

ORIGINAL ARTICLE

Modelling the photosensitization-based inactivation of *Bacillus cereus*

Y.L. Marc¹, I. Buchovec², S.M. George¹, J. Baranyi¹ and Z. Luksiene²

¹ Institute of Food Research, Norwich Research Park, Norwich, UK

² Institute of Material Sciences and Applied research, Vilnius University, Vilnius, Lithuania

Keywords

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Correspondence

Yvan Le Marc, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK. E-mail: yvan.lemarc@bbsrc.ac.uk

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Abstract

Aims: To study and to develop a model for the photo-destruction of the food-borne pathogen *Bacillus cereus*, initially treated with a precursor of endogenous photosensitizers (5-aminolevulinic acid, ALA).

Materials and methods: The cells were incubated in the presence of ALA (3 or 7.5 mmol l⁻¹) for incubation times ranging from 2 to 60 min, inoculated onto the surface of LB Agar plates and submitted to light irradiation. The Weibull model was used to describe the survival curves of *B. cereus*. Quadratic equations were used to describe the effects of ALA concentration and incubation time on the Weibull model parameters.

Results: ALA-based photosensitization proved to be an effective tool for inactivation of *B. cereus*. The decrease in viable counts observed after 20 min of irradiation, ranged from 4 to 6 log CFU g⁻¹.

Conclusions: The developed model proved to be a parsimonious and robust solution to describe the observed data.

Significance and Impact of the Study: The study demonstrates the effectiveness of photosensitization on *B. cereus* on agar plates. The model developed may be useful to optimize inactivation treatments by photosensitization.

Introduction

Bacillus cereus is a widespread foodborne pathogen which can cause illness by producing emetic and diarrhoeal enterotoxins. Strains producing diarrhoeal toxin can be found in a variety of foods from rice and vegetables to meat. Because of the ability of *B. cereus* to survive mild heat treatments and dried storage, the use of alternative nonthermal technologies for its inactivation are of great interest. Among these emerging technologies, photosensitization is a pioneering approach for surface decontamination of foods and materials along the food chain (Luksiene *et al.* 2004; Luksiene 2005). The method is based on the principle of activation of photosensitizers by visible light. After accumulation of photosensitizers inside the cell and following irradiation, the singlet oxygen generated interacts with bacterial structures, which lead to series of cytotoxic reactions and microbial death. This technique has already proved to be effective against some

highly proliferating cells and is a promising treatment for cancer. It has also been found that photosensitization is effective against various micro-organisms such as *Escherichia coli* O157 : H7 and *Listeria monocytogenes* (Romanova *et al.* 2003; Luksiene 2005). Some photosensitizers available are food additives or food constituents which do not induce any toxic effects in foods (Buchovec *et al.* 2009). One considerable advantage of photosensitization is that, according to Nitzan and Ashkenazi (2001), bacteria do not develop resistance after this treatment.

From the beginning of the twentieth-century, microbial inactivation has drawn a major interest from food researchers. The first models were developed on the assumption that bacterial inactivation follows a first order kinetics. However, deviations from log-linear curves have been observed by numerous authors (e.g. Cerf 1977; Peleg and Cole 1998; Xiong *et al.* 1999; Geeraerd *et al.* 2000). In a survey of 120 inactivation curves, van Boekel (2002) found that only 5% of the inactivation curves actually

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followed a first order kinetics. Three kinds of deviation have been observed: sigmoid, concave and convex shapes. Since the shape of sigmoid survival curves are similar to those of growth curves, some authors adapted growth models such as the logistic, the Gompertz or the Baranyi equations to model bacterial inactivation (Chen and Hoover 2003). However, these models are unsuitable for nonsigmoid survival curves.

In recent years, the Weibull model has received much attention as an alternative. Due to its flexibility, it can describe concave, convex and linear shapes. This model has been successfully used in a number of studies to model thermal (Fernandez *et al.* 2002; Mafart *et al.* 2002; Albert and Mafart 2005; Couvert *et al.* 2005) or nonthermal bacterial inactivation (Chen and Hoover 2003).

While a considerable amount of work has been carried out on nonthermal inactivation (e.g. dried storage, high pressure) only a few studies have focused on modelling the effect of photosensitization. Luskiene *et al.* (2009), however, showed that *B. cereus* can effectively synthesize endogenous porphyrins from 5-aminolevulinic acid (ALA) at low concentrations (3–7.5 mmol l⁻¹), confirming the possibility of this pathogen to be inactivated by ALA-based photosensitization (Luskiene 2005). Previous studies (Luskiene *et al.* 2009) indicated that concentrations of ALA lower than 3 mmol l⁻¹ would be ineffective. In this work, we studied the antibacterial effectiveness of ALA-based photosensitization against *B. cereus in vitro* at two levels of ALA concentrations, three and 7.5 mmol l⁻¹.

Materials and methods

ALA preparation

A stock solution of 5-aminolevulinic acid hydrochloride (ALA) (Fluka, Israel) was prepared by dissolving ALA in phosphate-buffered saline (0.1 mol l⁻¹ PBS, pH 7.2) up to the concentration 200 mmol l⁻¹. NaOH was used to adjust pH of the solution to 7.2. ALA stock solutions were made immediately before use and sterilized by filtration through 0.2 µm filter (Roth, Germany) (Luskiene *et al.* 1999).

Micro-organism and culture conditions

The target bacterium, *B. cereus* ATCC 12826, was kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland). The bacterial culture was grown at 37°C and maintained onto Luria Bertani Agar (LBA) (Liofilchem, Italy).

The bacterial culture was grown overnight (~14 h) at 37°C in 20 ml of Luria-Bertani medium (LB) (Liofilchem), with aeration of 120 rev min⁻¹ (Environmental Shaker – Incubator ES – 20; Biosan, Latvia). The over-

night bacterial culture grown in LB medium was diluted 20-fold with fresh LB medium (OD₅₄₀ = 0.164) and grown at 37°C to the mid-log phase (~6 × 10⁷ CFU ml⁻¹, OD₅₄₀ = 1) in a shaker (120 rev min⁻¹). Bacterial optical density was determined in a 10.01 glass cuvette at λ = 540 nm (Helios Gamma & Delta spectrophotometers; ThermoSpectronic, UK). Cells were then harvested by centrifugation (6 000 rev min⁻¹, 20 min) and resuspended in a small volume of PBS, to give approx. 3 × 10⁸ CFU ml⁻¹. This stock suspension was diluted to ~1 × 10⁷ CFU ml⁻¹ and immediately used for the photosensitization experiments.

Photosensitization and bacterial survival assay

Aliquots (10 ml) of bacterial suspension (~1 × 10⁷ CFU ml⁻¹ in 0.1 M PBS buffer) with appropriate concentration of ALA (3 and 7.5 mmol l⁻¹) were incubated in a plastic 50 ml bottle in the dark in the shaker (120 rpm) at 37°C for (0–60 min). After corresponding incubation time, 150 µl aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for different time (0–20 min) (Nitzan *et al.* 2004). The LED based light source used for illumination was developed by the Institute of Applied Sciences of Vilnius University. The surface of samples were illuminated at λ = 400 nm with an intensity of 20 mW cm⁻². Light dose was calculated as light intensity multiplied by illumination time. Light power density measurements were performed with a light energy measured by three Sigma meter ('Coherent') equipped with piro-electrical detector J25LP04. No thermal effect was detected for the experimental conditions tested.

The antibacterial effect of photosensitization on *B. cereus* was evaluated by the spread plate method; 100 µl of appropriate dilutions of bacterial test culture after photosensitization were inoculated onto the surface of LB Agar plates. The bacteria were enumerated after incubation at 37°C for 24 h. Duplicate experiments were carried out for each set of exposures.

Mathematical model

The modelling was carried out into two stages. In the first step (primary modelling), the survival curves were fitting with the Weibull model, as proposed by Peleg and Cole (1998):

$$\ln(N/N_0) = -kt^p \quad (1)$$

where t is the time of exposure to light, N is the bacterial concentration at time t , N_0 is its initial value at time 0, k and p are the scale and shape parameters of the model. Equation (1) describes a concave downward survival

curve if $P > 1$, a convex function if $P < 1$ and a linear function if $P = 1$. In the second stage (secondary modelling), polynomial equations were used to describe the effects of ALA concentration and incubation time on the parameters k and p of the Weibull model. As the parameters k and p are strongly correlated (see e.g. Couvert *et al.* 2005), in this study, only the shape parameter p was modelled directly as a function of the ALA concentration and the incubation time, the parameter k was calculated from the observed strong correlation. The choice of the parameter to be modelled directly was based on sensitivity analysis; namely the shape parameter was more affected by the measurement errors of the raw data (i.e. the sampling errors in the log cell concentrations), therefore it was important to minimize the error on p .

Bacterial growth curves were fitted using a nonlinear regression module (NLINFIT) of Matlab 7b (The MathWorks, USA). Linear regressions were performed with the Essential Regression and Experimental Design Version 2.2, a Microsoft Excel Add-In available at <http://www.geocities.com/SiliconValley/Network/1032/>. A backward elimination procedure was adopted to eliminate the insignificant terms.

Results

Effectiveness of ALA-based photosensitization

Following irradiation, the survival fraction decreased sharply (Figs 1 and 2). A clear dependence of photoinactivation

efficiency on irradiation time (or total energy dose) as well as incubation time with ALA was observed. The number of pathogens killed reached six orders of magnitude, after 20 min irradiation time and 60 min incubation time.

Modelling of the inactivation of *Bacillus cereus*

The survival curves as a function of irradiation time exhibit different shapes: linear, convex, and concave (Figs 1 and 2). At a low concentration of ALA (3 mmol l^{-1}) and for short incubation times (2 and 15 min), the survival curves have convex or linear shapes. However, for incubation times longer 15 min, all the curves showed a concave shape. The same observation was made with 7.5 mmol l^{-1} ALA: as the incubation time increases, the curves have a more pronounced concave shape. Increasing incubation time induces an increase of the mortality in the first minutes of irradiation. In this case, however, surviving cells are also more resistant to further irradiation. For example, a reduction of 5.1 log is observed following 5 min of irradiation after incubation for 60 min in the presence of 7.5 mmol l^{-1} ALA, compared with only 2.2 log reduction after incubation for 30 min. A subsequent irradiation of another 15 min induces a further reduction of only 0.6 log (incubation time 60 min) against 2.8 log (incubation time 30 min).

As can be seen in Figs 1 and 2, eqn (1) successfully described the different shapes of the observed survival curves. Table 1 gives the fitted parameters for k and p . It

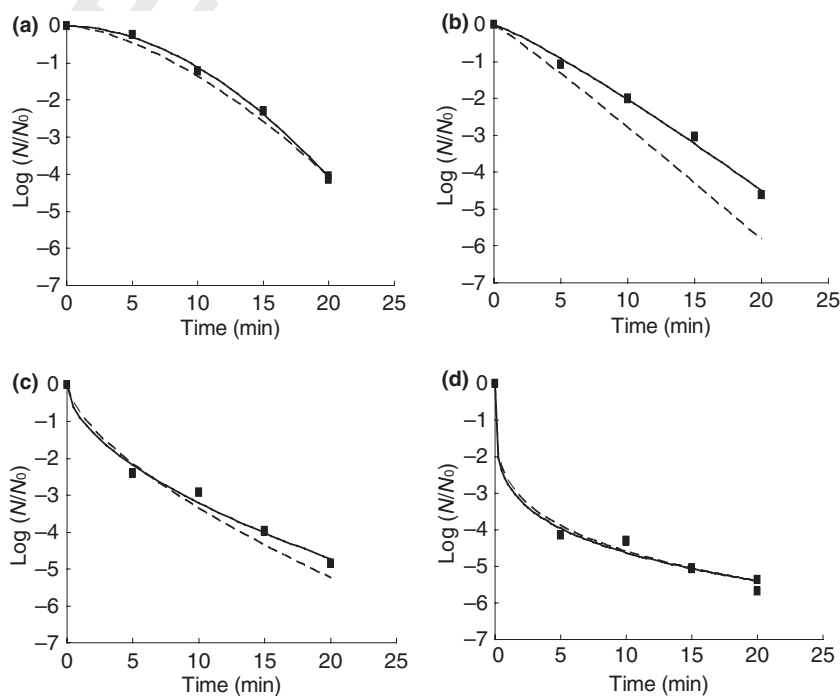


Figure 1 Survival of *B. cereus* after incubation in the presence of 3 mmol l^{-1} ALA for (a) 2 min, (b) 15 min, (c) 30 min and (d) 60 min. Straight line: fit of the Weibull model (eqn 1), dotted line: prediction of eqn (2) and (3), ■ bacterial log counts.

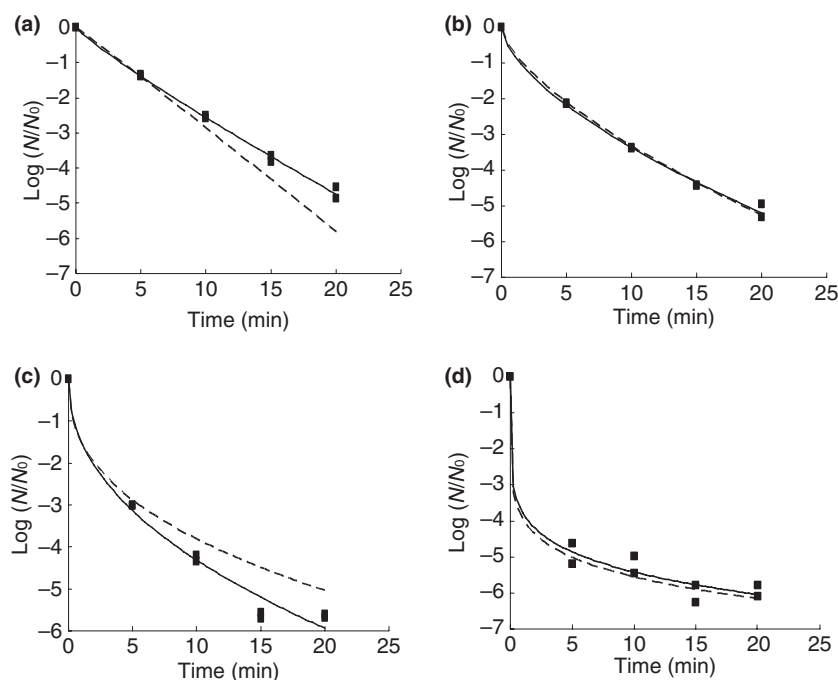


Figure 2 Survival of *B. cereus* after incubation in the presence of 7.5 mmol l⁻¹ ALA for (a) 2 min, (b) 15 min, (c) 30 min and (d) 60 min. Straight line: fit of the Weibull model (eqn 1), dotted line: prediction of eqn (2) and (3), ■ bacterial log counts.

Table 1 Fitted parameters of eqn (1)

ALA concentration (mmol l ⁻¹)	Incubation time (min)	Fitted values	
		<i>k</i>	<i>p</i>
3	2	0.034 (0.020–0.049)	1.87 (1.73–2.01)
3	15	0.33 (0.19–0.47)	1.14 (1.00–1.29)
3	30	2.03 (1.40–2.67)	0.56 (0.44–0.67)
3	60	6.37 (5.04–7.70)	0.22 (0.14–0.30)
7.5	2	0.76 (0.59–0.93)	0.89 (0.81–0.97)
7.5	15	1.83 (1.52–2.14)	0.63 (0.56–0.69)
7.5	30	3.43 (2.33–4.54)	0.46 (0.34–0.58)
7.5	60	8.68 (6.64–10.72)	0.16 (0.06–0.25)

can be observed that *k* increases with decreasing *p* and suggests a strong correlation between the two parameters. This is confirmed by Fig. 3 which clearly shows a linear correlation between ln(*p*) and sqrt(*k*). These results are in accordance with those of Couvert *et al.* (2005) who found a structural correlation between *p* and *k* in their study on the thermal resistance of *Bacillus pumilus*. The quadratic model was fitted for the effects of ALA concentration and incubation on ln(*p*) (*R*²_{adj} = 0.972). Square terms and interaction terms proved to be insignificant ($\alpha = 5\%$) and the final equation is:

$$\ln(p) = 0.877 - 0.105 \text{ Conc_ALA} - 0.0333 \text{ Inc_Time} \quad (2)$$

where Conc_ALA is the concentration of ALA (mmol l⁻¹) and Inc_Time is the incubation time (min). As shown in Fig. 4, the model provides an accurate description of the

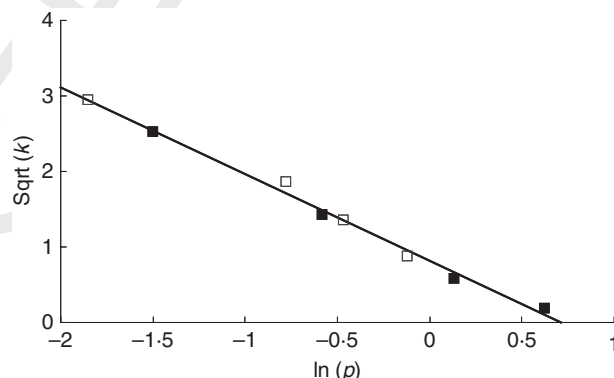


Figure 3 Linear relationship between ln(*p*) and sqrt(*k*), where *p* and *k* are the scale and shape parameters of the Weibull model (eqn 1). ■ and □ are the observations after incubation in the presence of 3 and 7.5 mmol l⁻¹ ALA, respectively.

effect of incubation time at ALA concentrations of 3 and 7 mmol l⁻¹. Sqrt(*k*) was modelled, using the linear correlation between ln(*p*) and sqrt(*k*). The fitted equation (*R*²_{adj} = 0.972) is written as:

$$\text{Sqrt}(k) = 0.816 - 1.151 \ln(p) \quad (3)$$

Equations (2) and (3) were used to predict the survival curves for the experimental conditions studied in this work. Comparison between the predicted and fitted survival curves shows that the model usually provides a good overall description of the data (Figs 1 and 2). Note that -

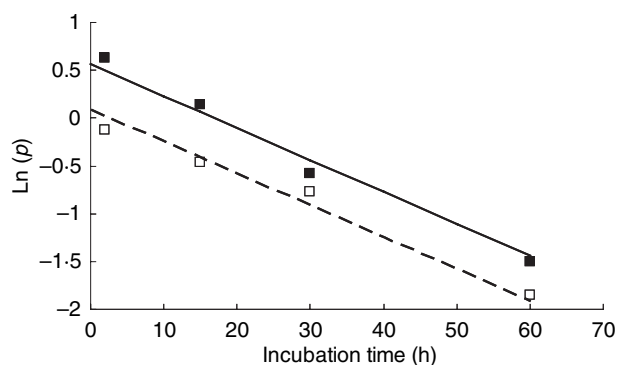


Figure 4 Predicted effect of incubation time on the natural logarithm of p , the shape parameter of the Weibull model (eqn 1) at 3 (solid line) and 7.5 mmol l⁻¹ (dashed line) ALA. ■ and □ represent the values of $\ln(p)$ at 3 and 7.5 mmol l⁻¹ ALA respectively.

when we modelled \sqrt{k} and then calculate $\ln(p)$ by using eqn (3), the predictions for the log cell concentrations were much poorer. The reason is that the shape parameter p is more sensitive to errors than k . Therefore, if the modelling follows the $k - p$ order, not only the error in the observed log counts affects p but also the error in the $p = f(k)$ function.

Discussion

The inactivation of pathogens on food or food-related surfaces by photosensitization is a novel and pioneering approach. The selected photosensitizer must have suitable photophysical and photochemical properties and be easy to produce and effective against foodborne pathogens. It must be either a food constituent or at least be nontoxic at the concentrations required for microbial inactivation. The study shows that *B. cereus* can be inactivated by an order of 4–6 log by photosensitization with 3 and 7.5 mmol l⁻¹ ALA. ALA is a naturally occurring substance, produced by e.g. mammals and plants. As ALA is planned to be used as a photosensitizer on the surface of ready to eat food products before the product is washed, these concentrations of ALA must not be toxic for humans (Luksiene 2003). However, food additives should be used in as low concentrations as possible. Considering the satisfactory six log reduction observed after exposure to concentrations of 7.5 mmol l⁻¹ ALA, the effects of higher concentrations were not investigated in this work. However, further research will investigate the applicability of the protocol used in this work to the decontamination of the surface of fruits and vegetables.

The Weibull model was able to describe the different shapes of the curves encountered in the experiments. A concave shape is generally the result of rapid elimination of the sensitive members of the population, leaving the

remaining cells with an increased resistance (Corradini and Peleg 2007). Our results suggest that increasing ALA concentration and incubation time increases the rates at which the sensitive cells are killed in the first minutes of irradiation. However, the survivors also become more resistant to light irradiation. Mathematical modelling can help selecting the combination of ALA concentration, incubation and irradiation times to reach the desired log reduction of pathogens.

Secondary modelling focuses on the effect of the environmental factors on the main parameters of the primary model. One problem which arises in the secondary modelling is the correlation between the parameters of primary models. This correlation is frequently observed in predictive microbiology, for example, between growth rate and lag time (e.g. Cooper 1963; Chandler and McMeekin 1985; Smith 1985; Fu *et al.* 1991; Baranyi and Roberts 1994; Rosso 1995). These authors reported that for the same pre-incubatory conditions the lag times are inversely proportional to the specific growth rates. This simple relationship has often been utilized to estimate the lag from the growth rate model (e.g. Rosso 1995; Ross and Dalgaard 2004).

A similar problem arises in the secondary modelling for bacterial inactivation with the correlation between the parameters of the Weibull model. Couvert *et al.* (2005) solved this problem by using a single P -value estimated from the whole data set (therefore, the secondary model developed by the authors describes only the effects of temperature on the scale parameter k). This was possible since the temperature has little effect on the shape parameter (van Boekel 2002). Despite a slight loss of goodness of fit, this modification improved the robustness of the model. This technique was also suggested by other authors (Mafart *et al.* 2002; Corradini and Peleg 2007). However, in our work the shape parameter p varies greatly with the experimental conditions (with values ranging from 0.15 to 1.15). It was therefore impossible to use a single P -value for the whole data set. Instead of modelling independently two parameters mutually dependent on each other, we modelled only one and then estimated the second parameter from their correlation. To our best knowledge, it is the first time that a secondary model has been developed for both parameters of the Weibull model, p and k , using the correlation observed between those two parameters. In this work, the best results were obtained by (i) model $\ln(p)$ as a function of ALA concentration and incubation time and (ii) model \sqrt{k} as a function of $\ln(p)$.

Conclusion

The data presented clearly indicates for the first time that ALA-based photosensitization can be effective against the

1 foodborne pathogen *B. cereus*. Survival curves of *B. cereus*
 2 estimated after inactivation by ALA-based photosensitiza-
 3 tion can be successfully described by the Weibull model.
 4 The secondary model developed can be a useful tool to
 5 determine the most effective combination of ALA concen-
 6 tration and incubation time to reach the desired target
 7 for the reduction of *B. cereus*. However, further work is
 8 needed to assess the applicability of the model and the
 9 effectiveness of the ALA-based photosensitization treat-
 10 ment in a real food scenario.

11 Acknowledgements

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Change italic to upright type	(As above)	⊕
Change bold to non-bold type	(As above)	⊖
Insert 'superior' character	/ through character or ∧ where required	Υ or Υ under character e.g. Υ or Υ
Insert 'inferior' character	(As above)	∧ over character e.g. ∧
Insert full stop	(As above)	⊙
Insert comma	(As above)	,
Insert single quotation marks	(As above)	Ƴ or ƴ and/or ƶ or Ʒ
Insert double quotation marks	(As above)	ƶ or Ʒ and/or Ʒ or ƶ
Insert hyphen	(As above)	⊥
Start new paragraph	┌	┌
No new paragraph	┐	┐
Transpose	└┐	└┐
Close up	linking ○ characters	Ⓞ
Insert or substitute space between characters or words	/ through character or ∧ where required	Υ
Reduce space between characters or words		↑