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

5
6 S. Gaillard⁽¹⁾, I. Leguérinel⁽¹⁾, N. Savy⁽²⁾, P. Mafart*⁽¹⁾

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8 *(1) : Laboratoire Universitaire de Microbiologie Appliquée de Quimper, Pôle Universitaire de Creach Gwen*
9 *29000 Quimper (France),*

10 *(2) : Institut de Recherche Mathématique de Rennes, Campus de Beaulieu 35042 Rennes cedex (France)*

11
12 **Abstract**

13
14 The purpose of this study was to quantify the lag time of re-growth of heated spores of
15 *Bacillus cereus* as a function of the conditions of the heat treatment: temperature, duration
16 and pH of the recovery medium. For a given heating temperature, curves plotting lag times
17 versus time of heating show more or less complex patterns. However, under a heating time
18 corresponding to a decrease of 2 decimal logarithms of the surviving populations of spores, a
19 linear relationship between the lag time of growth and the time of the previous heat treatment
20 can be observed. The slope of this linear relationship followed itself a Bigelow type linear
21 relationship, the slope of which yielded a ζ - value very close to the observed conventional z-
22 value. It was then concluded that the slope of the regrowth lag time versus the heating time
23 followed a linear relationship with the sterilisation value reached in the course of the previous
24 heat treatment. A sharp effect of the pH of the medium which could be described by a simple
25 “secondary” model was observed. As expected, the observed intercept of the linear

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

28

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

30

31 **1. Introduction**

32

33 *Bacillus cereus* is a pathogen that produces two heat resistant toxins which are likely to cause
34 more or less severe infections (Granum, 1994; Johnson,1984). The level of population which
35 can generate infections is in the magnitude of 10^5 - 10^6 spores per gram (Granum and Lund,
36 1997). This bacteria belongs also to the spoilage flora of dairy products because of the action
37 of its lecithinase on the globule membrane of milk fat (Jean Stone, 1952).

38 The heat resistance of *Bacillus cereus* spores is extremely variable, with a D-value (time of
39 decimal reduction) ranging from 5 to 108 minutes at 85°C and from 0.3 to 27 minutes at
40 100°C respectively (Bergère and Cerf, 1992; Picoche *et al.*, 1993). Because of their relatively
41 low heat resistance, most of strains are able to survive and grow after a pasteurisation .

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

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

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

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

59

60 **2. Material and methods**

61

62 *2.1. Microorganism and spore production*

63

64 The strain of *B. cereus* (Bce1), isolated from a dairy food line process, was supplied by
65 Danone (France). Spores were kept in distilled water at 4°C. Cells were precultivated at 37°C
66 for 24 h in Brain Heart Infusion (Difco). The preculture was used to inoculate nutrient agar
67 plates (Biokar Diagnostics BK021) added with MnSO₄ 40 mg l⁻¹ and CaCl₂ 100 mg⁻¹ on the
68 surface area. Plates were incubated at 37°C for 1 month. Spores were then collected by
69 scraping the surface of the agar and suspended in sterile Phosphate buffer M/15 at pH 7 and
70 washed three times by centrifugation (10 000 x g for 15 min) (Bioblok Scientific, model
71 Sigma 3K30). The pellet was then resuspended in 5 ml distilled water and 5 ml ethanol. The
72 suspension obtained was kept at 4°C for 12 h in order to eliminate vegetative nonsporulated
73 bacteria and washed again three time by centrifugation. Lastly, the final suspension (about
74 10¹⁰ spores ml⁻¹) was distributed in sterile Eppendorf micro-tubes and kept at 4°C.

75

76 *2.2. Thermal treatment of spore suspension*

77

78 First, 30 µl of spore suspension was diluted in 3 ml heating medium (Biokar nutritive broth,
79 20g / L) . Capillary tubes of 100 µl (vitrex) were filled with 100 µl of sample and submitted
80 to a thermal treatment in a thermostated bath. After heating, the tubes were cooled in
81 water/ice bath, washed in solution of soap and rinsed with sterile distilled water. Finally, ends
82 were flamed with ethanol. The capillary tubes were broken at both ends and their contents
83 poured into a tube containing 9 ml of sterile tryptone salt broth (Biokar Diagnostics) for an
84 appropriated series of decimal dilutions, by rinsing with 1 ml broth contained in a needle-
85 equipped syringe.

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

90

91 *2.3. Growth conditions and measurements*

92

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

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

104

$$105 \quad \text{Ln OD} = 0.793 \text{ Ln N} - 15.583 \quad (R^2 = 0.991)$$

106

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

109

110

111 *2.4. Lag time estimation*

112

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

117

118 *2.5. Parameters estimation*

119

120 The estimations of the parameters of the models are obtained by the least squares method *via*
121 the Matlab© function '*nlcurvefit*'. The criterion of Beale (Beale, 1960) provides us a tool
122 allowing construction, by numerical simulation (technic near to that of Lobry et al. (Lobry,
123 1991)), of a hypervolume of confidence to the risk 5% which is conventionally retained .
124 Projection on each axis of the space of the parameters gives us an interval of confidence of
125 this parameter.

126

127 3. Results

128

129 3.1. General pattern of curves

130

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

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

148

$$149 \lambda = \lambda_0 + mt \quad (1)$$

150

151 3.2. Secondary modelling of the initial linear phase

152

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

162

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

163

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

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

171

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

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

179

180

181 **4. Discussion**

182

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

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

199

200

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

207

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

208 Or (3)

$$m = m^* 10^{\frac{T - T^*}{z}}$$

209 (4)

210

211 The combination of Eq 1 and 4 yields:

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

212 (5)

213

214

215 herein F represents the well known sterilisation value (F -value) which is defined as the time
216 (in minutes) of a heat treatment at the constant reference temperature (generally, $T^* =$
217 121.1°C), or as any equivalent heat treatment which would cause the same destruction ratio.
218 Then, Eq 5 points out an interesting significance of m^* which would correspond to the slope
219 of a linear relationship between the lag time of growth and the sterilisation value submitted by
220 spores before growing.

221

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

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235

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279

280 Legend Table

281

282 Parameters estimates of equation 2.

283

284 Legend Figure

285

286 Fig 1 : λ values for different pH (\circ pH 7, \bullet pH 6.5, \blacktriangleright pH 6, \blacksquare pH 5.78 , Δ pH 5.75, pH 5.5)

287 Heating temperature 90°C

288

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

290

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

292

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

294

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

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297 Fig 6 : $\log \lambda_0$ values variation versus pH at 90°C

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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)	

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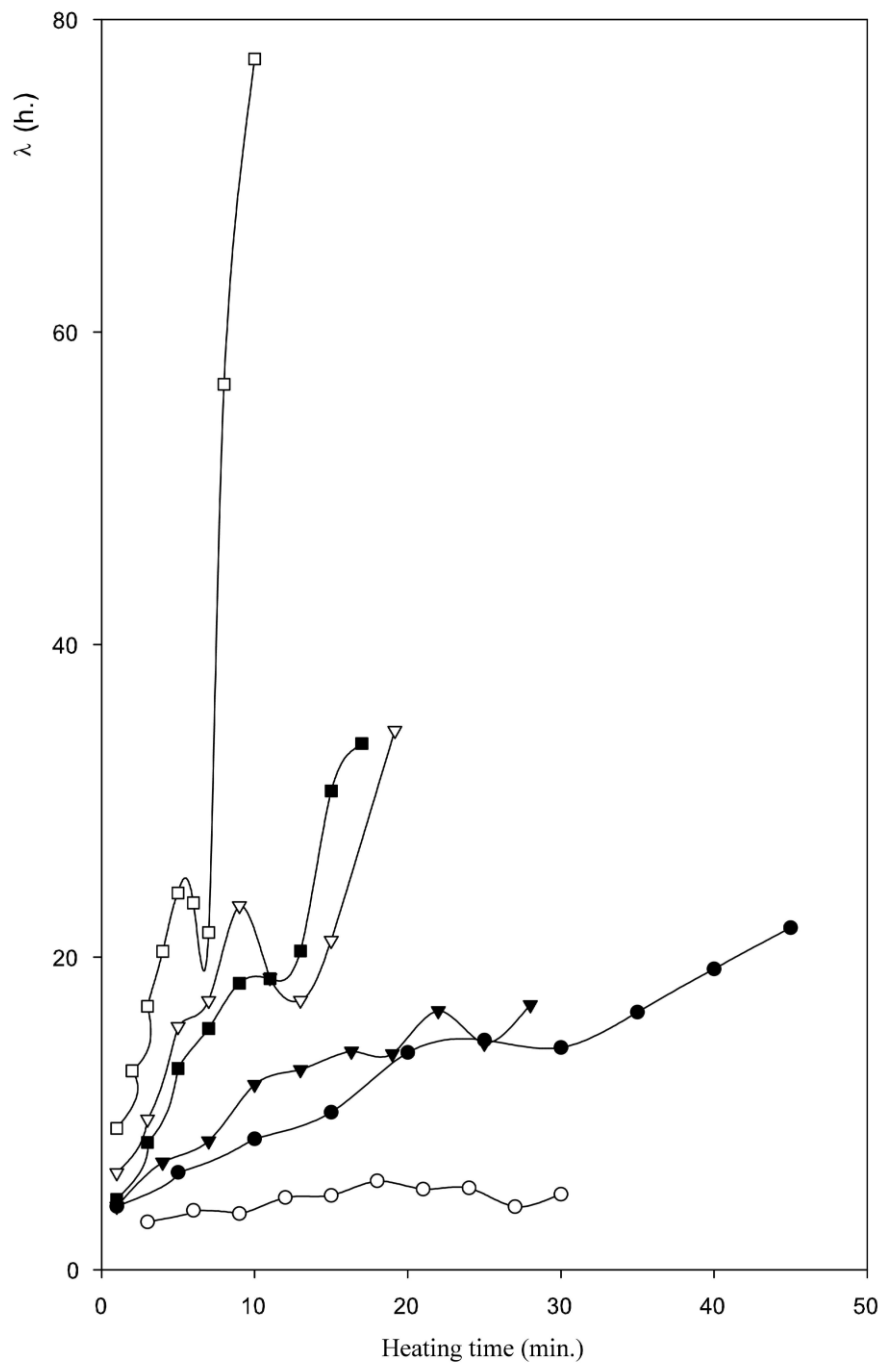
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316 **Fig 1**

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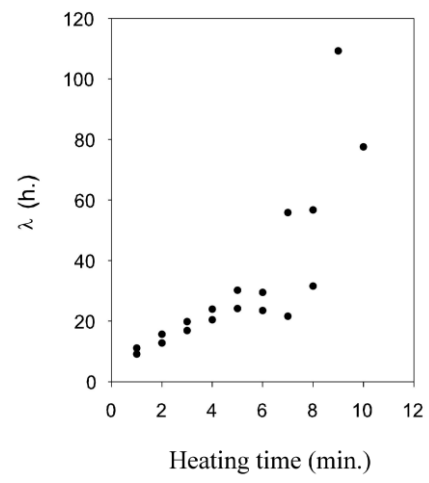
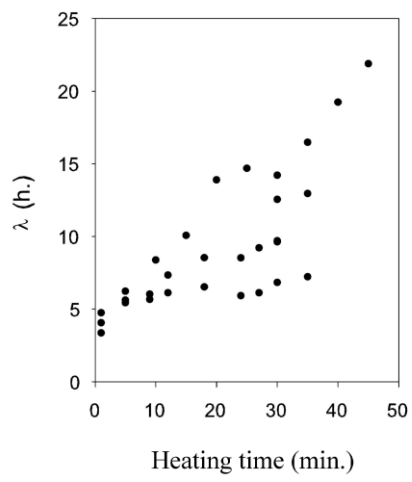


Fig 2a

Fig 2b

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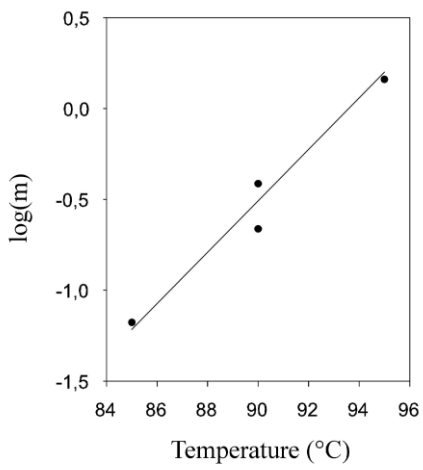


Fig 3

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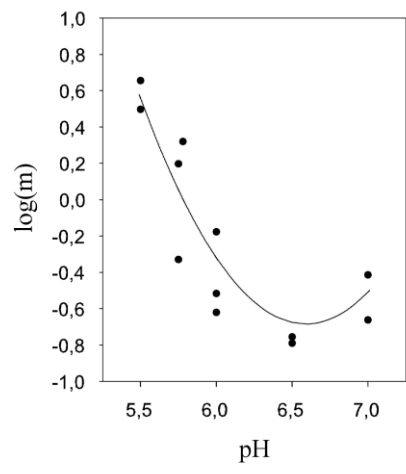


Fig 4

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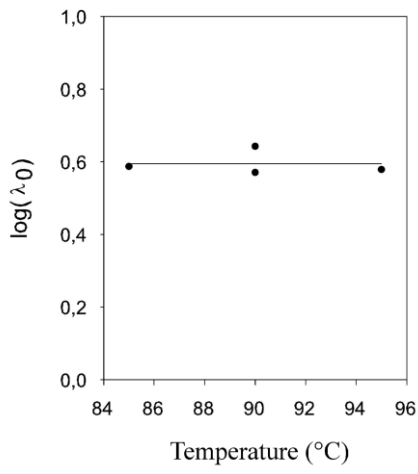


Fig 5

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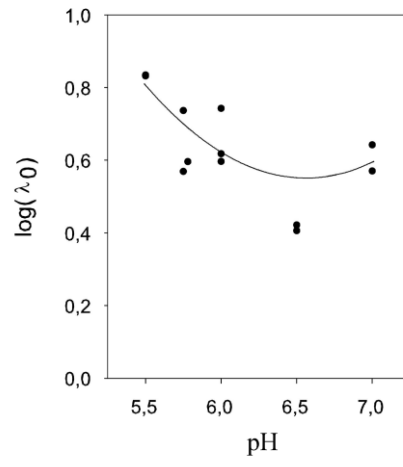


Fig 6

