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Modelling the influence of the sporulation temperature upon the bacterial spore heat resistance, application to heating process calculation

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Abstract

Environmental conditions of sporulation influence bacterial heat resistance. For different *Bacillus* species a linear Bigelow type relationship between the logarithm of *D* values determined at constant heating temperature and the temperature of sporulation was observed. The absence of interaction between sporulation and heating temperatures allows the combination of this new relationship with the classical Bigelow model. The parameters z_T and $z_{T_{spo}}$ of this global model were fitted to different sets of data regarding different *Bacillus* species: *B. cereus, B. subtilis, B. licheniformis*, *B. coagulans* and *B. stearothermophilus*.

The origin of raw products or food process conditions before a heat treatment can lead to warm temperature conditions of sporulation and to a dramatic increase of the heat resistance of the generated spores. In this case, provided that the temperature of sporulation can be assessed, this model can be easily implemented to rectify *F* values on account of possible increase of thermal resistance of spores and to ensure the sterilisation efficacy.

Keywords: Heat resistance; Temperature of sporulation; Predictive microbiology

1. Introduction

Pre-heating conditions, particularly the temperature of sporulation, influence the bacterial heat resistance. Palop et al. (1999) reviewed the influence of the temperature of sporulation on the heat resistance of *Bacillus* spores. Bacterial spores appear to be more heat resistant when they sporulate at higher temperature. However, the impact of the temperature of sporulation on the bacterial heat resistance appears more or less important depending on the bacterial species. *Bacillus stearothermophilus* heat resistance is strongly influenced by this factor. For example, an increase of the temperature of sporulation from 42 °C to 65 °C enhanced the *D* value by a factor 1000. *Bacillis subtilis*, *Bacillus coagulans*, *Bacillus cereus* and *Bacillus licheniformis* were less sensitive to this effect and results from literature showed that *D* values increase by a factor 10 for a jump of temperature of sporulation close to 25 °C (Condon et al., 1992; Sala et al., 1995; Raso et al., 1995; Palop et al., 1996; Gonzalez et al. 1999). Data regarding *Clostridium* species are very scarce. Rey et al. (1975) who studied 6 strains of *Clostridium perfringens*, did not detect any influence of the sporulation temperature on bacterial heat resistance. In most cases, a linear relationship appears between log(*D*) values and the sporulation temperature (Figs. 1, 2 and 3).

Generally, the temperature of sporulation is not a controlled environmental factor. However, in raw vegetables, high concentrations of *Bacillus* bacterial spores were observed. These spores can be considered to be formed in ground at ambient temperature close to 10 °C. Under particular conditions, such as in canned food processing, bacterial sporulation can be observed at higher temperature. In this case it would be necessary to evaluate the influence of the temperature of sporulation on bacterial spore heat resistance.

Many factors, other than the heat treatment temperature, influence the bacterial heat resistance such as the "real" effect of the heating medium pH or water activity and the "apparent" effect of the recovery medium pH or water activity. The influences of these environmental factors on *D* values were modelled by Mafart and Leguérinel (1998), Gaillard et al. (1998), Couvert

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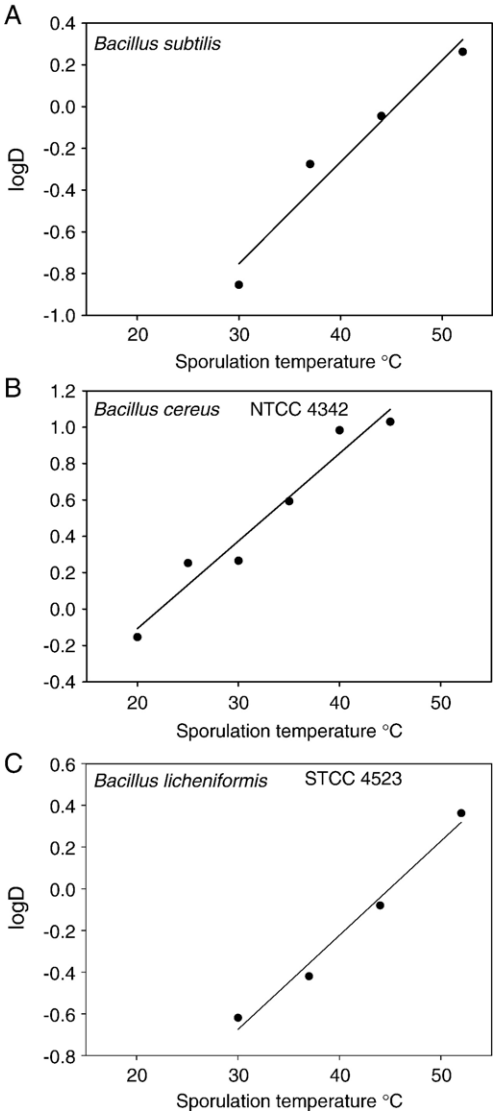

Fig. 1. log*D* (minute) as function of sporulation temperature for different *Bacillus* species, A: *B. subtilis* (Condon et al., 1992), heating temperature 112 °C; B: *B. cereus* ATCC 4342 (Gonzalez et al., 1999), heating temperature 98 °C; C: *B. licheniformis* STCC 4523 (Raso et al., 1995), heating temperature 109 °C.

et al. (1999) through extensions of the classical Bigelow model. In these models, the *z* parameters characterise the impact of these different environmental factors. Moreover, these models associated with the estimated *z* values are easily usable to optimize the heating time in canned food process while conserving quality and safety of foods (Couvert, 2002).

2. Materials and methods

2.1. Microorganism and spore production

B. licheniformis LS32 has been isolated from frozen onion, *B. stearothermophilus* ATCC 12980 was obtained from Institut Pasteur France. Cells were pre-cultivated at 37 °C for 24 h in Brain Heart Infusion (Difco, Detroit USA). The pre-culture was used to inoculate nutrient agar (Biokar Diagnostics, Beauvais France BK021) supplemented with sporulation salt added ($MnSO_4$ 40 mg l^{-1} and $CaCl_2$ 100 mgl^{-1}). Plates were incubated at different temperatures for 5 days ranging from 18 °C to 55 °C for *B. licheniformis* and 50 °C to 60 °C for *B. stearothermophilus*. Spores were then collected by scraping the surface of the agar, suspending in sterile distilled water and washing three times by centrifugation (10,000 *g* for 15 min) (Bioblock Scientific, Illkirch France, model Sigma 3K30). The pellet was resuspended in 5 ml distilled water and 5 ml ethanol. The obtained suspension was kept at 4 °C for 12 h in order to eliminate vegetative non-sporulated bacteria, and washed again three times by centrifugation. The final suspensions, about 10^{10} spores ml^{-1}, were distributed in sterile Eppendorfs microtubes and kept at 4 °C in distilled water.

2.2. Thermal treatment of spore suspension

30 µl of the spore suspension was diluted in 3 ml tryptone salt broth (10 g l^{-1} tryptone (Biokar Diagnostics, Beauvais France) and 10 g l^{-1} sodium chloride) pH 7. Capillary tubes of 200 µl (Hirschman Laborgerate, Eberstadt Germany) were filled with 100 µl of sample and submitted to a thermal treatment in a temperature controlled water glycerol bath, ranged from 95 °C to 121 °C for *B. stearothermophilus* and 95 °C to 105 °C for *B. licheniformis*. After heating, the tubes were cooled in water/ice bath, washed in a solution of soap and rinsed with sterile distilled water. The capillary tubes were broken and their contents poured into a tube containing 9 ml of sterile tryptone salt broth (Biokar Diagnostics, Beauvais France) by rinsing with 1 ml tryptone salt broth.

2.3. Viable spore count

Viable spores (CFU, colony forming unit) were counted by duplicate plating in nutrient agar (Biokar Diagnostic, Beauvais France) and incubated for 48 h at 37 °C for *B. licheniformis* or 55 °C for *B. stearothermophilus* ATCC 12980.

2.4. Data analysis

Experimental data and published data related to different Bacillus species and strains were analysed: *B. cereus* (Gonzalez

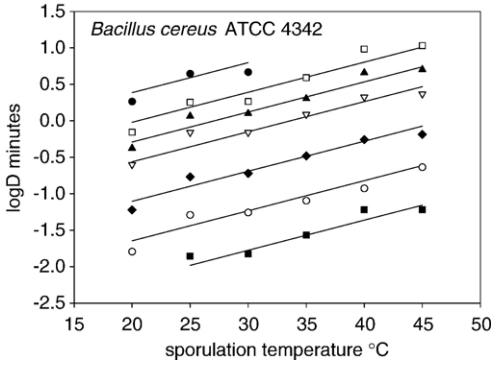

Fig. 2. log*D* values (minutes) for *B. cereus* ATCC 4342 as function of sporulation temperature for different heating temperatures: ● 95 °C, □ 98 °C, ▲ 100 °C, ▽ 102 °C, ◆ 106 °C, ○ 111 °C, ■ 114 °C (Gonzalez et al., 1999).

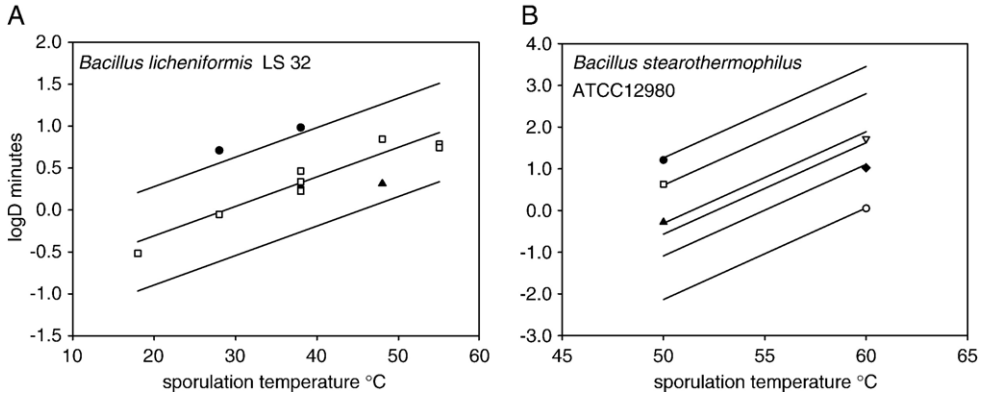

Fig. 3. log$D$ values (minutes) as function of sporulation temperature for different heating temperatures: A: *Bacillus licheniformis* SL32: ● 95 °C, □ 100 °C, ▲ 105 °C, B: *Bacillus stearothermophilus* ATCC 12980: ● 95 °C, □ 100 °C, ▲ 107 °C, ▽ 109 °C, ◆ 113 °C, ○ 121 °C.

et al., 1999), *Bacillus subtilis* (Koury et al., 1987; Condon et al., 1992; Sala et al., 1995; Palop et al. 1996), *B. coagulans* (Palop et al., 1996), *B. licheniformis* (Raso et al., 1995; our experimental data), *B. stearothermophilus* (Koury et al., 1987; Beaman et al., 1986; Palop et al., 1999; our experimental data).

Regarding the effect of the temperature of sporulation on the heat resistance of spores, an approximately linear relationship could be observed between the logarithm of D values and temperature (Figs. 1, 2 and 3).

From these results, we investigated the possibility of implementing a Bigelow type equation, according to the same approach as that followed in our previously cited models.

$$\log D = \log D^* + \left(\frac{T_{spo} - T^*_{spo}}{z_{T_{spo}}}\right) \quad (1)$$

T^*_{spo} is the reference sporulation temperature (37 °C), $z_{T_{spo}}$ is the distance of T_{spo} from T^*_{spo} which leads to a ten fold reduction in decimal reduction time. Lastly, D^* is the D value at T^*_{spo}. Providing that no significant interaction between the temperature of sporulation and the heating temperature will be observed, this model could be associated to the classical Bigelow model according to a multiplicative (or additive in the logarithmic form) combination, as it is frequently performed in the field of predictive microbiology. Such a combination yields the following equation:

$$\log D = \log D^* - \left(\frac{T - T^*}{z_T}\right) + \left(\frac{T_{spo} - T^*_{spo}}{z_{T_{spo}}}\right) \quad (2)$$

this model can be used to optimize heat treatment, so as to ensure can sterility.

The parameter values and their associated confidence interval were estimated using a non-linear module ("nlinfit" and "nlparci" Matlab 6.1, Optimization Toolbox, Math-works). The "nlparci" function used to evaluate confidence interval at 95% is based on the asymptotic normal distribution for the parameter estimates (Bates and Watts., 1988).

ANOVA analysis was performed using Minitab software. The weight of each factor and error was determined by the ratio: sum of square of factor by total sum of square.

3. Results and discussion

Because "Eq. (2)" neglects possible interactions between sporulation and heating temperatures, we checked whether $z_{T_{spo}}$ was independent of the heating temperature and whether z_T was independent of the sporulation temperature. Concerning *B. cereus* Gonzalez et al. (1999) and Condon et al. (1992) showed D values from a factorial design combining heating and sporulation temperatures related to different bacterial strains. Anova Analysis (Table 1) shows that the weight of errors including interactions and experimental errors represents less than 0.25%, 0.39% and 0.47% for *B. cereus* strains ATCC 9818, ATCC 4342, ATCC 7004 respectively for Gonzalez et al. (1999) data and less than

Table 1
ANOVA analysis and calculated weight of factors determined from log$D$ values for *Bacillus cereus* (Condon et al. 1992), *B. cereus* ATCC 4342, *B. cereus* ATCC 9818 and *B. cereus* ATCC 7004 (Gonzalez et al., 1999)

Factors	Degrees of freedom	Sum of square	F test	p values	Weight of factor (%)
Bacillus cereus (Condon et al. 1992)					
Heating temperature	2	1.473	39.62	0.000	49.84
Sporulation temperature	3	1.372	24.58	0.001	46.39
Error	6	0.112			3.77
Total	11	2.957			100.00
B. cereus ATCC 9818 (Gonzalez et al. 1999)					
Heating temperature	4	10.704	1348.68	0.000	85.55
Sporulation temperature	4	1.777	223.87	0.000	14.20
Error	16	0.032			0.25
Total	24	12.513			100.00
B. cereus ATCC 4342 (Gonzalez et al. 1999)					
Heating temperature	5	17.820	912.93	0.000	89.32
Sporulation temperature	4	2.053	131.46	0.000	10.29
Error	20	0.078			0.39
Total	29	19.951			100.00
B. cereus ATCC 7004 (Gonzalez et al. 1999)					
Heating temperature	3	5.859	767.73	0.000	72.17
Sporulation temperature	5	2.221	174.61	0.000	27.36
Error	15	0.038			0.47
Total	23	8.119			100.00

Table 2A
log$D$*, and $z_{T_{spo}}$ parameters and their associated confidence interval (95%) fitted on data with "Eq. (1)"

Species	Reference or origin	log$D$*	CI 95%	$z_{T_{spo}}$	CI 95%	r	T_{spo}°C range	Heating condition T*	Heating T°C range	
B. subtilis	NTCC 3610	1.90	0.07	78.61	33.87	0.936	20–45	T*=90 °C	90	Koury et al. (1987)
B. stearothermophilus	NRRLB 4419	1.61	0.06	131.34	46.93	0.996	45–70	T*=100 °C	100	Koury et al. (1987)
B. stearothermophilus	7953	1.33	4.99	29.54	167.1	0.914	45–75	T*=100 °C	100	Beaman et al. (1986)
B stearothermophilus	No reference	−2.66	2.73	6.39	7.06	0.94	40–62	T*=114 °C	114	Palop et al. (1999)

D* corresponds to fitted D estimated at heating temperature T* and sporulation temperature 37 °C.
$z_{T_{spo}}$ is the distance of T_{spo} from T_{spo}* which leads to a ten fold reduction in decimal reduction time.

3.7% for *B. cereus* strain for Condon et al. data. From these results, we deduced that the weight of interaction could be neglected in the model.

The model parameters from "Eqs. (1) and (2)" were fitted from published data and our own experimental data related to different *Bacillus* species and strains. Parameter $z_{T_{spo}}$ values were evaluated using "Eq. (1)" (Table 2A) when D values were given at a single heating temperature. Parameters (z_T and $z_{T_{spo}}$) of "Eq. (2)" were estimated from different sets of data (Table 2B). Classical z_T values are within the same order of magnitude and correspond to values currently given for *Bacillus* spores. Parameter $z_{T_{spo}}$ values for the different strains of *B. cereus*, *B. subtilis* and *B. licheniformis* species are close to 25 °C. Parameter $z_{T_{spo}}$ values determined for the species *B. coagulans* and *B. stearothermophilus* show that these species seem highly sensitive to the sporulation temperature. For these species the lower parameter $z_{T_{spo}}$ values estimates were 11.6 °C and 4.55 °C respectively. However, parameter $z_{T_{spo}}$ values present some differences according to authors: $z_{T_{spo}}$ determined from Koury et al. (1987) data presents high values whatever the studied species, which shows almost an absence of the sporulation temperature effect. These differences can be explained by D values determined by Koury et al. (1987) with less than two decimal reduction of population.

3.1. Discussion: the influence of sporulation temperature on the resulting process calculation

The model "Eq. (2)" can be applied to take the sporulation temperature into account in the determination of aimed F values for getting a target number of decimal reduction. It can be considered that bacterial spores in raw vegetables were formed at ground temperature close to 20 °C in temperate countries. During food process, waiting time at warm temperature in the order of 55 °C can be observed in sauce, for example. Such an increase of the temperature of sporulation highly increases the thermal bacterial resistance. Regarding our own data of *B. licheniformis* ($z_{T_{spo}}$: 28.5 °C) the ratio $D_{55\ °C_{spo}}/D_{18\ °C_{spo}}$ equals 19.5. Increases in sporulation temperature is also observed during compost process (Palmisano et al., 1996; Dees et al., 2001), regarding to *B. stearothermophilus* ($z_{T_{spo}}$: 6.4 °C (Palop et al., 1999) the ratio $D_{65\ °C_{spo}}/D_{40\ °C_{spo}}$ equals 8170.

Table 2B
log$D$*, z_T and $z_{T_{spo}}$ parameters and their associated confidence interval (95%) fitted on data with "Eq. (2)"

Species	Reference or origin	log$D$* T*121.1 °C T*$_{spo}$37 °C	CI 95%	z_T	CI 95%	$z_{T_{spo}}$	CI 95%	r	T_{spo}°C range	Heating condition	Heating T°C range	
B. cereus	ATCC 9818	−1.79	0.09	8.15	0.36	24.77	2.24	0.994	20–45		98–115	Gonzalez et al. (1999)
B. cereus	ATCC 4342	−2.43	0.1	7.42	0.31	23.97	2.3	0.993	20–45		92–106	Gonzalez et al. (1999)
B. cereus	ATCC 7004	−3.39	0.55	8.07	1.59	66.398	59.66	0.851	20–45		95–114	Gonzalez et al. (1999)
B. subtilis	Asparagus	−1.3	0.14	9.26	0.96	28.37	7.88	0.975	30–52		101–125	Condon et al. (1992)
B. subtilis	Asparagus	−1.69	0.04	8.66	0.25	25.95	2.16	0.997	33–52	pH 7	102–124	Sala et al. (1995)
B. subtilis	Asparagus	−1.69	0.12	9.65	0.82	33.18	7.54	0.994	32–52	pH 6	100–128	Sala et al. (1995)
B. subtilis	Asparagus	−1.76	0.1	10.2	0.78	28.97	5.41	0.995	32–52	pH 5	102–131	Sala et al. (1995)
B. subtilis	Asparagus	−1.93	0.1	10.8	0.7	21.3	3.15	0.991	32–52	pH 4	96–135	Sala et al. (1995)
B. subtilis	NTCC 4524	−2.71	0.28	9.61	1.41	14.08	3.15	0.993	35–52	pH 4 HCl	90–120	Palop et al. (1996)
B. subtilis	NTCC 4525	−2.59	0.63	10.7	3.19	20.69	13.37	0.988	35–52	pH 4 acetic acid	90–120	Palop et al. (1996)
B. subtilis	NTCC 4526	−2.45	0.99	10.2	3.93	16.42	12.54	0.994	35–52	pH 4 citric acid	90–120	Palop et al. (1996)
B. subtilis	NTCC 4527	−2.78	0.24	9.45	1.06	18.34	4.53	0.994	35–52	pH 4 lactic acid	90–120	Palop et al. (1996)
B. coagulans	NTCC 5422	−2.57	0.13	11.1	0.83	13.04	1.35	0.997	35–52	pH 4 HCl	90–123	Palop et al. (1996)
B. coagulans	NTCC 5423	−2.66	0.15	11.6	1.03	17.77	3.16	0.996	35–52	pH 4 lactic acid	93–129	Palop et al. (1996)
B. coagulans	NTCC 5424	−2.53	0.16	11.8	1.08	11.6	0.99	0.999	35–52	pH 4 citric acid	96–120	Palop et al. (1996)
B. licheniformis	STCC 4523	−1.79	0.18	8.33	0.83	22.8	3.98	0.987	30–52		99–118	Raso et al. (1995)
B. licheniformis	Onion	−2.18	0.09	8.52	3.01	28.34	7.73	0.943	18–55		95–105	
B. stearothermophilus	ATCC12980	−5.02	0.86	7.65	1.19	4.55	0.71	0.996	50–60	pH 6.5	95–121	

D* corresponds to fitted D value estimated at heating temperature 121.1 °C and sporulation temperature 37 °C.

The $D_{121.1\ °C}$ calculated from "Eq. (2)" is the input for determining the target F value as a function of the aimed decimal reduction ratio "n" (Eq. (3)).

$$F_{121.1\ °C} = nD_{121.1\ °C} \quad (3)$$

To keep the same microbial safety, the decimal ratio "n" has to be constant. When the sporulation temperature increases from T^* to T, $F_{121.1\ °C}$ value increases as a function of the ratio $D_{T_{spo}}/D_{T^*_{spo}}$.

For example, let us assume an $F_{121.1\ °C}$ value which would be fixed arbitrarily at 10 min for a target bacteria *B. licheniformis* sporulated at 18 °C. If the temperature of *s*porulation increases from 18 °C to 55 °C, the same safety level would require a new $F_{121.1\ °C}$ value of 195 min. Such a value is too high to be applied because the canned food would become overcooked. If the target bacteria considered is *B. stearothermophilus,* the new F value becomes 81,700 min (56.7 days). These examples show the impact of the temperature of sporulation on the F values, and consequently on the risk of non-sterility. On the other hand, the fact that the calculated F values, applied in industry, always input D values determined from spores sporulated at laboratory condition (37 °C or 55 °C according to species) ensure a certain safety if the spores were sporulated at lower temperature. However, in compost bacteria grow and sporulate at high temperature (60–80 °C) and this yields high heat resistance bacterial spores. Bacterial spores present in compost used in agriculture are collected with vegetables during harvest, so that the use of these vegetables, particularly mushrooms, in the can industry may lead to the risk of non-sterility when classical F values are applied.

4. Conclusion

Bacterial sporulation temperature highly influences bacterial heat resistance. Origin of raw product and food process conditions before heat treatment, with waiting time at warm temperature, can lead to non-sterility in can food. Generally, the canned food industry still considers that sterility can always be solved by an increase in sterilisation value, which can be wrong in these particular conditions. It is clear that canned food sterility requires the knowledge of both the origin of initial raw products and their microbiological quality, together with the control of food process, temperature, waiting and storage time.

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