Development and validation of a combined temperature, water activity, pH model for bacterial growth rate of \textit{Lactobacillus curvatus}

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Abstract

A model was established to predict growth rate as a function of temperature, pH and water activity. The model is based on two, earlier developed models, one for growth rate as a function of temperature and water activity and the other for growth rate as a function of temperature and pH. Based on the assumption that combinatory effects between pH and water activity do not exist, the two models were multiplied to produce one overall model. The overall model was then fitted to data sets measured earlier, and the parameters of the model were determined. A new data set with values for controlling variables outside the range of the earlier developed model was then used to validate the overall model statistically. The model was well able to extrapolate outside the measured data range. Finally, the model was updated with all measured data. No significant changes in the parameters were found. The approach followed underpins the gamma concept, since in the gamma concept it is assumed that the effects of controlling variables can be multiplied, and cardinal parameters are not a function of other variables (temperature, pH, and water activity). © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The quality and shelf life of foods is often determined by the presence and growth of bacteria. Foodborne bacteria can be divided into spoilage and pathogenic species. Bacterial growth is only allowed, to a certain extent, for spoilage organisms. The presence and growth of pathogenic organisms in foods should be avoided as much as possible by inactivating the living cells or altering the product formulation in such a way that growth of these organisms is slow or even absent.

Previous articles have described bacterial growth as a function of water activity, temperature or pH (Ratkowsky et al., 1982; McMeekin et al., 1987;...
Adams et al., 1991; Wijtzes et al., 1993). McMeekin et al. (1987), for instance, described the combined effect of water activity and temperature on bacterial growth rate. Wijtzes et al. (1995) described the growth of Lactobacillus curvatus as a function of temperature and pH. The effect of temperature, pH and water activity on the growth rate of Listeria monocytogenes was modelled previously by Wijtzes et al. (1993), and the performance of growth models for L. monocytogenes was evaluated with respect to independent literature data (Giffel and Zwietering, 1999). The models mentioned contain parameters that have an interpretable meaning. The model proposed by McMeekin et al. (1987) contains a theoretical minimal temperature and a minimal water activity. The model described by Wijtzes et al. (1995), apart from a theoretical minimal temperature, contains a theoretical minimal and maximal pH. Estimates of the values for these parameters can be obtained from the literature or assessed by relatively simple experiments; therefore, in this paper, this type of model will be used.

In this paper, models for bacterial growth rate as a function of temperature and water activity, and growth rate as a function of temperature and pH are combined into one model describing growth rate as a function of temperature, water activity and pH. The parameters of the model are calculated on the basis of earlier measured data. The predictions of the model are then used to validate the model with a newly measured growth rate data set where the values of the controlling variables lie outside the earlier measured temperature, water activity, and pH data range.

2. Models

Growth rate as a function of suboptimal growth temperature has been modelled by several researchers (Ratkowsky et al., 1982; McMeekin et al., 1987; Adams et al., 1991; Wijtzes et al., 1993):

\[
\mu = b_1(T - T_{\text{min}})^2
\]

where \( \mu \) (h\(^{-1}\)) is the specific growth rate of a microorganism at sub-optimal temperature \( T \) (°C), and \( b_1 \) (h\(^{-1}\) °C\(^{-2}\)) and \( T_{\text{min}} \) (°C) are regression coefficients. \( T_{\text{min}} \) represents the theoretical suboptimal temperature where the growth rate just equals 0 (h\(^{-1}\)). Since the value of \( T_{\text{min}} \) was found to be independent of pH, Wijtzes et al. (1995) proposed to substitute the effect of pH into parameter \( b_1 \) resulting in Eq. (2), where \( pH_{\text{min}} \) is the minimal pH, and \( pH_{\text{max}} \) the maximal pH at which growth stops. \( pH_{\text{min}} \) and \( pH_{\text{max}} \) were found to be independent of temperature. \( b_2 \) is a regression coefficient (h\(^{-1}\) °C\(^{-2}\)):

\[
\mu = b_2(pH - pH_{\text{min}})(pH - pH_{\text{max}})(T - T_{\text{min}})^2
\]

The same was done earlier by McMeekin et al. (1987); a water activity model was substituted into that have an interpretable meaning. The model proposed by McMeekin et al. (1992). Multiplicative models were used by Ratkowsky and Ross (1995) to describe the effects of \( T \), \( a_w \), pH and NO\(_2\) concentration on the growth of Shigella flexneri, and Presser et al. (1998) to describe the effects of \( T \), \( a_w \), pH and lactate concentration on the growth of E. coli. The principle of multiplication of effects is consistent with the earlier described hurdle concept (Leistner and Gorris, 1995). Each of the different effects, temperature, water activity, and pH, render their own specific hurdle. Here, the hurdles are multiplied to result in
an overall effect of the individual hurdles (Zwietering et al., 1996). The effect of, for instance, pH, \( \gamma(pH) \), can be made explicit as

\[
\gamma(pH) = \frac{\mu_{opt}}{\mu_{opt}} = \frac{b_2(pH - pH_{min})(pH - pH_{max})}{b_2(pH_{opt} - pH_{min})(pH_{opt} - pH_{max})} = \frac{(pH - pH_{min})(pH - pH_{max})}{(pH_{opt} - pH_{min})(pH_{opt} - pH_{max})}
\]

Eq. (5) has the advantage of containing only interpretable parameters because the regression coefficient \( b_2 \) is no longer part of the equation. If the effects of pH, temperature and water activity can be multiplied as described above, the individual gammas can be multiplied as well, resulting in an overall gamma:

\[
\gamma(\text{total}) = \gamma(T) \times \gamma(a_w) \times \gamma(pH)
\]

and the overall growth rate can be calculated as

\[
\mu(\text{total}) = \gamma(\text{total}) \times \mu_{opt}
\]

Therefore, if the multiplication of effects of temperature as described in Eq. (4) can be validated, the described gamma concept is also validated and can be used.

3. Materials and methods

To set up and validate the model, four independently measured data sets were used: three data sets were used to fit the parameters of the model and the fourth data set was used for validation. All measured growth rate data are based on plate counts, in order to measure the growth characteristics where growth is not yet affected by macroscopic effects such as pH changes, nutrient depletion and interactions between organisms. The first data set was previously measured to model the growth characteristics of *Lactobacillus curvatus* (LAB 962) as a function of temperature and pH. The acquisition of this data set was described by Wijtzes et al. (1995). The temperature range for the model is between 6 and 29°C, and the pH range is between 4.6 and 9.0. The measured water activity of the entire data set is 0.991.

The second data set was measured at a constant pH of 6.2 over the entire water activity and sub-optimal temperature range. MRS broth (Difco) was used as growth medium for *Lactobacillus curvatus* (LAB 962, LMG Culture Collection, Gent, Belgium, private collection). Water activity was set using NaCl (Merck) and measured using a Novasina (ER84/3H/63T; sensors: enBSK-4) calibrated with different salt solutions. The water activity range of this data set is between 0.964 and 0.979 and temperatures are between 1 and 25°C. Preincubation method, incubation, enumeration, plating and counting methods are described in Wijtzes et al. (1995).

The same was done for the third data set. In this data set, however, the pH was set to 5.8 using 2 N sterile HCl (Merck). The water activity range of this data set is between 0.932 and 0.990 and the temperature range is between 3 and 30°C. The preincubation method, incubation, enumeration, plating and counting methods are described by Wijtzes et al. (1995).

The three data sets are combined into one set, which consist of 164 growth curves. This combined data set is called the ‘data set for model fitting’ below.

The fourth data set was measured outside the temperature, water activity, and pH range where the model parameters are fitted. MRS (Difco) was used as growth medium for *Lactobacillus curvatus* (LAB 962). Water activity was set using NaCl (Merck) and measured using a Novasina (ER84/3H/63T; sensors: enBSK-4) calibrated with different salt solutions. pH was set using 2 N sterile HCl (Merck) and 2 N sterile NaOH (Merck). The chosen combinations at which growth was followed were, for temperature, 7, 10, 15, and 28°C, for water activity, 0.95, 0.96, and 0.97, and for pH, 5, 6, 7, and 7.5. The preincubation method, incubation, enumeration, plating and counting methods are described by Wijtzes et al. (1995). Various combinations were measured in replicate, resulting in a total of 47 growth curves. This is the ‘data set for validation’.

Fig. 1 shows the experimental setup at different constant temperatures. The lines represent the experiments of the data set for model fitting. The data points are the measured growth curves of the data set for validation. In all cases, the numbers of microorganisms in time are fitted to the modified Gompertz equation (Zwietering et al., 1990), resulting in estimates for lag time, growth rate, asymptote and initial number of organisms. A non-linear regression program with a Marquardt optimisation procedure.
was used (nlin procedure; SAS package; SAS Institute, USA).

3.1. Modelling procedure

Growth rates at different temperatures, water activities and pH values are fitted to Eq. (4) by means of a least square fitting routine that directly estimates the values of the parameters. To stabilise the variance of growth rate data, a square root transformation of growth rate was required. Since the model was set up for non-transformed growth rate, the right-hand side of Eq. (4) has to undergo the same transformation.

The data set for model fitting is fitted to the equation and the values for the parameters and 95% confidence intervals are estimated. Model predictions (fitted) are plotted against the measured values using the square root transformation method. The residuals, deviations from the diagonal, should be distributed homogeneously throughout the range of observed values and the values should not be too far away from the diagonal. Furthermore, the 95% prediction interval of each of the fitted growth rates is calculated (Lindgren, 1976) and plotted. Finally, an F-ratio test is carried out to assess the statistical quality of the model predictions.

Next, a comparison of the measured growth rates from the data set for model validation with the predictions of the fitted model is performed. The statistical acceptability is assessed in a predicted versus measured plot. The calculated 95% prediction interval of the equality line is used to assess the acceptability of the data points of the validation data set; if at least 95% of the measured data points fall inside the plotted 95% prediction interval, the model is accepted statistically. Furthermore, an F-ratio test is used to calculate the statistical acceptability of the predictions of the model.

In the last stage of the modelling procedure, the growth rate model (Eq. (4)) is fitted to all measured data. A fitted versus measured plot gives a graphical representation of the statistical acceptability of the model predictions. An F-ratio test is used to calculate the statistical acceptability of the predictions of the model.

3.2. F-ratio test

To assess the statistical quality of a model, an F-ratio test can be used. Two variances are compared, the variance of the fitted model and a typical \( \sqrt{\mu} \) variance \( (5.11 \times 10^{-3} \) at 20 degrees of freedom; Zwietering et al., 1994). The model is found to apply.
if the variance of the model is not significantly larger than the typical growth rate variance. This can be assessed by means of an $f$-ratio value, which is calculated as

$$f = \frac{\text{var(model)}}{\text{var(reference)}} = \frac{\text{RSS/df}}{5.11 \times 10^{-3}}$$

where RSS is the residual sum of squares of the fitted model and df is the degrees of freedom of the model. This $f$-ratio value should be smaller than a reference 95% $F_{df}^{159}$ value to obtain statistically significant equal model predictions.

### 4. Results and discussion

The data set for model fitting is fitted to the overall model resulting in an estimate for the parameters of the overall model (Eq. (4)) as given in Table 1. Fig. 2 shows the square root of the predicted growth rate versus the square root of the observed growth rate. As can be seen the distribution of errors is homogeneous throughout the entire range of measured growth rates. The calculated 95% prediction interval of the fitted growth rates is also shown. Of the measured data set, 95% of the data has to fall within the prediction intervals. Of the data set for model fitting of 164 data points, 156 points fall inside the 95% prediction interval, which is exactly 95% of the data points.

The degrees of freedom of the fitted model are 159, the RSS of the fitted model is 0.827, and the variance of the model is $5.20 \times 10^{-3}$. The calculated $f$ value equals 1.02, whereas the reference $F$ value equals 1.88 ($F_{20}^{159} = 1.88$), therefore the variance of the model equals the reference variance so the model predictions are acceptable statistically. This second form of validation agrees with the first described in the previous paragraph.

### Table 1

Values of the estimated parameters from Eq. (4) and their 95% confidence intervals; data set for model fitting

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated value</th>
<th>95% lower confidence interval value</th>
<th>95% upper confidence interval value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b$ (h$^{-1} \cdot ^{o} C^{-1}$)</td>
<td>$-2.34 \times 10^{-3}$</td>
<td>$-2.74 \times 10^{-3}$</td>
<td>$-1.94 \times 10^{-3}$</td>
</tr>
<tr>
<td>$T_{min}$ ($^{o} C$)</td>
<td>$-3.63$</td>
<td>$-4.66$</td>
<td>$-2.61$</td>
</tr>
<tr>
<td>$pH_{min}$</td>
<td>$4.24$</td>
<td>$4.11$</td>
<td>$4.37$</td>
</tr>
<tr>
<td>$pH_{max}$</td>
<td>$9.53$</td>
<td>$9.33$</td>
<td>$9.73$</td>
</tr>
<tr>
<td>$a_{w_{min}}$</td>
<td>$0.928$</td>
<td>$0.925$</td>
<td>$0.931$</td>
</tr>
</tbody>
</table>

Fig. 2. Fitted versus measured transformed growth rate ($\sqrt{\mu}$) and 95% prediction interval of the model based on the data set for model fitting.
The model with the fitted parameters, based on the data set for model fitting, is now used to predict growth rates of the validation data set. The measured growth rates from the data set for model fitting and the data set for model validation are plotted against the predicted growth rates (Fig. 3). The closer the data points are to the diagonal, the better the model predicts the measured growth rates from the validation data set. Only one data point of the validation data set falls outside the 95% prediction interval calculated with the model based on the data set for model fitting. This is only 2% of the data, whereas 5% is acceptable. The measurements of the validation data set, therefore, cannot be distinguished statistically from the predictions of the model based on the data set for model fitting.

The degrees of freedom for the prediction of the validation data set are 47, since no parameters are estimated, the RSS of the model predictions is 0.188, and the variance of the model predictions, therefore, is $4.0 \times 10^{-3}$. The calculated $f$ value equals 0.783, whereas $F_{20.42} = 2.03$. The model predictions are therefore acceptable statistically. This supports the conclusion in the previous paragraph that the measurements are not statistically dissimilar.

The growth rate from the data set for model validation can be predicted with the model developed for the data set for model fitting. Extrapolation should not be considered good practice, since models are only valid in the range where actual data were gathered, although in this case it is statistically allowed to extrapolate.

Finally, to refine the developed model, all data sets are used to re-estimate the value of the parameters. The parameter estimates are shown in Table 2. The degrees of freedom of the entire model are 206, the RSS of the model is 0.995, and the variance of the model is $4.83 \times 10^{-3}$. The calculated $f$ value equals 0.945, whereas the reference $F_{20.42}$ = 1.87, so the model is acceptable statistically. Fig. 4 shows the square root of the fitted growth rate versus the square root of the measured growth rate. All points are close to the diagonal, which was also indicated by the results of the $F$-ratio test.

As can be seen from Tables 1 and 2, the estimated parameters remain almost constant and the confi-
Table 2
Values of the estimated parameters from Eq. (4) and their 95% confidence intervals; all measured data sets

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated value</th>
<th>95% lower confidence interval value</th>
<th>95% upper confidence interval value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b_1$ (h^{-1} °C^{-1})</td>
<td>$-2.35 \times 10^{-3}$</td>
<td>$-2.72 \times 10^{-3}$</td>
<td>$-1.97 \times 10^{-3}$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (°C)</td>
<td>3.32</td>
<td>-4.20</td>
<td>-2.43</td>
</tr>
<tr>
<td>pH_{max}</td>
<td>4.23</td>
<td>4.10</td>
<td>4.35</td>
</tr>
<tr>
<td>pH_{min}</td>
<td>9.53</td>
<td>9.34</td>
<td>9.72</td>
</tr>
<tr>
<td>$a_{w,min}$</td>
<td>0.926</td>
<td>0.923</td>
<td>0.929</td>
</tr>
</tbody>
</table>

Even with more extreme values of the controlling variables, no interacting effects are necessary to describe the data. The preliminary assumption, that the effects of water activity, pH and temperature on bacterial growth rate can be multiplied over a fairly wide range of the controlling variables, remains likely. The described gamma concept can therefore also be applied over a wide range of controlling variables.

References


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