Short communication

Comparison of the thermal inactivation of *Bacillus subtilis* spores in foods using the modified Weibull and Bigelow equations

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Abstract

The association of a modified Weibull model and Bigelow model was applied to the thermal inactivation of *Bacillus subtilis* spores heated in phosphate buffer, milk, *kayu* (a Japanese style rice porridge) and soy sauce as well. The inactivation kinetics presented a light downward concave profile, the acidic pH increased the efficiency of the heat treatment but on the opposite, lesser the water activity, weaker was the efficiency. The heat treatment kinetics observed in milk, soy sauce and *kayu* were greatly different from each other, while no large difference between sterilized whole milk, UHT whole milk, sterilized skim milk and UHT skim milk, were observed. The model established in buffer system allowed heat treatment in milk products to be simulated although it could not be employed to describe the inactivation of *B. subtilis* spores in soy sauce and *kayu*. For these two latter products, the food itself had to be introduced in the model as a parameter. Finally, this approach combining primary model (to simulate inactivation kinetics) and secondary model (to introduce temperature, pH, a_w and food matrix effect) seemed available for food application, nevertheless validations of results such as challenge-tests, must be performed before it is put to routine use.

1. Introduction

Bacillus subtilis is a common cause of food spoilage and their heat resistant spores, often pose a challenge to the thermal efficacy of heat processes resulting in reduced shelf life of many processed foods. Higher heat treatments alter the organoleptic properties of food and hence the use of a combination of antibacterial agents has been suggested. These factors may not be able to inhibit the microbial growth individually, but can operate in an interactive way and in combination may restrict the growth of micro-organisms in foods. This is the basis of "hurdle technology" (Leistner, 2002) and in the last decade there has been an explosion of research interest in realizing the potential benefits of this technology. However a thorough understanding of the physiological responses of micro-organisms to stresses imposed during food preservation is essential if novel combination systems based on milder food processing procedures are to be developed effectively (Roller, 1999) and hence heat treatments continue to be still the most preferred method of ensuring safety and quality of foods. Consequently, the field of thermobacteriology has seen a variety of research on the inactivation of cells and spores of bacteria.

Bigelow was the first to apply bacteriological and physical data to the thermal calculation of processes for canned foods (Bigelow et al., 1920) and to later demonstrate the logarithmic nature of the death time curves (Bigelow, 1921). This concept has served well the food industry in general and the canning industry in particular since the days when it was first demonstrated

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for spore-forming bacteria. Recently, the original equation has been modified to model the combined effects of up to three variables on the inactivation of spores (Gaillard et al., 1998; Mafart and Leguerinel, 1998; Coroller et al., 2001). The Weibull equation has been also adapted to nonlinear survival curves and applied to several micro-organisms (Mafart et al., 2002; Van Boekel, 2002; Collado et al., 2003; Peleg, 2003).

In the present study a two-stage modeling approach is used to simulate the thermal inactivation of B. subtilis spores heated in phosphate buffer. The logarithm of the bacterial count is fitted by the Weibull equation, including three parameters $\log N_o$, m and δ. Thus, the logarithm of δ (equivalent to the D value, Mafart et al., 2002) is modeled using a secondary model based on the Bigelow equation. Based on the model results, a comparison between thermal inactivation of B. subtilis spores in buffer system, milk and certain Japanese foods is made. The foods selected were soy sauce which is a high salt containing popular condiment used in oriental cuisine and kayu which is cooked, boiled rice, without sodium chloride. The Japanese porridge kayu bears an analogy to the western cooked or boiled rice which has been the vehicle of many food poisoning incidents worldwide mainly because of the survival of spores during the cooking procedure.

2. Materials and methods

2.1. Spore production

Bacillus subtilis 168 stock culture was maintained on nutrient agar slants (Becton Dickinson, Sparks, Maryland, USA). An overnight culture isolated from nutrient agar slant was transferred to 100 ml of trypticase soy broth (Becton Dickinson). The flask was incubated at 37°C for 16 h in a rotary shaker. These cells at the stationary phase were inoculated into another flask containing the sporulation medium of double strength nutrient broth supplemented with 0.01% $MnCl_2 \cdot H_2O$ (Wako Pure Chemicals Industries Ltd., Osaka, Japan). The flask was vigorously shaken during incubation at 37°C for 72 h and the sporulation was monitored microscopically under a phase contrast microscope at 12 h intervals. Spores were harvested (when 90% of the cells had sporulated) by centrifugation at is 7000g for 20 min at 4°C. The resultant spores were treated with lysozyme (1 mg ml^{-1}, Sigma Chemical Co. St. Louis, Missouri, USA) and washed with 50 mmol l^{-1} potassium phosphate buffer at pH 7.0 as described frozen and stored at −84°C until use. The lysozyme treatment was for a short duration of time and was meant to facilitate release of spores by the breakage of cell wall. The residual lysozyme was subsequently removed by successive washings with sterile distilled water. Later the

spores were seen under a phase contrast microscope and no germination was visible. Ultra freezing of spores in distilled water at −84°C is a routine technique employed in the Department of Biotechnology of the Faculty of Engineering at Osaka (Japan). A part of the thawed spore suspension was examined under phase bright microscope before using for the experiments to ensure the phase bright nature of mature spores. However, as long duration at −84°C was seen to decrease the spore viability, the spore suspensions were monthly prepared.

2.2. Heat treatment

One hundred microlitres of the thawed spore suspension was added to test tubes ($12 \times 90 \, mm^2$) and was mixed with 900 μl of either, 50 mmol l^{-1} phosphate buffer, various milk samples, soy sauce or diluted kayu to obtain an initial spore density of 10^8 spores ml^{-1}. Sonication was carried out to prevent clumping of the spores. The tubes were then covered with aluminum caps and heated indirectly on an aluminum block heater (Dry Thermo Unit DTU-1C, Taitec Corporation, Koshigaya, Japan). The spore suspensions were held at different temperatures (89°C, 92°C, 95°C and 98°C) for varying periods of time, to obtain finally at least 2–4 log$_{10}$ reduction from the initial count. Samples were removed from the block heater at appropriate intervals, plunged into an ice-water bath, and plated out within 30 min. By preliminary experiments, the come up time was found to be 2 min and hence the zero time was considered after the initial 2 min come up in all our experimental set.

2.3. Viability assay

A 0.1 ml of the treated suspension was serially diluted with sterile deionized water and plated out in duplicate on nutrient agar to enumerate survivors. A 0.1 ml aliquot of undiluted suspension was also plated, where necessary. The plates were incubated overnight at 37°C and the colony counts were expressed as survivors (log$_{10}$ cfu ml^{-1}).

2.4. Experimental design

A $4 \times 3 \times 6$ full factorial design of temperature of heat treatment (89°C, 92°C, 95°C and 98°C), pH of buffer (6.0, 6.5 and 7.0) and concentration of sodium chloride (0%, 1%, 2%, 3%, 4% and 5% w/v) was carried out. In buffer system, the equivalence of sodium chloride and water activity was 0% as 1, 1% as 0.987, 2% as 0.982, 3% as 0.977, 4% as 0.971 and 5% as 0.965. Giving the large number of experiments in buffer system, only certain combinations of factors were randomly selected and duplicated. In this latter case, the mean D value (or more exactly δ value, see below) was determined.

2.5. Trials on milk, soy sauce and kayu

All the foods used in the study were obtained from a local supermarket. Sterilization of milk was done in the laboratory at 120°C, 15 psi pressure for 15 mn while UHT treatment was done by the processors at 120°C for 2 s. The whole milk (3.6% fat) and skim milk (0.6% fat) had a solids not fat (SNF) content of 8.3%. The pH of the whole sterilized and UHT milk were 6.7 and 6.5, respectively, while the corresponding values for skim milk were 6.7 and 6.6, respectively. The water activity was considered as 1, based on sodium chloride concentration.

The soy sauce was a brewed variety and had a pH of 4.8 and sodium chloride concentration of 16.3%. The water activity was considered as 0.88 based on sodium chloride concentration.

Kayu, Japanese porridge used in this study was a plain variety with 0% sodium chloride and a pH of 6.8. Forty mililitres of *kayu* was diluted with 60 ml of sterile distilled water, mixed well and used for the experiments. The water activity was considered as 1 based on sodium chloride concentration.

All experiments carried out in milk products, soy sauce and *Kayu* were replicated twice. The heating conditions for the selected foods were similar to those adapted for the suspensions in buffer (see Section 2.2).

2.6. Modeling of thermal inactivation kinetics

The model suggested by Mafart et al. (2002), Collado et al. (2003) or Peleg (2003) based on Weibull frequency distribution (Eq. (1)) was used throughout the study, both to describe thermal inactivation observed in buffer system and in foods.

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p, \qquad (1)$$

where, $\log N$ is the logarithm of surviving *B. subtilis* spores after a heat treatment, t the time (min) and $\log N_0$ the logarithm of the number of spores at time 0. Since $\log N$ was the value measured experimentally, it was considered as the statistical response, and $\log N_0$, δ and p, as estimated parameters. Moreover, if $\log N_0$ was fluctuated with the experimental conditions, δ was assumed to be strictly dependent on bacterial cells and matrix environment (buffer, milk, *kayu*, soy sauce). On the other hand, the parameter p represented the shape of the curves, with $p = 1$ corresponding to a straight line, $p > 1$ for downward concave survival curves, and, $p < 1$ for upward concave curves. To build a predictive model, p was estimated as a non-environmental-factor-dependent parameter throughout the study.

2.7. Modeling of temperature, pH and water activity influence on thermal inactivation

The modified Bigelow equation proposed by Gaillard et al. (1998), Mafart (2000) or Mafart et al. (2001) was used throughout the study, to describe the temperature, pH and water activity influence on δ-value, in both buffer system and foods (Eq. (2)).

$$\begin{cases} \log \delta = \log \delta_{\text{matrix}}^* - \dfrac{T - T^*}{z_T} - \left(\dfrac{\text{pH} - \text{pH}*}{z_{\text{pH}}}\right)^2 - \dfrac{a_\text{w} - 1}{z_{a_\text{w}}}, \\ T^* = 121.1°C, \text{pH}^* = 7, \end{cases}$$

$$(2)$$

where T^* was the temperature of reference, fixed traditionally to 121.1°C (Mafart et al., 2002; Peleg, 2003). Likewise, the pH of reference, pH*, was chosen to 7. The term Z_T could be assimilated the conventional thermal Z value while Z_{a_w} was the distance of a_w from 1 which led to a tenfold reduction of the decimal reduction time (still called "rate constant" since the term δ replaced the traditional D-value in Eq. (1), Mafart et al., 2002). Concerning pH, Z_{pH} was the distance of pH from pH* which led to a tenfold reduction of the square root of the decimal reduction time. It was assumed that Z_T, Z_{pH} and Z_{a_w} depend on bacterial species but not on matrix, i.e. the terms Z_T, Z_{pH} and Z_{a_w} in buffer medium were similar to those in milk products, soy sauce or *kayu*. Consequently, the terms Z_T, Z_{pH} and Z_{a_w} were obtained by fitting the Eqs. (1) and (2) on the set of data carried out in buffer system ($R^2 = 0.90$, $\text{Sd}_{\text{error}} = 0.029$).

On the other hand, the term δ_{matrix}^*, corresponding to the δ-value at $T = T^*$ and pH = pH* and $a_\text{w} = 1$, was suggested as matrix-dependent parameter, which meant its value was different when *B. subtilis* spores were heat treated in buffer medium, milk products, soy sauce or in *kayu*. Therefore, in Eq. (2), the parameters, δ_{buffer}^*, $\delta_{\text{milkproducts}}^*$, $\delta_{\text{soysauce}}^*$, δ_{kayu}^* were estimated by modeling the data obtained on buffer, milk products, soy sauce and *kayu*, respectively.

The non-linear regression was computed with S-plus (AT&T Bell Laboratories, Murray Hill, New Jersey, USA).

3. Results and discussion

3.1. Comparison of temperature, pH and a_w effect in buffer medium

The 72 kinetic curves obtained in buffer system were adjusted with the modified Weibull equation including a shoulder (Eq. (1)). By this way, 3×72 parameters (72 N_0, 72 δ and 72 p) were generated. Concerning the p parameter, no great difference on its

value among the 72 experimental conditions was found, with a 25th percentile of 1.09 and a 0.75th percentile of 1.56. Therefore, in order to use the results in the next step as a predictive model, the term p was considered as a constant and fixed to 1.325 (50th percentile or median value). Modified Weibull equation with shoulder described relatively accurately the data, whatever the temperature, pH or a_w values. The median value, $p = 1.325$, indicated that the inactivation kinetics present a light downward concave profile, even if the value is not far from 1 corresponding to a straight line.

Secondly, the 72 δ-values were analysed by including the effect of temperature, pH and water activity (Eq. (2)). By combination of Eqs. (1) and (2), the decline curve could be predicted whatever the temperature, pH and a_w, in the buffer medium within the experimental domain limits. Results are illustrated in Figs. 1 and 2.

Values of estimated parameters Z_T, Z_{pH} and Z_{a_w} are given in Table 1. The temperature effect is illustrated in each plot of Figs. 1 and 2, the pH effect is illustrated in making a comparison between Figs. 1a and 2a (or Figs. 1b and 2b) and a_w effect is illustrated with a comparison of Figs. 1a and b (or Figs. 2a and b). The effect of pH on heat treatment has been already described on spores of *Bacillus cereus* (Gaillard et al., 1998; Leguerinel and Mafart, 2001; Collado et al., 2003), *Clostridium sporogenes* (Ocio et al., 1994) and *Bacillus stearothermophilus* (Fernandez et al., 1994). The comparison of Z_T, Z_{pH} and Z_{a_w} values led to conclude that the temperature effect in buffer system was the preponderant one, which meant that spores of *B. subtilis* were found to be very sensitive to heat treatment. In fact, to decrease two times the δ value (for instance to obtain 5 min instead of 10 min), an increase of the temperature up to 2.4°C was sufficient.
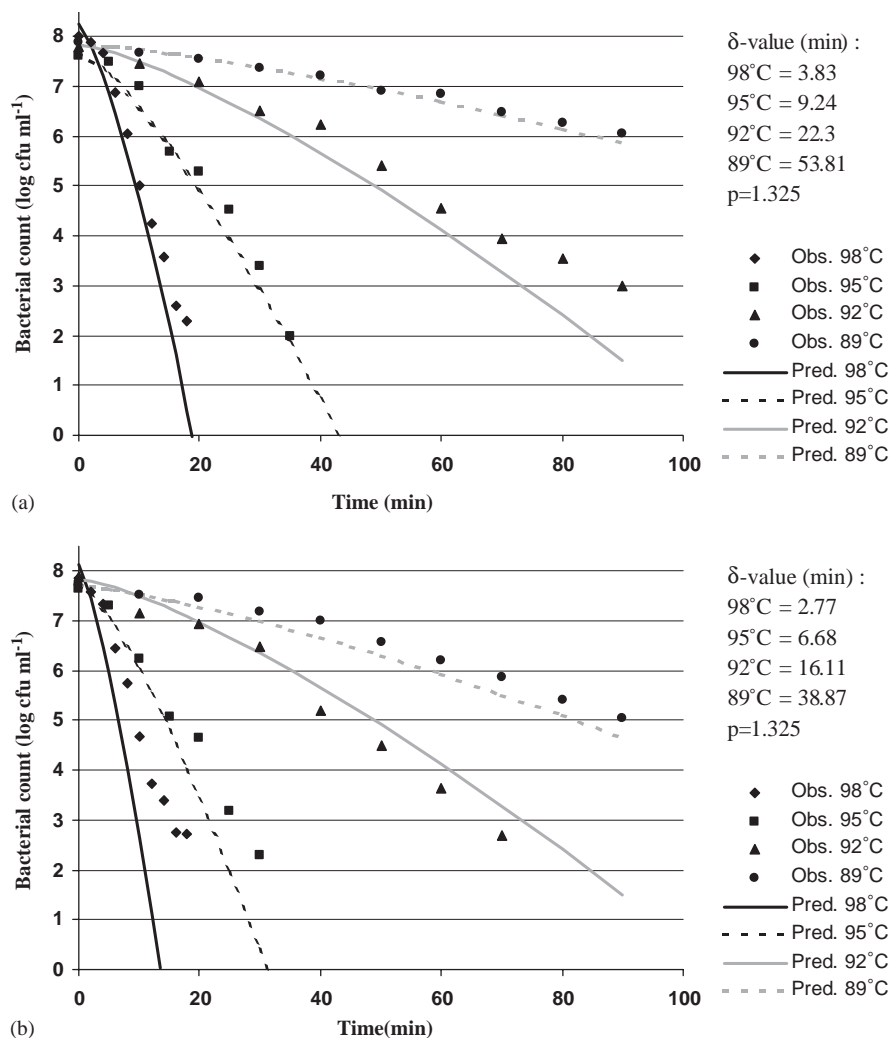

Fig. 1. Survival curves for *Bacillus subtilis* spores in buffer system at pH 6.0, in the presence of 5% NaCl (1a) or in presence of 3% NaCl (1b). Experimental data (symbols) and predicted results by combining Eqs. (1) and (2) (lines).
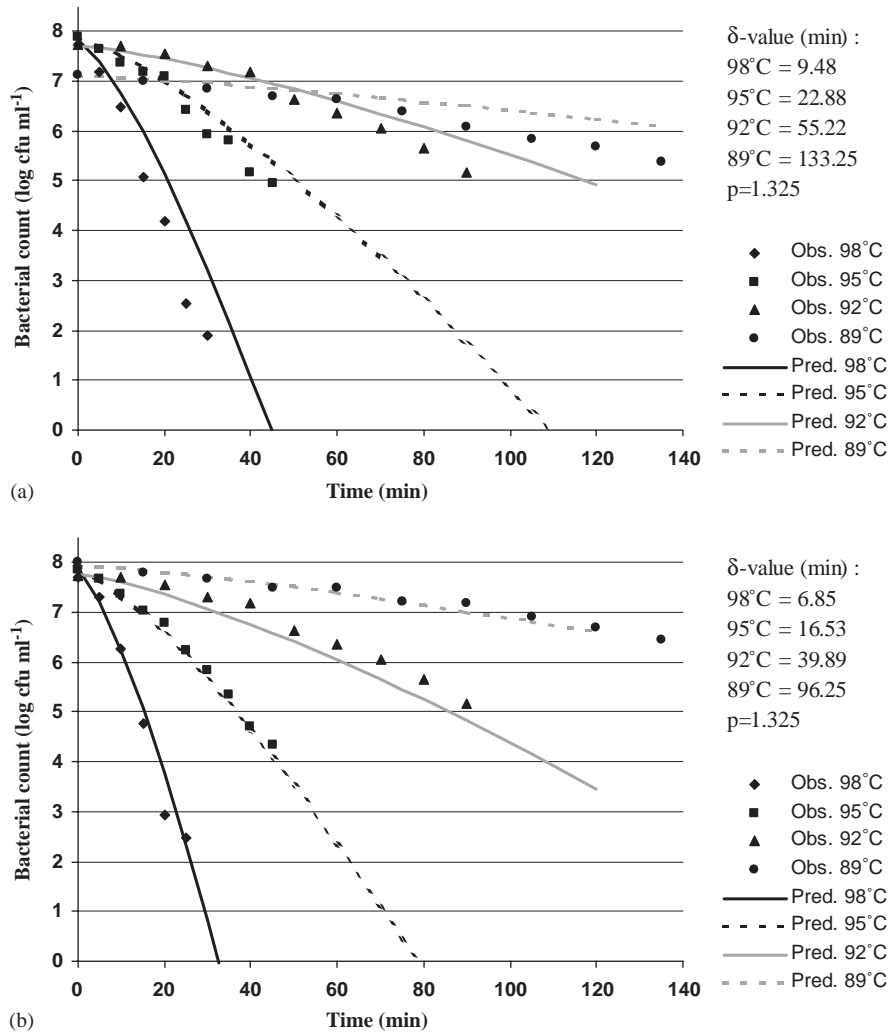
δ-value (min) :
98°C = 9.48
95°C = 22.88
92°C = 55.22
89°C = 133.25
p=1.325

◆ Obs. 98°C
■ Obs. 95°C
▲ Obs. 92°C
● Obs. 89°C
— Pred. 98°C
-- Pred. 95°C
— Pred. 92°C
-- Pred. 89°C

(a)

δ-value (min) :
98°C = 6.85
95°C = 16.53
92°C = 39.89
89°C = 96.25
p=1.325

◆ Obs. 98°C
■ Obs. 95°C
▲ Obs. 92°C
● Obs. 89°C
— Pred. 98°C
-- Pred. 95°C
— Pred. 92°C
-- Pred. 89°C

(b)

Fig. 2. Survival curves for *Bacillus subtilis* spores in buffer system at pH 6.5, in the presence of 5% (2a) or 3% NaCl (2b). Experimental data (symbols) and predicted results by combining Eqs. (1) and (2) (lines).

Table 1
Model parameter estimates, corresponding to heat treatment inactivation kinetics (Eq. (1)) and environmental factor and matrix effects on δ-value (Eq. (2))

No matrix-dependent parameters		Matrix-dependent parameters	
p	1.325		
T^*	121.1°C		
PH*	7	δ^*_{buffer}	0.0056 min
Z_T	7.8°C	$\delta^*_{\text{milkproducts}}$	0.0054 min
Z_{pH}	1.38	$\delta^*_{\text{soysauce}}$	0.0311 min
Z_{a_w}	0.085	δ^*_{kayu}	0.0029 min

3.2. Comparison of thermal inactivation of Bacillus subtilis spores in buffer, milk products, soy sauce and kayu

A comparison of food products in terms of their influence on thermal inactivation of *B. subtilis* spores,

could be performed based on modified Bigelow equation (Eq. (2)), and particularly on matrix-dependent parameter, δ^*_{matrix}. In Table 1, the terms δ^*_{buffer}, $\delta^*_{\text{milkproducts}}$, $\delta^*_{\text{soysauce}}$, δ^*_{okayu}, obtained by fitting the Eq. (2) for buffer, milk, soy sauce and *kayu*, respectively, using Z_T, Z_{pH} and Z_{a_w} derived from buffer, are indicated.

If a food matrix has no effect, the Eq. (2) could be employed with δ^*_{buffer} as δ^*_{matrix} parameter. In other words, when δ^*_{matrix} is close to δ^*_{buffer}, the thermal inactivation can be explained by the environmental factors, temperature, pH and a_w, without any particular food influence. Consequently, a model built with data obtained in buffer system can be then employed to predict the thermal inactivation in a such a food. This situation was observed with milk products. In fact, in this case, only one δ^*_{matrix} was estimated for all the set of data obtained with sterilized whole milk, UHT whole milk, sterilized skim milk and UHT skim milk. Moreover, the $\delta^*_{\text{milkproducts}}$ value was found to be very close to
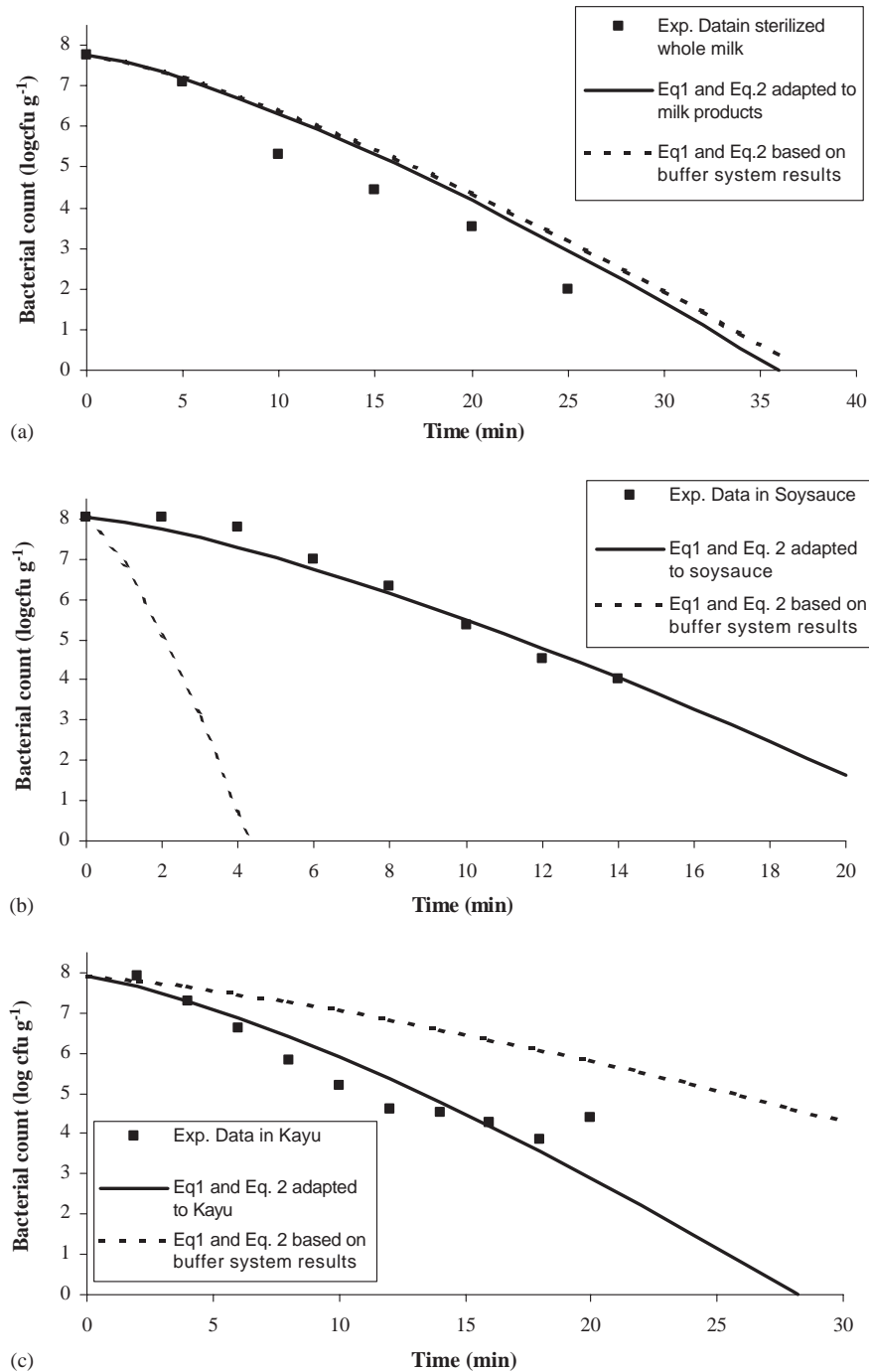
Fig. 3. Illustration of modeling of survival curves, by using matrix-dependent parameter adjusted on buffer system data (dotted line) or in milk products (3a), in soy sauce (3b) or in *kayu* (3c). Experimental data (symbols) were obtained at 95°C.

the δ^*_{buffer} value (0.0054 min (0.32 s) and 0.0056 min (0.33 s) for milk products and buffer, respectively). The consequences in term of thermal inactivation kinetics are shown in Fig. 3a. On the opposite, the soy sauce and *kayu* products were fitted with a δ^*_{matrix} parameter significantly different from the δ^*_{buffer} value (Table 1), which means that the thermal inactivation kinetics observed in soy sauce and *kayu* could not be predicted by using a model established in laboratory medium

(Figs. 3b and c). The heat treatment in soy sauce is less efficient than in buffer system at the same levels of temperature, pH and a_w (for instance, at 89°C, pH = 4.8, $a_w = 0.88$, $\delta = 29$ mn in soy sauce instead of 5 min in laboratory medium). On the contrary, the heat treatment in *kayu* is more efficient than predicted with buffer system results, with for instance, at 89°C, pH = 6.8, $a_w = 1$, $\delta = 34$ min in *kayu* instead of 66 min in laboratory medium. Fernandez et al. (1994) has already

described this food effect in literature, with a study on heat resistance of *Bacillus stearothermophilus* spores in alginate-mushroom puree mixture.

4. Conclusions

The association of a modified Weibull and Bigelow equations enabled to build a model for the thermal inactivation, taking the pH and water activity into account. The inactivation kinetics presented a light downward concave profile, the acidic pH increased the efficiency of the heat treatment but on the opposite, lesser the water activity value, weaker was the efficiency.

The heat treatment kinetics observed in milk, soy sauce and *kayu* were greatly different from each other, while no large difference between sterilized whole milk, UHT whole milk, sterilized skim milk and UHT skim milk, were observed. The model established in buffer system allowed heat treatment in milk products to be simulated while it could not be employed directly to describe the inactivation of *B. subtilis* spores in soy sauce and *kayu*. For these two latter products, the food itself had to be introduced in the model as a parameter. The advantage of the modified Bigelow equation is to allow such a parameterization, through the δ^*_{matrix} value. Finally, if this approach combining primary model (to simulate inactivation kinetics) and secondary model (to introduce temperature, pH, a_w and food matrix effect) seems available for some foods (such as milk products), it should be employed carefully with others. In other words, validation of model with experiments in/on foods must be performed before they can be routinely used.

Acknowledgements

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