Modelling the influence of pH and organic acid types on thermal inactivation of Bacillus cereus spores

Ivan Leguérinel, Pierre Mafart

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LEGUERINEL I.*, MAFART P.

Laboratoire Universitaire de Microbiologie Appliquée de Quimper, Quimper, France

* Corresponding author : IUT Quimper, 2 rue de l'Université, F29334, Quimper cedex, France Tel +33 02 98 90 02 27, Fax +33 02 98 90 85 44

E mail address: guerinel@univ-brest.fr (I. Leguérinel)

Abstract

A model is proposed to describe the influence pH on the heat resistance of Bacillus cereus spores. In addition to the conventional z value, the effect of pH on the thermal resistance of spores is characterised by a z_{pH} value (z_{pH} is the distance of pH from a reference pH*, which leads to a ten fold reduction of D-value). The type of organic acid used for acidifying the heating medium, influences the z_{pH} value. For nine organic acids, a linear relationship between the calculated z_{pH} value and its lower acid pKa is observed. This relationship showed that the acid form (dissociated or undissociated) modifies the thermal spore resistance in addition to the H^+ ion. The influence of acetic acid concentration on the D value at pH 7 shows the protective effect of the dissociated acid form on the heat resistance of spores. The acid concentration in the medium modified the heat resistance of spore and the z_{pH} value.

Keywords : Organic acids, pH, heat resistance, Bacillus cereus

Introduction

The effect of pH on the heat resistance of bacterial spores in food systems were extensively studied for different species Clostridium botulinum (Xezones and Hutching 1965), Clostridium sporogenes (Cameron et al. 1980), Bacillus stearothermophilus (Lopez et al. 1996). It has been recognised for several years that low pH reduces spores resistance. This effect is used to reduce heat treatment with microbiological safety and preservation of nutritive and sensorial properties.

However, according to the type of acid used, the level of pH influence changes (Blosher and Busta 1983). For Bacillus thermoacidurans spore Anderson et al. (1949) showed that at the same pH 3.8 in tomato juice acetic acid was more effective than lactic or citric acid in reducing heat resistance at various temperatures. The same influence of these two acids and malic acid on D-values was observed for Bacillus stearothermophilus and Bacillus coagulans spore in frankfurter emulsion (Lynch and Potter 1988). Pontius et al. (1997) for Alicyclobacillus acidoterrestris spores reported the effect of malic, citric and tartaric acids on $D_{91°C}$ values. According to these published data, the level of influence of acid type can be graded as follows : acetic > lactic > malic >

tartaric. These hierarchies correspond to the values of their lower dissociation constant (pKa) which are respectively 4.75, 3.86, 3.40, 3.00.

The aim of this paper is to study the heat resistance of spores of Bacillus cereus CNRZ 110 at 95°C in heating medium acidified at different pH level with different organic acid types The influence of pH on the heat resistance is differentiated from acid specific effect. The study also investigates the acid form (dissociated or undissociated) which influences the heat resistance of spores of Bacillus cereus.

Materials and methods

Micro-organism and spore production

The strain of Bacillus cereus (CNRZ 110) was obtained from the Institut National de Recherche Agronomique (INRA / France). Spores were kept in distilled water at 4°C.

Cells were pre-cultivated at 37°C for 24 hours in Brain Heart Infusion (Difco 0037). The preculture was used to inoculate nutrient agar (Biokar Diagnostics, Beauvais / France) supplemented with added sporulation salt ($MnSO_4$ 40mg l^{-1} and $CaCl_2$ 100 $mgl^{-1)}$. Plates were incubated at 37°C for 5 days. Spores were then collected by scrapping the surface of the agar, suspending in sterile distilled water and washed three times by centrifugation (10000xg for 15 min) (Bioblock Scientific, model Sigma 3K30). The pellet was resuspended in 5 ml distilled water and 5 ml ethanol. The obtained suspension was kept at 4°C during 12 hours in order to reduce the number of vegetative non sporulated bacteria, and washed again three times by centrifugation. The final suspension (about 10^{10} spores ml^{-1}), containing more than 99% refractile spores and no visible vegetative cells, was finally distributed in sterile Eppendorf microtubes and kept at 4°C.

Thermal treatment of spore suspension and Viable spore count

The concentrated spore suspension (10^{10} spores per ml) 30µl were diluted in 3 ml of acidified tryptone salt agar (Biokar Diagnostics). The tryptone salt broth has a slight buffer effect due to the tryptone. The pH of the heating medium was adjusted by addition of the test organic acid in tryptone salt broth. Table 1 shows the relation between pH and the concentration of each organic acid in tryptone salt broth. Capillary tubes of 25 µl (Vitrex) were filled with 10µl of sample and submitted to a thermal treatment at 95°C in a temperature controlled glycerol bath (± 0.01°C). After heating, the tubes were cooled in water/ice bath, washed and rinsed with sterile distilled water. The capillary tubes were broken 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 syringe equipped with a needle.

After dilution in tryptone salt broth, heated spores suspension were included in nutrient agar (Biokar Diagnostic) and incubated at 25°C for 6 days to count colony.

Heat resistance parameters

Decimal reduction time D-values are calculated as the inverse negative of the slope of the regression line of the straight survivor curve part with a

coefficient of correlation (r_0)≥ 0.98,. Only survival curves with more than four values and more than one log reduction in the straight portion, are used.

Experimental set-up

For the first part of our study nine different organic acids were considered : L glutamic acid (Sigma, >99%), malonic acid (Merck, >99%) , citric acid (Merck, >99.5%), malic acid (Prolabo, >99%), glucono-δ-lactone (Prolabo, >99.5%), lactic acid (Prolabo, ≅ 90%), succinic acid (Merck, >99.5%), adipic acid (Sigma, >99%) and acetic acid (Carlo Erba, >99.9%). The organic acid types were chosen as according to their lowest pKa ranging from 2.15 to 4.75. The pKa constant quantifies the acid strength. When the organic acid has several acid functions, the lowest pKa is corresponding to the major acidic function. The used pKa values at 25°C (Dean, 1985) are given in Table 1.

For each acid, the tryptone salt was adjusted at pH levels ; 4, 4.5, 5, 5.5, 6 and 6.5. The heating media acid molarity (mMolar) for condition of acid type and pH are shown in Table 1. The D values were determinated in duplicate for each combination

A second experimental set-up was carried out for tryptone salt broth with different acid acetic concentration (1M, 0.1M and 0.01M) were adjusted with NaOH 0.1M to pH 4.3, 5, 6 and 7.

Results and discussion

Survival curves at each combination of pH and type of acid were obtained by plotting the decimal logarithm of colony forming unit versus heating time. Figure 1 shows an example of curves obtained from heating spores at 95°Cin acidified tryptone salt solution at different pH.

The D values at 95°Cdetermined for each condition of heat treatment media, tryptone salt broth acidified at different pH with different acids, are shown in Table 2.

Figure 2 illustrates the influence of pH of the heating medium adjusted with citric acid and acetic acid on the $D_{95°C}$ value for Bacillus cereus spores.
For a given acid, D-values were markedly reduced when the pH decreased. The plot of log D-values vs heat media pH yielded a straight line for the range studied.

Mazas et al. 1998 reported the same linear relationship by for three strains of Bacillus cereus, while a quadratic relationship was found by Davey et al. (1978) and Mafart and Leguerinel (1998) for some other spore species and for Bacillus cereus CNRZ 110 heated in citrate-phosphate buffer (Gaillard et al., 1998 a , 1998 b). Data related with Bacillus cereus were then fitted according to the following modified model of Mafart and Leguerinel (1988) at isothermal conditions

$$\log D = \log D^* - \frac{1}{z_{pH}} \left| pH - pH^* \right| \quad \text{(eq 1)}$$

where D* is the D value at pH*, pH* (7) is the optimum pH of heating menstruum corresponding to the maximum heat resistance, z_{pH} is the distance of pH from pH*, which leads to a ten fold reduction of D-value and quantifies the effect of pH on the thermal resistance of bacterial spores. For each acid type the z_{pH} value is determined and shown in Table 3.

For the nine studied organic acids studied, the relation between the calculated z_{pH} values and the lower pKa of acids (table 3 and Fig 4) shows that the z_{pH} values decrease when the acid pKa value increases. The relationship between z_{pH} and pKa values was fitted according to the following by linear regression :

$$z_{pH} = -3.37\ pKa + 21.23 \text{ , r = } 0.965$$

This relationship showed that the acid form (dissociated or undissociated) should modify the thermal spore resistance in addition to the H^+ ion.

The second experimental design aimed to determine the acid form which is responsible. The spores were heated in tryptone salt broth added with different acetic acid concentration adjusted to different pH with NaOH. The percentages of the dissociated and undissociated acetic acid at given pH are calculated by Henderson Hasselbach equation ($100/(1+10^{(pH-pKa)}$) and are presented in Table 4.

Figure 4 shows a clear effect of the acid molarity on D-values at pH 7 (3.4 min., 5.7 min. and 6 min. for 0.01M, 0.1M and 1M respectively). This differences decreased when pH decreased to pH 4.3. At pH7, 99.6% of the acid is dissociated and 29.1% at pH 4.35. These results show a protective effect of the dissociated acid form on the heat resistance of spore. The lowest D value difference observed for heating medium containing 0.1M and 1M acetic acid apparently indicates a saturation of this influence for the highest concentrations. The protective influence of the dissociated acid form in the heat resistance of spore can help explain different observations.

When the acidified medium is adjusted with NaOH, the pH influence on the D value, characterised by z_{pH}, changes with the acid concentration : the z_{pH}

value equals respectively 4.32 and 2.76 for 0.01M and 1M acid concentration. For citric acid a similar influence is observed (data not shown).

The dissociated acid form influence in the heat resistance of spore explains the relation between zpH and pKa. The organic acid with a lower pKa value is more dissociated at lower pH than organic acid with higher pKa, then the pH influence on the D-value decreases when the used acid has a lower pKa ; in this case the z_{pH} value increases.

For acetic acid, the z_{pH} value obtained when the acidified medium is adjusted with NaOH (z_{pH} equal 2.76 to 4.32) is lower than the z_{pH} value calculated when the medium is acidified with added acid (z_{pH} equal 5.8). Indeed, when organic acid is added in the medium, the H^+ ion concentration and the dissociated acid form increase although the ratio dissociated / undissociated acid form decreases. The influence of H^+ ion is preponderant, however, when the pH decreases, the increase of dissociated acid form concentration reduces the pH influence, then the z_{pH} value increases

As far as we know, the specific effect of acid concentration (at fixed pH and with a specific acid type) on the heat resistance of spores, was pointed out in this work for the first time. Then it would be simplistic to consider a z_{pH}

value related to a strain regardless not only of the type of acid, but also, of its concentration in the medium.

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Legends of tables

Table 1.

Acid molarity (mMolar) in heating media (tryptone salt broth) for each condition of pH and acid type and their lowest pKa value.

Table 2

D values (minutes) at 95°C for various pH for each organic acid type.

Table 3

Z_{pH} determined for each acid type. (z_{pH} : distance of pH from pH*, which leads to a ten fold reduction of D-value).

Table 4

Percentage of dissociated and undissociated acetic acid according to pH calculated by Henderson Hasselbach equation.

Legends of figures

Fig. 1. log C.F.U. vs heating time (min.) for different pH heating medium acidified with acetic acid at pH 6.5■, 6□, 5.5●, 5○, 4.5◆.(C.F.U. : forming colony units of Bacillus cereus in tube after dilution).

Fig. 2. log D (min.) vs pH for citric ● and acetic ○ acids.(pH*=7).

Fig. 3. z_{pH} value vs pKa for different organic acids.

Fig. 4. Log D (min.) values as function of pH for different acetic acid molarities 1M ▲, 0.1M □ and 0.01M ◆ (résults of duplicate trials are shown).

Table 1

Acid type		Glutamic acid	Malonic acid	Citric acid	Malic acid	G.D.L	Lactic acid	Succinic acid	Adipic acid	Acetic acid
pH	pKa	2.15	2.82	3.13	3.4	3.8	3.86	4.16	4 4	4.75
6.5		0.07	0.08	0.06	0.06	0.31	0.10	0.10	0.09	0.06
6		0.23	0.15	0.12	0.16	0.60	0.23	0.22	0.19	0.22
5.5		0.39	0.23	0.20	0.25	0.84	0.35	0.35	0.30	0.38
5		0.61	0.38	0.29	0.37	1.10	0.47	0.58	0.48	0.64
4.5		0.96	0.64	0.47	0.58	1.48	0.67	1.07	0.92	1.22
4		2.08	1.03	0.82	1.01	2.13	1.07	2.23	2.11	3.21

Table 2

pH	L Glutamic	Malonic	Citric	Malic	GDL	Lactic	Succinic	Adipic	Acetic
6.5	[1] 1.18	1.68	0.98	0.89	1.32	1.16	1.11	1.45	1.16
	[1] nd [2]	nd	1.07	1.03	1.11	1.01	1.44	1.28	1.26
6	0.95	1.44	0.97	0.99	1.12	0.80	0.86	1.24	0.89
	nd	nd	0.92	0.92	0.87	0.90	nd	1.14	1.08
5.5	0.87	1.32	0.81	0.76	0.94	0.69	0.73	1.02	0.76
	nd	nd	nd	0.78	nd	0.94	0.96	0.89	0.88
5	0.84	1.17	0.74	0.66	0.88	0.59	0.62	0.75	0.63
	nd	nd	0.73	0.72	0.69	0.73	nd	nd	0.70
4.5	0.82	1.19	0.58	0.61	0.58	0.51	0.55	0.66	0.58
	nd	nd	0.70	0.63	0.73	0.57	0.77	0.66	0.51
4	0.71	nd	0.61	0.59	nd	0.46	0.43	0.63	nd
	nd	nd	0.62	0.59	nd	0.53	nd	nd	nd

[1] Duplicate determinations

[2] nd : not determined

Table 3

Acid type	pKa	z_{pH}[1]	r
L glutamic	2.15	13.52	0.947
Malonic	2.82	12.76	0.953
Citric	3.13	10.49	0.964
Malic	3.40	10.58	0.956
GDL	3.80	7.78	0.894
Lactic	3.86	7.31	0.936
Succinic	4.16	6.25	0.899
Adipic	4.40	6.88	0.953
Acétic	4.75	5.79	0.971

[1] z_{pH} is defined as the distance of pH from pH*, which leads to a ten fold reduction of D-value

Table 4

pH solution	% dissociated form (A-)	% undissociated form (AH)
4.35	29.1%	70.9%
5	63.7%	36.3%
6	94.6%	5.4%
7	99.4%	0.6%

Figure 1

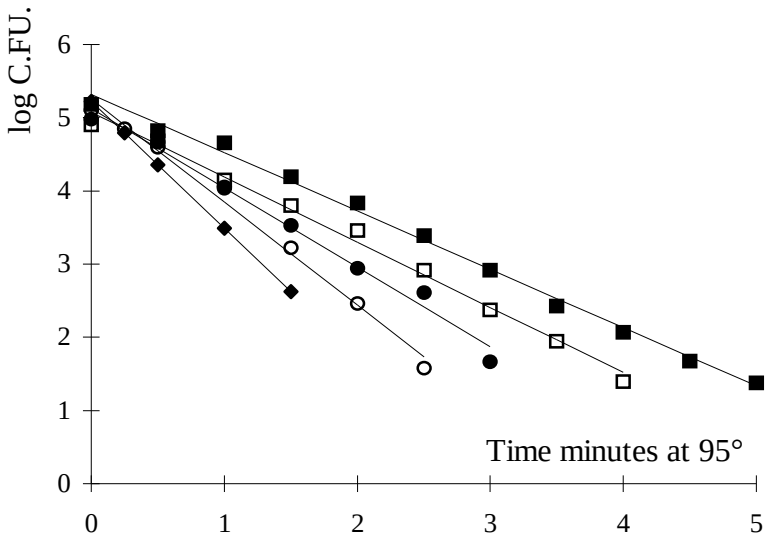

Figure 2

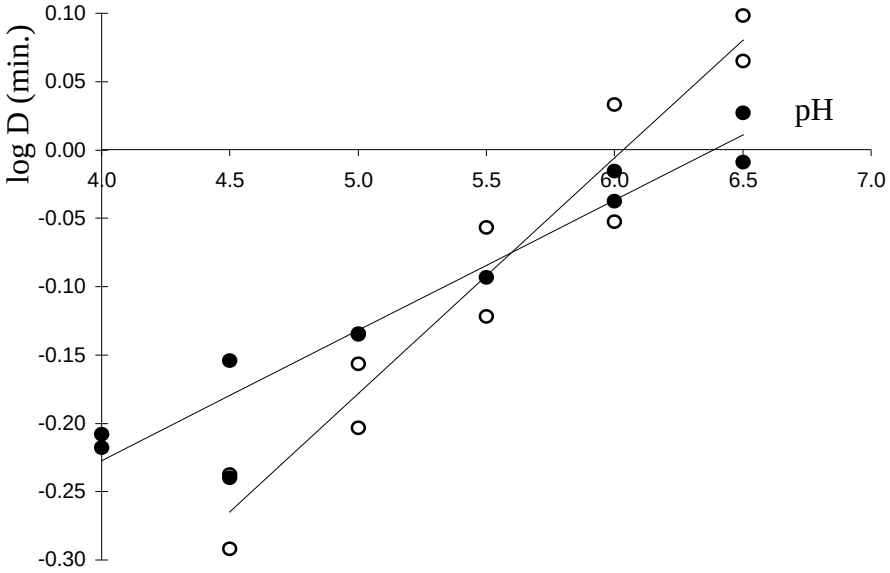
log D (min.)
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
4.0
4.5
5.0
5.5
6.0
6.5
7.0
pH

Figure 3

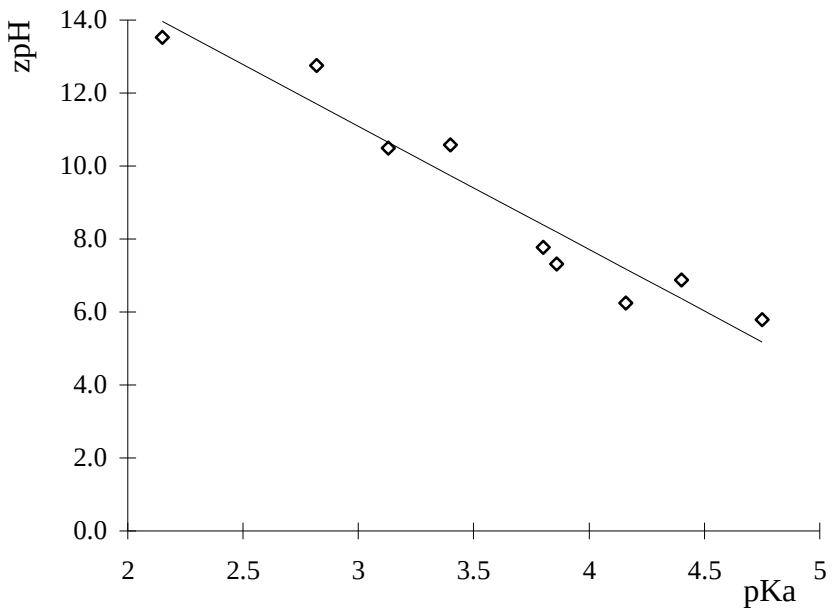

Figure 4