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Modelling the overall effect of pH on the apparent heat resistance of Bacillus cereus spores

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Introduction

Among environmental conditions that affect spore thermoresistance, pH is a major factor in microbial destruction. It has been recognised for several years that low pH values reduce spore resistance

(Alderton et al., 1976; Townsend et al., 1938; Tsuji et al., 1960). However, available information related to the quantitative effect of pH is scarce. Jordan and Jacobs (1948) observed a linear relationship between the D-value (decimal reduction time) of Escherichia coli and the pH of the heating menstruum. Davey et al., (1978) and, more recently, Mafart and Leguérinel, (1998), proposed a model to describe the combined effect of temperature and pH on heat resistance of spores.

These models were developed from data where D-values were estimated by recovering surviving cells at optimum conditions and, particularly, at optimal pH of the recovery medium. However, it is well known that counts of survival spores after a heat treatment are greatly influenced by the characteristcs of the recovery medium (temperature, pH, water activity, composition). At non optimum recovery conditions, both a decrease in the number of viable cells capable of producing colonies and a decrease in the estimated decimal reduction time are observed. It is generally accepted that the pH of the recovery medium exerts a great influence on the apparent heat resistance of spores: D-values decrease as pH is reduced (Cook and Brown, 1965; Yokoya and York, 1965;

Cook and Gilbert, 1968; Mallidis and Scholefield, 1986; Santos and Zarzo, 1996; Lopez et al., 1997).

This paper aims to present a simple overall model which takes into account both the effect of the pH of the heating menstruum and that of the recovery medium upon the observed D-value.

Assuming a "multiplicative" effect of the pH on the D-value, the influence of the heating menstruum pH can be written:

$$D = f(pH).D^* \qquad (1)$$

where D* is the maximum D-value at the optimum pH. Similarly, the effect of the recovery medium pH can be written:

$$D' = f'(pH').D \qquad (2)$$

where D' is the apparent decimal reduction time at pH' (pH of the recovery medium). The overall model is provided by combining Eqn (1) and (2):

$$D' = f(pH).f'(pH').D^* \qquad (3)$$

Model (3) can be linearized by the following logarithmic transformation:

$$\log D' = \log D^* + \log f(pH) + \log f'(pH') \qquad (4)$$

The destruction factor f (pH) which is related to Mafart and Leguérinel's model (1998) corresponds to:

$$f(pH) = 10^{-\frac{(pH-pH^*)^2}{z_{pH}^2}} \qquad (5)$$

in which pH* is the optimum pH of the heating menstruum corresponding to the maximum heat resistance. On account of the similitude of patterns which can be

observed between curves plotting D *vs* pH and D' *vs* pH' respectively, and in order to keep the homogeneity of the overall model, we tested the following stress factor:

$$f'(pH') = 10^{-\frac{(pH'-pH'^*)^2}{z'^2_{pH}}} \qquad (6)$$

where pH'* is the optimum pH of the recovery medium corresponding to the maximum D' value. Then, Eqn (4) was transformed into:

$$\log D' = \log D^* - \frac{(pH-pH^*)^2}{z^2_{pH}} - \frac{(pH'-pH'^*)^2}{z'^2_{pH}} \qquad (7)$$

Materials and methods

Micro-organism 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 $MnSO_4$ 40 mg l^{-1} and $CaCl_2$ 100 mgl^{-1} on the surface area. Plates were incubated at 37°C for 5 days. Spores were then collected by scraping the surface of the agar, suspended in sterile distilled water, and washed three times by centrifugation (10000xg for 15 min) (Bioblock Scientific, model Sigma 3K30). The pellet was then suspended

againin 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 finally distributed in sterile Eppendorf microtubes and kept at 4°C.

Thermal treatment of spore suspension

D values in citrate-phosphate buffer adjusted were determined at 95°C with one replicate at each pH ranging from 5 to 7.

First, 30µl of spore suspension was diluted in 3 ml buffer. Capillary tubes of 25 µl (vitrex) were filled with 10µl of sample and submitted 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, the 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 rinsing with 1 ml tryptone salt broth contained in a needle-equipped syringe.

Recovery conditions

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 incubated at 25°C for 6 days. The pH medium

ranging from 5 to 7 was adjusted with H_2SO_4 1N before autoclaving. After autoclaving the pH was measured and the final pH value was kept for calculations.

For media whose pH was lower than 5.5, 10g/l of agar was added. A solution of Na_2SO_4 was added to adjust the medium at the same ionic strength.

Data analysis

D values were based on the reciprocal of slopes obtained when the log number of survivors was plotted against time.

Parameters of the models were fitted by simple or multiple linear regressions carried out with the STAT-ITCF software (Institut Technique du Fourrage France).

The goodness of fit of the model was evaluated by using the accounted for per cent variance (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. Complementarily, mean square errors were determined.

Results

In a first set of experiments spores were heated at 95°C in media ranging from pH 5 to 7 and recovered at 25°C , at the optimum recovery pH (6.7). Observed D-values are shown in table 1. logD values were then plotted *vs* $(pH-pH^*)^2$ (fig1). The pH of maximal thermal resistance related to the studied strain of *B. cereus* was close to 7.5 (Gaillard et al., 1998). The model was then fitted with $pH^* = 7.5$ according to a linear regression (table 2).

In a second set of experiments, spores were heated at 95°C in a medium of pH 7 and recovered at 25°C in media ranging from pH 5 to 7. Observed apparent decimal reduction times are shown in table 3. log D' values were then plotted *vs* (pH'-pH'*) with $pH'^* = 6.7$. Fitted parameters according to the linear regression are presented in table 4. The observed satisfactory goodness of fit allows to adopt the same function for the destruction factor as for the stress factor (Eqn 5 and 6 respectively).

As foods make up the heating menstruum and the recovery medium, a third set of experiments, in which spores were recovered at the same pH as those of the heating menstruum, was carried out in order to validate the overall model. Parameters of the overall model were not fitted but parameters'values obtained from the first two independent sets of data (tables 2 and 4) were used in order to compute D' values. Squares on Figure 3 represent observed log D' values while the continuous line corresponds to the calculated values according to the model.

The correlation between observed and calculated values gives a R^2 value of 0.968 and a Mean Square Error of $8.83.10^{-3}$.

Discussion

Currently the D-values used to establish sterilisation process were not taken into account in the recovery phase. The overall model (Eqn 7) is proving to describe successfully the influence of the pH on the heat resistance of spores both during the heat treatment and the recovery phase. However its lack of robustness, due to overparameterization, makes it difficult for it to be used for calculating heat food processes. Lack of robustness is pointed out by the following test: if instead of calculating D' values with parameters separately estimated from the first two independent sets of data, parameters are fitted according to the actual observed D' values from the third set of data (pH* and pH'* are then held to be fitted parameters and not fixed values), quite different values are obtained from those previously determined (table 5). On account of the great variability of microbial species and strains, it seems worthwhile, in the framework of standard calculations, to fix pH* and pH'* values to 7.Consequently, when pH' = pH , Eqn (7) is reduced to

$$\log D' = \log D^* - \left(\frac{1}{z_{pH}^2} + \frac{1}{z'^2_{pH}}\right)(pH - pH^*)^2 \qquad (8)$$ or

$$\log D' = \log D^* - \frac{1}{Z_{pH}^2}(pH - pH^*)^2 \qquad (9)$$

Fitted parameters of this simplified model are respectively D*=2.27 min and Z_{pH}=1.69, with R^2=0.976 and a Mean Square Error of $4.34.10^{-3}$. The robustness of the new overall parameter Z_{pH} was checked through the following test: z_{pH} and z'_{pH} were fitted again while standard values were affected to pH* and pH'* (7 instead of 7.5 and 6.7 respectively). As expected, new values were clearly different from previous ones (z_{pH}=2.62 instead of 3.10 and z'_{pH}=2.04 instead of 1.75). However, when calculating the overall Z_{pH} value from the following relationship

$$\frac{1}{Z_{pH}^2} = \frac{1}{z_{pH}^2} + \frac{1}{z'^2_{pH}} \qquad (10)$$

Z_{pH}=1.61 was found, which is close to the value determined from the last set of data.

It is clear that substituting model (7)(5 parameters when pH* and pH'* are fitted instead of being fixed) for the more parsimonious model (9) (only 2 parameters) is likely to lead to some lost of goodness of fit : a R^2 value of 0.976 instead of 0.983 and a Mean Square Error of $4.34.10^{-3}$ instead of $2.99.10^{-3}$ are obtained. However, this drawback is largely set off by the simplification and the improvement of the model's robustness.

When a n decimal reduction ratio is aimed, at the reference temperature (121.1°C) and at pH 7, the sterilisation value must be:

$$F = nD^*_{121.1°C} \qquad (11)$$

At 121.1°C but at a pH different from 7, the time of heat treatment leading to the same destruction ratio will be:

$t = nD'_{121.1°C}$ (12)

Combining Eqn (11) and (12) gives:

$$F = \frac{D^*_{121.1°C}}{D'_{121.1°C}} t = L_{121.1°C}(pH)t \quad (13) \quad \text{with}$$

$$L_{121.1°C}(pH) = 10^{\frac{(pH-pH^*)^2}{Z_{pH}^2}} \quad (14)$$

The function L(pH) must be regarded as a partial apparent Biological Destruction Value (partial because pH is here the only considered factor).

Obviously, the studied strain of B.cereus which is very sensitive to recovery medium pH, is not a representative strain likely to be used as an indicator strain in order to develop standard heat process calculations. From data of Lopez et al., (1996), it was possible to fit z_{pH} values of 4 strains of Bacillus stearothermophilus which were heated at 120°C. Another set of data from the same laboratory (Lopez et al., 1997) allowed to fit z'_{pH} values related to the same strains. For each strain, overall Z_{pH} values were deduced from Eqn (10), (see Table 6). Table 7 shows the magnitude of partial apparent Biological Destruction Values related to B. stearothermophilus with an average Z_{pH} value of 3.4. Further works would be needed in order to validate the model more generally and to determine Z_{pH} value related to other representative species, especially Clostridium botulinum.

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Abstract

A simple overall model was proposed to describe the effect of both pH of the heating menstruum and pH of the recovery medium on apparent spore heat resistance of Bacillus cereus. Applied to foods making up both heating and recovery media, the model can be reduced to only 2 parameters. Its goodness of fit and its robustness enable it to be applied for the optimisation of heat treatments. However, further experiments should be undertaken to validate the model for other species and to determine parameters related to reference species such as Clostridium botulinum.

KEYWORDS: pH, heat resistance, recovery, Bacillus cereus.

Legends of tables

Table 1. The effect of the heating medium pH on the heat resistance of Bacillus cereus spores. D_{95} (min)

Table 2. Rate coefficient related to equation 5 (pH*=7.5)

Table 3. The effect of the recovery medium pH on the apparent heat resistance of Bacillus cereus spores. D'_{95} (min)

Table 4. Rate coefficient related to equation 6 (pH'*=6.7)

Table 5. Rate coefficient related to equation 7

Table 6. z_{pH}, z'_{pH} and Z_{pH} values related to Bacillus stearothermophilus , according to Lopez et al.'s data (1996, 1997)

Table 7. Partial apparent Biological Destruction Values *vs* medium pH with Z_{pH}=3.4

Legends of figures

Fig. 1. Fitting of log D *vs* $(pH-pH^*)^2$ with $pH^*=7.5$

Fig 2. Fitting of log D' *vs* $(pH'-pH'^*)^2$ with $pH'^*=6.7$

Fig 3. Observed (squares) and calculated (continuous line) D' values according to equation 7 and parameters values indicated in Tables 2 and 4.

Table 1.

pH	$D_{95°C}$(min)
5	0.64
5.25	0.79
	0.76
5.5	0.98
	1.10
6	1.71
	1.62
6.5	1.91
	2.07
7	2.52
	2.79

Table 2.

Number of data	11
R^2	0.981
Mean Square Error	$6.78.10^{-4}$
$D_{95°C}$*	2.71 min
z_{pH}	3.10

Table 3.

pH'	$D_{95°C}$' (min)
5	0.33
5.15	0.26
5.25	0.42
5.5	0.59
5.8	1.22
5.9	1.65
5.95	1.81
6.2	1.72
6.35	1.58
6.35	1.93
6.7	2.27
6.75	2.13
6.9	2.17

Table 4.

Number of data	13
R^2	0.942
Mean Square Error	$5.77.10^{-3}$
$D_{95°C}$	2.17 min
z'_{pH}	1.74

Table 5.

Number of data	20
R^2	0.983
Mean Square Error	$2.99.10^{-3}$
pH*	7.87
pH'*	7.38
$D_{95°C}$*	3.36 min
z_{pH}	3.59
z'_{pH}	2.53

Table 6.

Strain	z_{pH}	z'_{pH}	Z_{pH}
12980	3.82	3.06	2.39
7953	3.97	3.80	2.75
15951	3.45	3.21	2.35
15952	4.12	2.94	2.39

Table 7.

pH	$L_{120°C}$(pH)
7	1.00
6.5	1.11
6	1.49
5.5	2.46
5	4.95

Figure 1

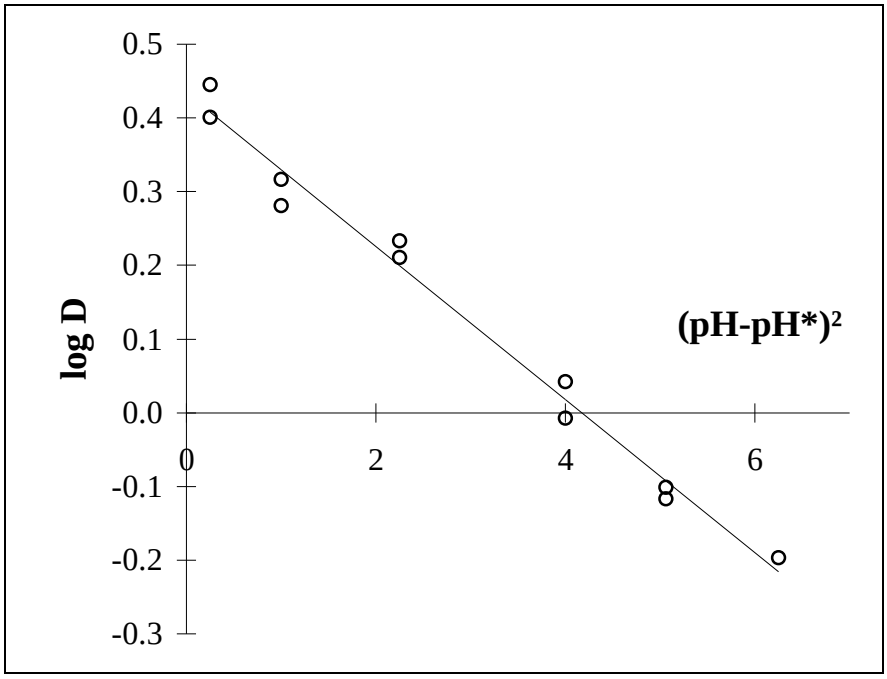

Figure 2

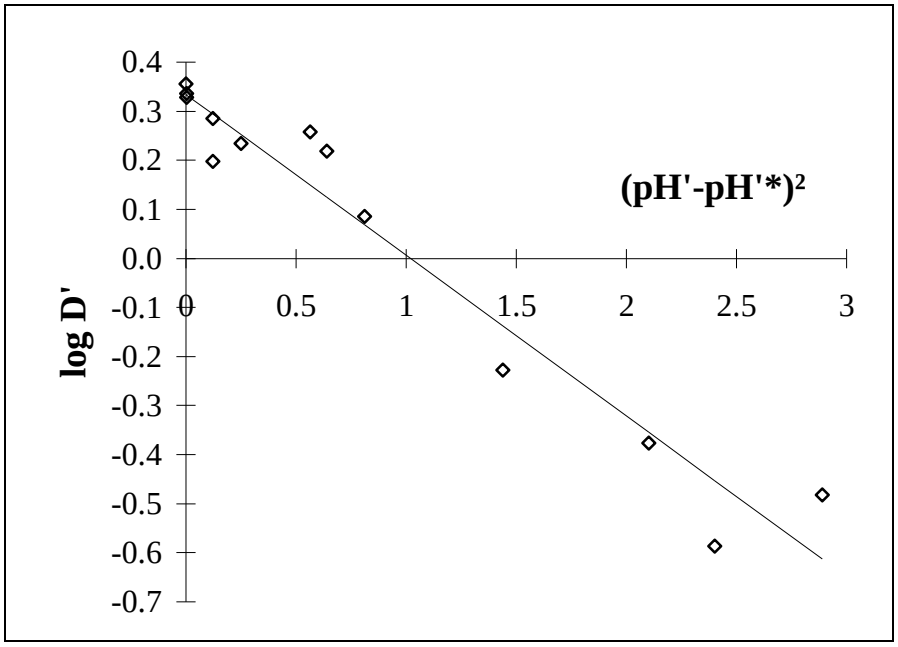
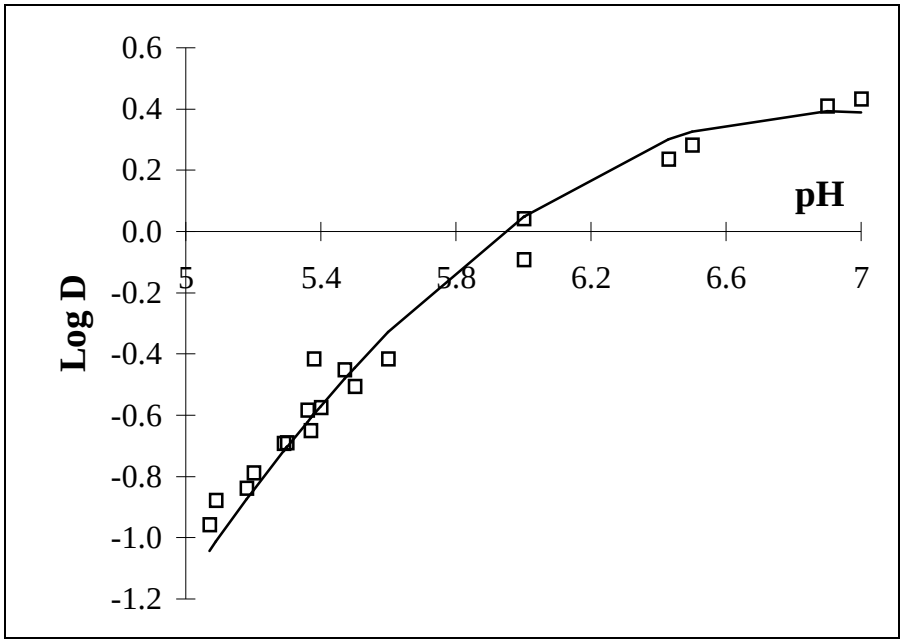
Figure 3