# Validation of an overall model describing the effect of three environmental factors on the apparent D-value of Bacillus cereus spores

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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 mo D

16 el parameters characterising the environmental factor influences (pH, Temperature, aw) 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, aw, *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; Fernadez 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°C) and $pH*$ is the reference $pH$

35    fixed at =7, $D*$ is the $D$-value at $T*$, $pH*$ and $aw = 1$, $z_T$ 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, $z_{aw}$ is the

37    difference of aw from $aw* = 1$ 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    $$\log D' = \log D'_{opt} - \left(\frac{pH' - pH'_{opt}}{z'_{pH}}\right)^2 \text{ Eq. 2}$$

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

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

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 $10^{10}$ 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 ( 10g$l^{-1}$ tryptone

81    Biokar and 10g$l^{-1}$ 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 <u>et al.</u> 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 aw: 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 aw 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 aw 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, $aw^*$: 1 and the evaluated optimal

140 recovery conditions $pH'_{opt}$ and $aw'_{opt}$. 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

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243                           Fig1

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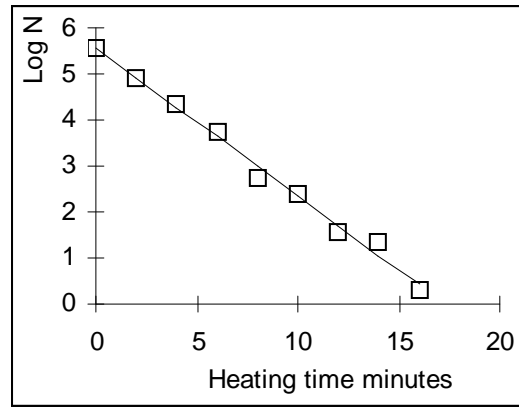256

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Fig2

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Fig2

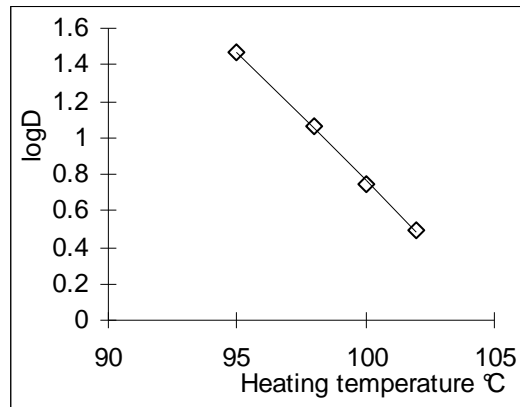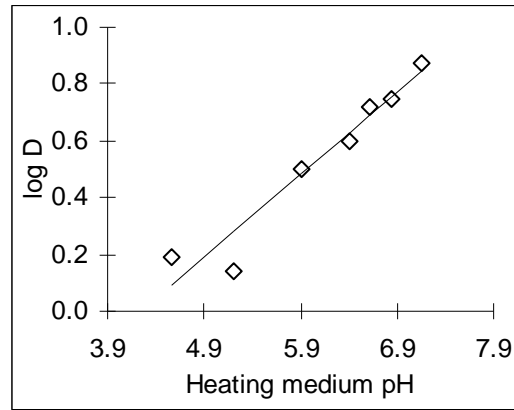279                                Fig3

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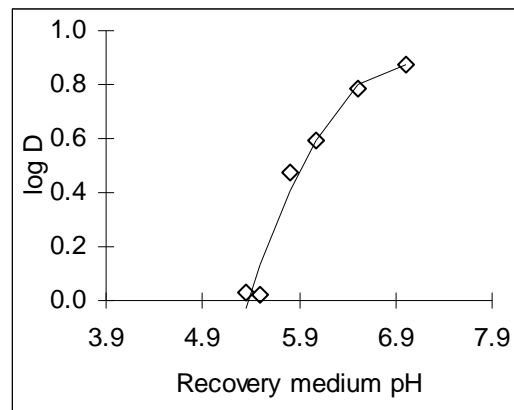
298                                    Fig4

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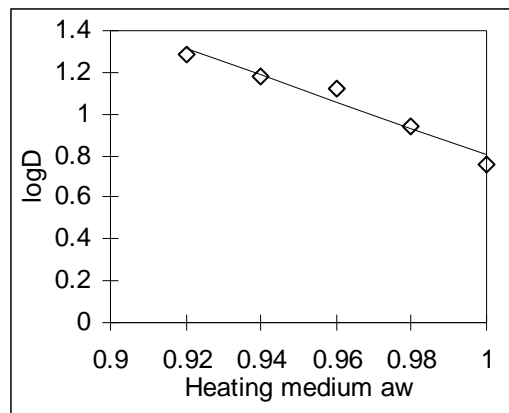328

330                           Fig6

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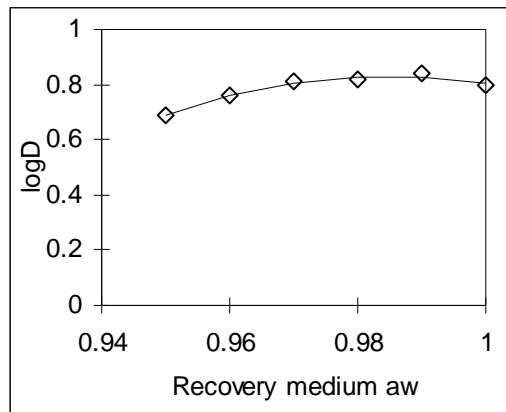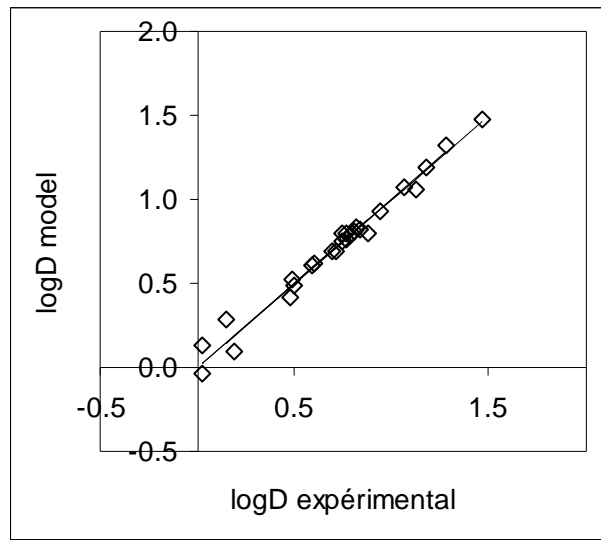
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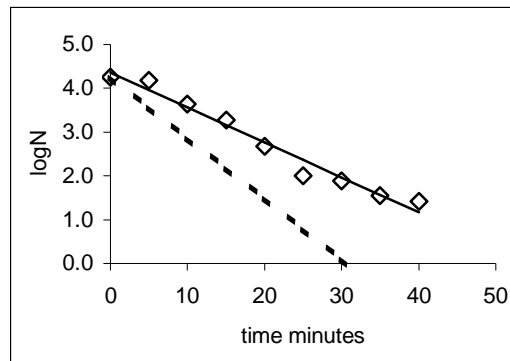
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| Heating temperature °C | Heating medium pH | Recovery medium pH' | Heating medium aw | Recovery medium aw' | D value minutes | Confidence interval |
|---|---|---|---|---|---|---|
| 95 | 7 | 7 | 1 | 1 | 29.52 | ± 2.31 |
| 98 | 7 | 7 | 1 | 1 | 11.54 | ±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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387 Table1
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| | Values | r |
|---|---|---|
| $z_T\,°C$ | 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 | |

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Table 2

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|  | 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 |

400

401                          Table3

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

404 Table4