versus heating medium pH

224 Fig 4: Log D versus recovery medium pH

225 Fig 5: Log D versus heating medium aw

226 Fig 6: Log D versus recovery medium aw

227 Fig 7 : correlation between experimentally log D values and theoretically log D values calculated
228 from the overall model

229 Fig 8: comparison of the experimental (—) and calculated (---) *Bacillus cereus* death kinetics,
230 heating in chocolate cream and recovery in nutritive agar pH7, aw1.

231

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233

234

235 Table of legends

236 Table 1 Effects of heating temperature, heating and recovery medium pH and aw on D-values (min) of
237 *Bacillus cereus*

238 Table 2: models parameters

239 Table 3: fitting parameters on the whole set of data

240 Table 4: comparison of the experimental and calculated *Bacillus cereus* growth in capillary
241 tube after heating and recovery in chocolate cream for different heating times

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Fig1

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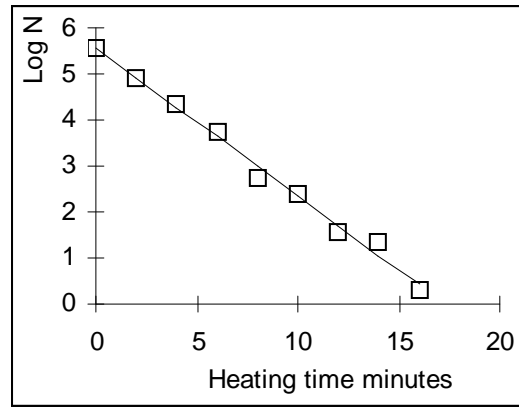
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Fig2

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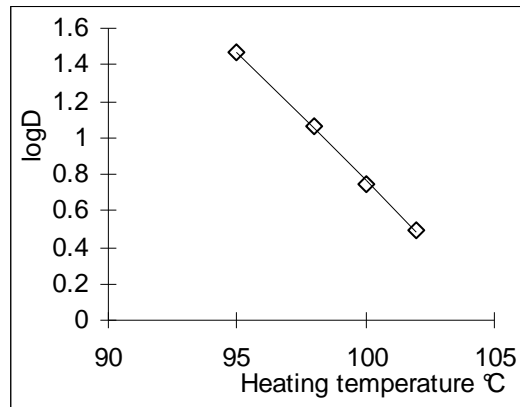
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Fig3

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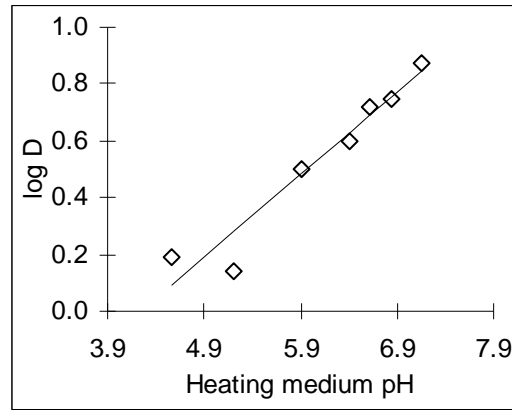
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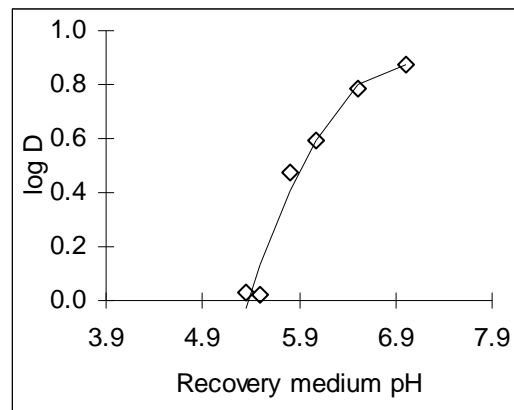
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Fig4



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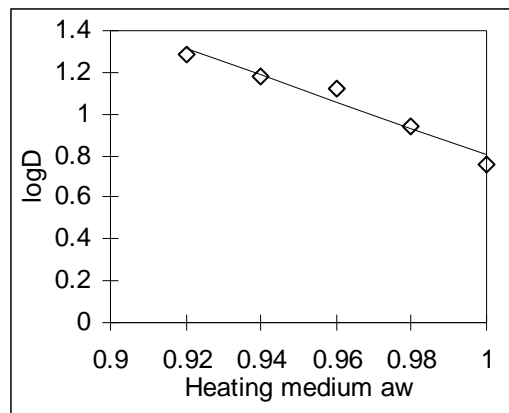
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Fig5



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Fig6

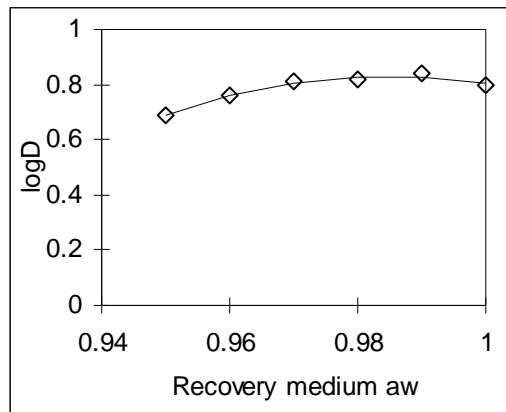
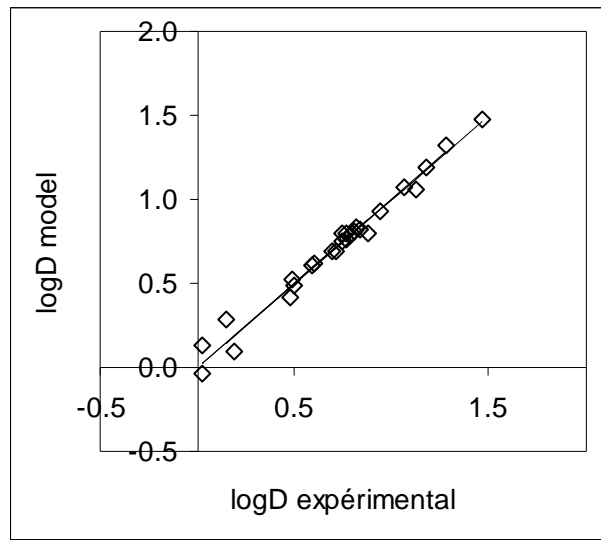


Fig7



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Fig8

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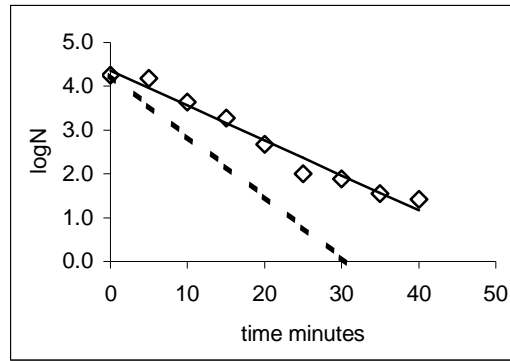
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Heating temperature °C	Heating medium pH	Recovery medium pH'	Heating medium aw	Recovery medium aw'	D value minutes	Confidence interval
95	7	7	1	1	29.52	± 2.31
98	7	7	1	1	11.54	±0.78
100	7	7	1	1	5.61	±0.46
102	7	7	1	1	3.11	±0.19
100	6.85	7	1	1	5.57	±0.57
100	6.62	7	1	1	5.22	±0.60
100	6.41	7	1	1	3.99	±0.32
100	5.92	7	1	1	3.14	±0.22
100	5.21	7	1	1	1.61	±0.19
100	4.56	7	1	1	1.89	±0.16
100	7	7	1	1	7.53	±0.73
100	7	6.52	1	1	6.06	±0.54
100	7	6.07	1	1	3.92	±0.44
100	7	5.8	1	1	2.98	±0.33
100	7	5.5	1	1	3.59	±0.37
100	7	5.35	1	1	1.04	±0.14
100	7	7	1	1	5.78	±0.48
100	7	7	0.98	1	8.71	±1.39
100	7	7	0.96	1	13.32	±1.67
100	7	7	0.94	1	15.07	±1.66
100	7	7	0.92	1	19.10	±2.31
100	7	7	1	1	6.55	±0.53
100	7	7	1	0.99	6.93	±0.61
100	7	7	1	0.98	6.58	±0.49
100	7	7	1	0.97	6.44	±0.60
100	7	7	1	0.96	5.79	±0.38
100	7	7	1	0.95	4.90	±0.47

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Table1

389

390

	Values	r
$z_T^{\circ\text{C}}$	7.09	0.999
z_{pH}	3.44	0.967
z'_{pH}	2.18	0.920
pH'_{opt}	6.96	
z_{aw}	0.156	0.979
z'_{aw}	0.092	0.989
aw'_{opt}	0.985	

391

392

Table 2

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398

Fitting on the whole set of data	
D* minutes	0.009
logD*	-2.02
z_T °C	7.32
z_{pH}	3.48
z'_{pH}	1.55
z_{aw}	0.153
z'_{aw}	0.088
pH'opt	6.78
aw'opt	0.983
r	0.990

402

Heating time minutes	Tubes with observed growth	Calculated N value in tube
20 min	2 / 2	54
25 min	2 / 2	11
30 min	1 / 2	2
35 min	1 / 2	0
40 min	0 / 2	0
45 min	0 / 2	0

403

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Table4