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Practical application of dynamic temperature profiles to estimate the parameters of the square root model

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Abstract

Optimal experimental design for parameter estimation (OED/PE) is a promising method to improve parameter estimation accuracy and minimise experimental effort in the field of predictive microbiology. In this paper, the OED/PE methodology was applied on two practical examples: the growth of *Bacillus cereus* and *Enterobacter cloacae* in liquid whole egg product. Both strains were recovered from samples of a commercial product. The goal of the modelling exercise was to quantify the influence of temperature on bacterial growth. The Baranyi-model for bacterial growth combined with the Ratkowsky square root model to describe temperature dependence was used. Using this model, a temperature step profile was calculated based on the optimal D-criterion. The model was then fitted against the experimental bacterial growth curve measured under the dynamic temperature conditions. This process was repeated until the parameters could be estimated with sufficient accuracy, apparent by the model prediction errors. For *B. cereus*, prior information could be extracted from the literature, allowing calculating a dynamic temperature profile directly. Two-step profiles were sufficient to obtain a good estimation for the model parameters. No prior information could be found for *E. cloacae*. Therefore, a limited series of static experiments had to be conducted to obtain usable prior model parameters estimates. Only one dynamic experiment was then needed to achieve a good estimation.
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Keywords: Optimal experimental design; Predictive microbiology; Square root model; Dynamic experiments

1. Introduction

Predictive modelling of bacterial growth in foods remains an important research topic among food microbiologists. A good predictive model should allow predicting bacterial growth in a certain food matrix as function of environmental conditions, enabling application in shelf-life estimation and microbiological risk assessment (Buchanan and Whiting, 1996). Predictive microbiological models can be subdivided in two categories: primary and secondary models (Whiting, 1995). Primary models describe the growth of bacteria under static conditions; the secondary models describe the primary model param-

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eter-dependence on environmental factors. This subdivision reflects the traditional way of calibrating predictive models: a series of growth or inactivation curves is measured; the parameters of the primary model are fitted to each curve, after which the secondary model parameters can be estimated. This represents a substantial experimental effort.

The reliability of the model parameters determines to a large extent the value of model-based predictions. It requires considerable effort to collect the necessary data to allow for good parameter estimation (McMeekin et al., 1993). Optimal experimental design (OED) can help to optimise available resources. Two types of OED can be distinguished: OED for model discrimination and OED for parameter estimation (OED/PE). The former is used when different competing models are available and the aim is to find the best one for the task at hand. Despite the fact that numerous types of predictive models are available, primary as well as secondary, this type of OED has not been applied in the field of predictive microbiology to the best of our knowledge. OED/PE presumes a fixed model structure; the aim is now to estimate the model parameters as accurately as possible by manipulating the available experimental degrees of freedom. Note that these methodologies are quite common in fields like chemical, biochemical and environmental engineering (Froment and Bischoff, 1990; Vanrolleghem, 1994).

Pioneering work on OED/PE in the field of predictive microbiology has been done by Van Impe and co-workers (Versyck et al., 1999; Bernaerts et al., 2000; Bernaerts et al., 2002; Bernaerts et al., 2003). They have published results on OED/PE for estimating model parameters of growth as well as inactivation temperature models and have demonstrated convincingly that OED/PE can be used successfully to improve the estimation properties and optimise the experimental efforts by directly estimating the secondary parameters from a carefully designed dynamic experiment. However, the methodology appears not to be applied in practice in recent predictive modelling exercises. The aim of this paper is to utilize the OED/PE techniques to two real-life examples in order to assess their practical usefulness. Therefore, an OED/PE exercise was carried out for two bacteria isolated from commercial liquid whole egg products, *Bacillus cereus* and *Enterobacter cloacae*.

2. Materials and methods

2.1. Inoculation and bacterial count

Bacterial growth curves for *B. cereus* and *E. cloacae* were measured in sterilised liquid whole egg from the same producer. Sterilisation of egg products is not possible by heat processes due to the texture changes of the product; therefore, the sterilisation was done by radiation with gamma beams from a cobalt-60 source with a radiation intensity of 4 kGy at Ion Beam Applications (Fleurus, Belgium). It was verified that the radiated product was indeed sterile. This was done by submitting not-inoculated product to the same conditions as inoculated product for all experiments. Microbiological growth was never observed in any of these blanks.

The strains recovered from samples of the commercial product, stored on Protect Beads at −80 °C, were resuscitated overnight at 30 °C in Brain Heart Infusion (BHI, Oxoid, Hampshire, UK). This culture was diluted with 1/4-strength Ringer solution (Oxoid). A suitable dilution was used to inoculate the sterile whole egg product with a practical concentration of the bacteria of interest. The inoculated whole egg product was placed at a preset temperature. Samples were taken at pre-calculated times (see further), put in sterile test tubes. and put on ice. This sample was then plate counted. *B. cereus* was counted by spread plate method on Cereus selective agar base according to MOSSEL (MYPagar, Merck, Darmstadt, Germany), *E. cloacae* by pour plate method on Nutrient agar (NA, Oxoid). Agar plates were incubated for 2 days at 30 °C. All samples were taken in duplicate at each sampling time and were independently processed.

2.2. Bacterial growth model

2.2.1. Primary growth model

The bacterial growth model used in this study was developed by Baranyi and Roberts (1995). To simulate dynamic temperature conditions the model must be used in its differential equation form:

$$\frac{dn}{dt} = \frac{q(t)}{1+q(t)}\mu_{max}(T(t))\left(1-e^{n(t)-n_{max}}\right)$$

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$$\frac{dq}{dt} = \mu_{max}(T(t))q(t)$$

$$n(0) = n_0$$

$$q(0) = q_0 \quad (1)$$

Here, $n(t)$ denotes the natural logarithm of the cell density (ln(cfu/ml)), n_{max} is the natural logarithm of the maximum population concentration (ln(cfu/ml)), $q(t)$ (−) is a measure of the physiological state of the cell and is closely related with the lag phase, and $\mu_{max}(T(t))$ (h^{-1}) is the maximum specific growth rate, dependent on temperature (T (°C)) and consequently on time (t (h)).

2.2.2. Secondary temperature dependence model

The square root model (Ratkowsky et al., 1982) has proven its efficiency to describe the temperature dependence of bacterial growth in the sub-optimal temperature range.

$$\sqrt{\mu_{max}} = b(T - T_{min}) \quad (2)$$

b ($h^{-0.5} \cdot °C$) and T_{min} (°C) are the model parameters, with T_{min} defined as the theoretical minimum growth temperature.

2.3. Optimal experimental design

The basic concepts of optimal experimental design are described elsewhere (Froment and Bischoff, 1990; Grijspeerdt and Vanrolleghem, 1999; Bernaerts et al., 2000). In predictive microbiology, the secondary model parameters are traditionally estimated from a series of static experiments. An experiment under dynamic temperature conditions contains potentially more information, which could result in a more efficient parameter estimation process. The possible dynamic temperature conditions are infinite, but practical considerations impose some constraints:

- Only temperature steps are considered. Other possibilities such as temperature pulses or sinusoidal profiles are more difficult to implement; Bernaerts et al. (2000) showed that a step profile was the most informative among a series of possible temperature profiles.
- The temperature difference between 2 successive steps should preferably not be larger than 5 °C to avoid the causation of a new lag time (Zwietering et al., 1994; Bernaerts et al., 2002; Swinnen et al., 2003).
- The total time of an experiment should be kept within practical limits.

2.3.1. Temperature profile

Even when limiting ourselves to step profiles, there remains an infinite choice in possible temperature profiles. Preliminary selection is necessary. Two or more temperature steps are beneficial for improving inter-parameter correlation (Bernaerts et al., 2002), but they make planning the experiments more difficult. For practical reasons, the calculations in this paper were limited to a single temperature step, within the constraints outlined before. The procedure is then to find the optimum time at which the temperature step should be placed and the magnitude of the temperature shift, which is a priori limited to a maximum of 5 °C.

The criterion to be calculated involves the computation of a characteristic of the Fisher information matrix **F** (Bernaerts et al., 2000). Maximising det(**F**) leads to minimising the estimation errors on the parameters (D-optimal criterion). Alternatively, maximising the smallest eigenvalue of **F** leads to a maximum decorrelation of the parameter estimates (E-optimal criterion). In this paper, the D-optimal criterion will be used.

2.3.2. Optimal sampling

Grijspeerdt and Vanrolleghem (1999) described an optimal method for sampling growth curves for experiments at constant temperature. This procedure was extended for dynamic experimental conditions allowing determining optimal sampling points of a bacterial growth curve for a temperature step profile. The original method also comprised a Monte Carlo simulation step to quantify the uncertainties of the optimal sampling points. Although theoretically straightforward to extend for dynamic conditions, the computing requirements made it practically impossible to assess the uncertainty of the optimal sampling times. The results should be interpreted accordingly, and it is strongly advisable to sample more points spread around the predicted optimal

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timings. An experimental sampling plan should keep this in mind.

Due to the long experimental times needed, it is not trivial to work out the most efficient experimental sampling scheme. This scheme should take working hours into account, combined with the fact that the sampling times positions have a certain degree of uncertainty. A spreadsheet application was developed to assess alternative starting points. As mentioned before, these times are approximate. To increase the reliability of the results all the samples were done in twofold. Moreover, because of the uncertain basis of the design, it is advisable to take more samples if possible, in order to minimise the risk that a crucial time zone would be missed. These additional samples would be preferably spread over the day.

3. Practical examples

3.1. Bacillus cereus

3.1.1. Preliminary data

The starting point for any optimal experimental design for parameter estimation requires a minimal degree of a priori knowledge about the parameter values. There is considerable work done on predictive modelling of *B. cereus* growth (e.g., Baker and Griffiths, 1993; Benedict et al., 1993; Chorin et al., 1997), but we could not locate any in eggs. Based on the work of Chorin et al. (1997), the following preliminary Ratkowsky-parameters were obtained: b=0.036 $h^{-0.5} \cdot °C$ and T_{min}=6.26 °C.

3.1.2. Dynamic temperature profile experiments

An optimal single-step experiment was calculated, using the preliminary Ratkowsky values as a starting point, according to the D-optimal criterion. The calculations were done assuming a starting concentration of 10^3 cfu/ml, an asymptotic concentration of 8.5×10^9 cfu/ml and q_0=e (Baranyi and Roberts, 1995). The three best conditions are summarized in Table 1, together with the total required experiment time.

Table 1
Optimal temperature shift for the *B. cereus* single-step experiment

t_{switch} (h)	T_1 (°C)	T_2 (°C)	t_f (h)	Det (**F**)
40	15	20	114	36.99
35	15	20	111	32.81
30	15	20	108	28.13

t_{switch} is the switching time, T_1 and T_2 are the temperatures before and after the switch, respectively, and t_f is the predicted running time of the experiment.

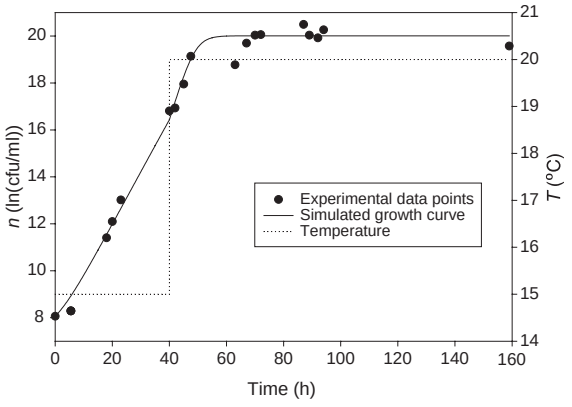

Fig. 1. Experimental results and best model fit for the first single step experiment for *B. cereus*.

The optimising process tends to favor long running experiments at relatively low temperature, making it more difficult to do the experiment in practice. The experimental data points and the best fitted Baranyi-model for the most optimum temperature profile in Table 1 are shown in Fig. 1. The main source of uncertainty is the q_0-parameter. An ad hoc value was presumed for the calculation of the temperature profile and the optimal sampling points. This is visible in Fig. 1; the lag phase is

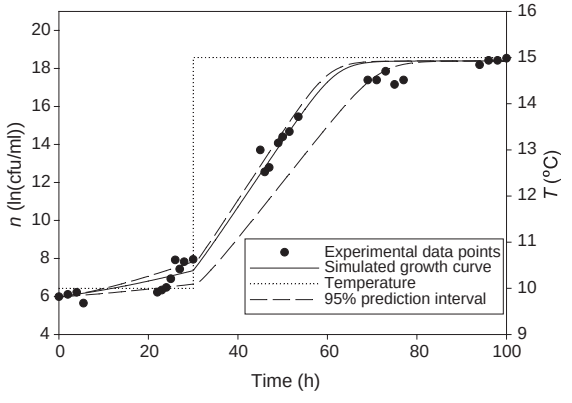

Fig. 2. Experimental results and best model fit for the second single step experiment for *B. cereus*.

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Table 2
Parameter estimates and joint confidence limits for the *B. cereus* second temperature step experiment

Parameter	Estimate	Standard error	95% confidence interval
b ($h^{-0.5} \cdot °C$)	0.0637	3.24×10^{-6}	0.0552–0.0723
T_{min} (°C)	5.76	0.182	4.25–7.27
q_0	2.10	0.161	0.201–4.004

clearly not sufficiently covered by the experimental points. Therefore the Baranyi-model was fitted to the experimental data points using three degrees of freedom: q_0, and the Ratkowsky parameters b and T_{min}. The parameters could not be estimated significantly, so another experiment was necessary. The estimates obtained in this first experiment (b=0.032 $h^{-0.5} \cdot °C$, T_{min}=0.18 °C, and q_0=1.32) were used to calculate the next optimal temperature profile.

The best combination according to the optimal D-criterion was a temperature step from 10 to 15 °C after 30 h, with a predicted running time of 94 h. The resulting growth curve, which was sampled according to an optimal sampling scheme as outlined before, is shown in Fig. 2.

Again, three degrees of freedom were used for the fitting process. The resulting parameter estimates had much better properties this time, as can be seen in Table 2 where the 95% confidence intervals are shown. Significant estimation was possible for all three parameters. It has to be noted that a limited lack of fit can be observed at the end of the first temperature step and in late exponential phase. This

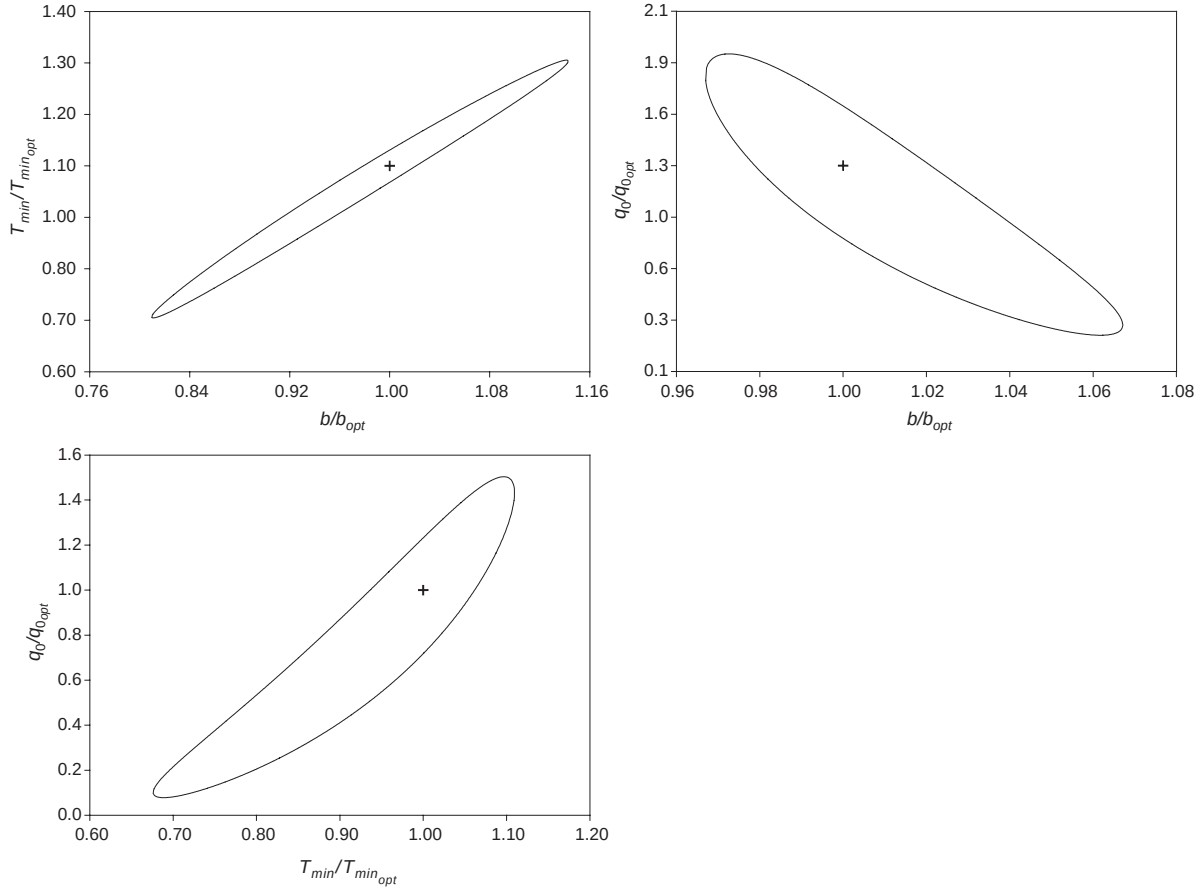

Fig. 3. 2D projections of the 95% joint confidence region for the different parameter combinations. The crosshair denotes the optimal parameter estimates.

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is probably due to fixing the shape parameters of the Baranyi-model, a procedure suggested by Baranyi and Roberts (1995) and applied in numerous predictive modelling studies. As the lack of fit is very limited, the impact on the other parameter estimates can be expected to be limited. Including the shape parameters in the fitting process would only marginally improve the model fit not justifying the introduction of two extra parameters.

The joint confidence regions of the parameter estimates are well shaped, as can be seen in Fig. 3. The correlation matrix shown in Eq. (3) reveals a relatively high inter-parameter correlation of 0.97 between b and $T_{\min}$. This is a known artefact of square root type models (Rosso et al., 1995). The obtained value of 0.97 is in the same order of magnitude as reported by Bernaerts et al. (2002), who obtained the same correlation for an E-optimally designed experiment for *Escherichia coli*.

$$\begin{pmatrix} 1 & 0.97 & 0.78 \\ 0.97 & 1 & 0.91 \\ 0.78 & 0.91 & 1 \end{pmatrix} \tag{3}$$

3.2. Enterobacter cloacae

3.2.1. Static experiments

No useful experimental data could be located for this particular spoilage organism. To obtain preliminary parameter values, a limited number of static growth curves were determined at only three temperatures: 10, 25, and 30 °C (Fig. 4). From these results,

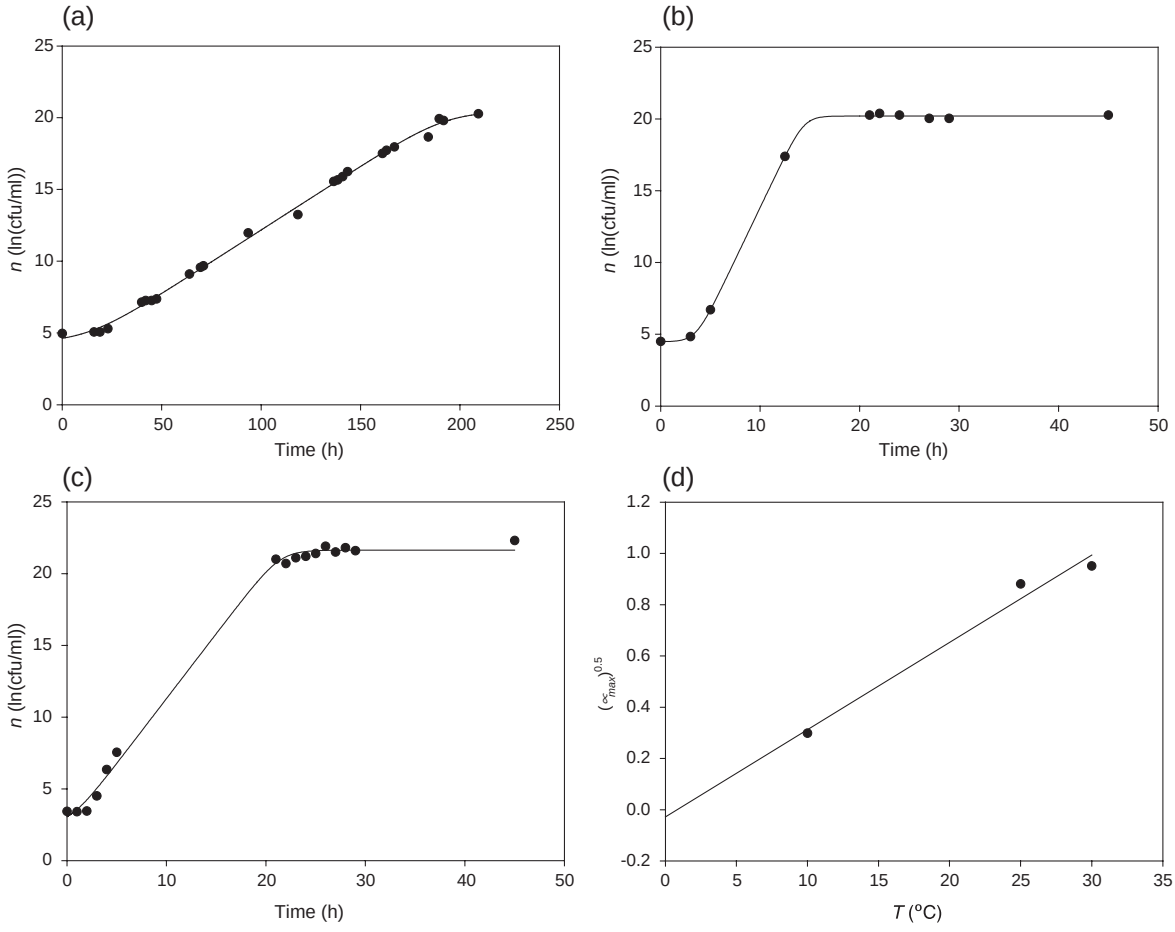

Fig. 4. Static experiments with the respective model fit at 10 (a), 25 (b), and 30 °C (c) and the resulting square root plot (d).

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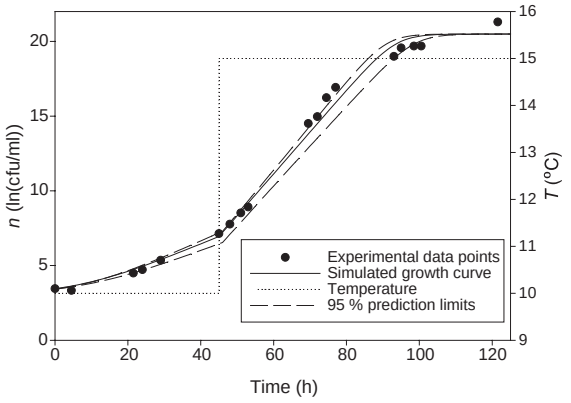

Fig. 5. Experimental results and best model fit for the *E. cloacae* single-step temperature experiment.

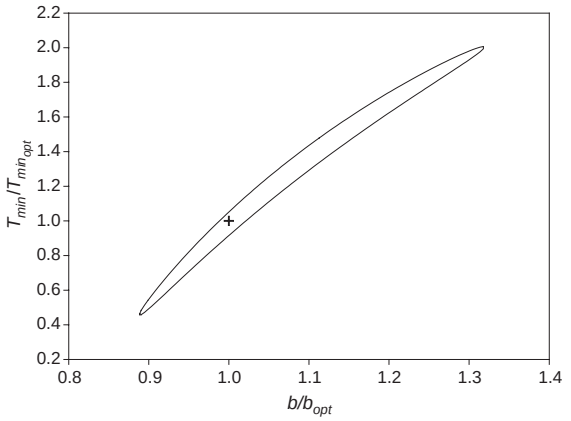

Fig. 6. The 95% joint confidence region for the parameter estimates. The crosshair denotes the optimal parameter estimates.

the square root parameters were calculated in the traditional way, resulting in b=0.0341 and T_{min}=0.821 °C. Since only three points were used to estimate these parameter values, it is not surprising that they could not be estimated significantly. However, a good estimation was obtained for q_0 for all three cases, which were all within the individual 95% confidence limits. Therefore the average of the three q_0 estimates (0.494) was assumed to be a fixed value for the dynamic temperature experiment. This is justified by the standardised preparation procedure of the different cultures, so that these cultures have the same history when starting the experiments and consequently q_0 can be considered to be constant (Baranyi and Roberts, 1995).

3.2.2. Dynamic temperature experiments

The optimal single-step temperature profile was calculated starting from the preliminary values obtained from the static experiments. The best single temperature step experiment according to the optimal D-criterion was a temperature step from 10 to 15 °C at a switching time of 45 h and a predicted running time of 120 h. This experiment was carried out analogously as for *B. cereus* and is shown in Fig. 5. Obviously, the fit is quite good resulting in good estimations for b and T_{min}.

The estimation errors are smaller than for the *B. cereus* case, and significantly different from zero (Table 3). The joint confidence region again reveals the estimation problems typically associated with the square root model, but to a lesser extent than the *B. cereus* case, as is evident from the lower inter-parameter correlation of 0.90. Note that comparison of Figs. 3 and 6 is not straightforward due to the difference in parameter dimensionality of both cases.

Table 3
Parameter estimates and joint confidence limits for the *E. cloacae* dynamic temperature experiment

Parameter	Estimate	Standard error	95% confidence limits
b ($h^{-0.5} \cdot °C$)	0.0421	1.78×10^{-7}	0.0402–0.0439
T_{min} (°C)	2.42	1.04×10^{-2}	1.98–2.87

4. Model prediction errors

Using the optimal experimental designs, the model parameters have been estimated with a certain precision. In order to assess the impact of the parameter estimates precision on the practical use of the models, the prediction errors needs to be analysed. This can be done using Monte Carlo simulation (Poschet and Van Impe, 1999; Poschet et al., 2003): the parameter estimates distribution is sampled for a large number of iterations and for each parameter combination, the model output is calculated. The result is a large number of output curves from which simulated quantiles can then be calculated to visualize the prediction error limits. An important aspect for the case presented here is the inter-parameter correlation. As these are relatively high, they should be taken into account in the Monte Carlo simulation (Haas et al., 1999; Verdonck, 2003). Different methodologies exist

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to incorporate correlation in a Monte Carlo simulation, the most frequent one being the empiric method of Iman and Conover (1982) based on rank-order correlations, or bootstrap-based methods (Davison and Hinkley, 1997). The correlation information available here is in the format of Pearson's correlation coefficients as opposed to rank-order correlation, and the computational requirements for a bootstrap-based method turned out to be too computing intensive to be practically feasible. An elegant solution can be found in the following method developed by Scott (Richard T. Scott, Kodak, Rochester, New York, personal communication).

Suppose that x and y are two normally distributed variables with mean μ_x and μ_y, and variance σ_x and σ_y, respectively. There exists a correlation ρ_{xy} between x and y. To generate two random variables X and Y with the same correlation by resampling x and y, the following procedure is followed:

$$X = N(\mu_x,\sigma_x)$$

$$Y = \left(\rho_{xy}\left(\frac{X-\mu_x}{\sigma_x}\right) + \sqrt{1-\rho_{xy}^2}N(0,1)\right)\sigma_y + \mu_y \quad (4)$$

$N(\mu, \sigma)$ is a normal random generating function with mean μ and variance σ. The extension to three variables, like the *B. cereus* case is straightforward.

The normal distribution parameters μ and σ can be directly obtained from the estimation results (Tables 2 and 3), as is the case for the correlation coefficients. Due to the dependent sampling, it is not possible to use Latin Hypercube Sampling (Vose, 1996) and the less-efficient Monte Carlo sampling has to be utilised. Therefore, the number of iterations was taken sufficiently high. 100 000 iterations were carried out for both the *Bacillus* and the *Enterobacter* models and the resulting 95% prediction intervals are indicated in Figs. 2 and 5. The limits are not symmetric against the model output using the optimal parameter estimates, due to the inter-parameter correlation. It is known that the effect of correlations on resampling procedures is mostly felt at the boundaries of the resulting distribution (Verdonck, 2003), such as the 95% limits calculated here. The prediction limits are wider for the *B. cereus* case, in line with the higher inaccuracy of the parameter estimates. The range of the 95% interval remains below 2.3 on natural logarithmic scale, a normal variation on microbial count data in practice (Jarvis, 1989).

The above outlined procedure uses the individual parameter distributions as a starting point. This introduces a minimal bias as opposed to using the full multivariate joint parameter distribution accessible by bootstrapping methods (Haas et al., 1999).

5. Discussion

The square root model has its flaws, as is demonstrated by the high inter-parameter correlations. The cardinal model as presented by Rosso et al. (1995) was demonstrated to have better parameter estimating properties, but has the disadvantage that it contains five model parameters and needs full temperature range data for calibration. It is unlikely that eggs will be stored at super-optimal temperatures, so it does not seem worthwhile to conduct experiments at these high temperatures. Other types of temperature models exist, such as the Arrhenius type models presented by Davey et al. (Davey, 1989; Daughtry et al., 1997), neural networks (Geeraerd et al., 1998; Hajmeer and Basheer, 2003), and polynomial models. These models have not yet found widespread use in predictive microbiology. In practice, the square root model still appears to be the most often used for predictive modelling in the sub-optimal temperature range; the main reason why it was used in this paper.

Despite being a quite established methodology, OED/PE has not been frequently applied in the field of predictive microbiology. The examples presented in this paper show it to be a means to optimise available resources. Both cases presented required a minimum number of experiments to obtain reasonable and usable estimates for the square root model parameters.

For the *B. cereus* case, where preliminary information was available, two experiments already sufficed. These experiments, all under dynamic temperature conditions, are more elaborate than experiments at constant temperature in the sense that one has to be able to impose the dynamic environ-

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ment. Otherwise, the needed efforts to obtain a growth curve are quite comparable in terms of sampling and experimental running times. The *E. cloacae* example required four experiments; three static and one dynamic were sufficient to obtain significant parameter estimates. The lack of prior knowledge translated in more required effort. Nevertheless, using the traditional approach would probably require more growth curves to obtain comparable results.

The model prediction errors fall between reasonable limits, as was verified using Monte Carlo simulations. The parameter estimates could probably have been refined in terms of accuracy and inter-parameter correlation by planning extra experiments, especially for the *B. cereus* case. However, in view of the considerable variability inherently present when dealing with microbial counts data, there is a limit to the information that can be gained by doing extra experiments. Future research could aid on finding the optimal number of experiments in terms of parameter estimation gain versus experimental effort for some practical examples.

Comparing to traditional method of modelling the temperature influence on primary predictive microbiological models, it is clear that OED/PE can be beneficial in terms of the necessary experimental effort. An even more clear advantage would emerge when more factors are considered, such as pH and water activity. The amount of experimental work following the traditional method tends to get very large in these cases (Devlieghere et al., 2000; Wijtzes et al., 2001). This could enhance the potential gain of OED/PE, despite the fact that the required calculations to determine optimal profiles would be more involved than in the one-dimensional case. Future research should focus on expanding OED/PE to such multi-dimensional cases.

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