Modelling of microbial activity and prediction of shelf life for packed fresh fish

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Abstract

Prediction of shelf life based on growth of specific spoilage organisms (SSO) in model substrates was studied. The effect of CO$_2$ on the growth kinetics for *Photobacterium phosphoreum* and *Shewanella putrefaciens* was quantified and modelled. Results showed that microbial spoilage of packed cod stored with various concentrations of CO$_2$ was accurately predicted from the effect of CO$_2$ on *P. phosphoreum* grown in model substrates. The short shelf life extensions previously reported for packed cod therefore can be explained by the high CO$_2$ resistance of this Gram negative organism. *S. putrefaciens* was very sensitive to CO$_2$ and growth rates could not be related to the shelf life of packed cod.

Growth curves without lag phases were found for all concentrations of CO$_2$ and for both the microorganisms studied. For the fitting of these growth curves the log-transformed Logistic models were selected after comparison with the 'modified Gompertz' models and with the model of Baranyi et al. (1993). The effect of CO$_2$ on $\mu_{\text{max}}$ was well described by a 2 parameter square root model. Validation of kinetic models by comparison of shelf life predictions with shelf life determined by sensory evaluations in product experiments was preferred for comparison of microbial growth rates determined in product and model system experiments. Kinetic modelling was found to be valuable for both evaluation and prediction of microbial fish spoilage and an iterative approach for development of kinetic shelf life models was suggested.

Keywords: Kinetic modelling; CO$_2$; *Photobacterium phosphoreum*; *Shewanella putrefaciens*; Shelf life prediction; Fish spoilage

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1. Introduction

The shelf life of fresh fish products depends on storage conditions (temperature and atmosphere) and of the initial fish quality. Based on storage trials, empirical shelf life models of the effect of temperature and of the effect of initial product quality have been suggested. These models were not based on knowledge of spoilage reactions but useful shelf life predictions were obtained from time-temperature profiles or rapid methods of initial product quality (Spencer and Baines, 1964; Olley and Ratkowsky, 1973; Gibson, 1985; Bremner et al., 1987; Jørgensen et al., 1988). No empirical product prientated models, however, have been suggested for the effect of storage atmosphere on shelf life or for the keepability of lightly preserved fish products.

Microbial activity limits the shelf life of packed as well as unpacked fresh fish products (Herbert et al., 1971; Jørgensen et al., 1988; Dalgaard, 1995). It is therefore possible that kinetic models of spoilage organisms grown in model substrates can be used for prediction of shelf life. This of course will require that SSO are known and that growth kinetic parameters can be estimated accurately.

*Shewanella putrefaciens* was found important for spoilage of packed cod (Lee, 1981; Cann et al., 1983; Jørgensen et al., 1988). Dalgaard (1995), however, concluded that *S. putrefaciens* was not important for spoilage of packed cod and that *Photobacterium phosphoreum* were responsible for spoilage of these products.

For spoilage organisms grown in model substrates, the effect of environmental conditions on the microbial activity should correspond to the effect of the same storage conditions on product shelf life. Kinetic models therefore can be used, for shelf life prediction but also as a tool to test the importance of different potential spoilage microorganisms.

The purpose of the present study was to evaluate the possibility of predicting shelf life of packed cod from growth and activity of specific spoilage organisms in model substrates. Different growth models for estimation of kinetic parameters were compared and the effect of CO₂ on the maximum specific growth rate of *P. phosphoreum* and *S. putrefaciens* was quantified and modelled. Two methods of model validation were compared and an iterative approach for development of kinetic spoilage models was suggested.

2. Materials and methods

2.1. Bacteria and media

Strains of *P. phosphoreum* and *S. putrefaciens* were isolated from packed cod fillets stored at 0°C with five different concentrations of CO₂ (Dalgaard et al., 1993; Dalgaard, 1995). A mixture of five strains of *P. phosphoreum* (MIX-P), a mixture of five strains of *S. putrefaciens* (MIX-S) and two individual strains of *S. putrefaciens* (S-0 and S-100) were studied. The mixtures contained strains isolated
from products stored with different levels of CO₂ from 0 to 100%. S-0 and S-100 were from products stored with 0–5% CO₂ and with 90–100% CO₂, respectively.

Growth experiments were carried out in cooked fish muscle juice (FJ) (Dalgaard, 1995) and in Growth Medium Broth (GMB) (Dalgaard et al., 1994). Iron Agar (Oxoid, CM867) was used for determination of viable counts (VC).

2.2. Pre-culture and preparation of inoculum

Strains were inoculated individually in test tubes with growth medium. The tubes were incubated in 100% N₂ in anaerobic jars (Oxoid) at 0°C until an absorbance value of 0.1–0.5 indicating that the culture had not entered the stationary phase. For experiments with MIX-S and MIX-P approximately equal concentrations of the individual cells, determined from absorbance measurements, were transferred to a sterile test tube and mixed. MIX-S, MIX-P, S-0 and S-100 were then diluted in growth medium and used as inoculum.

2.3. Model system experiments

The effect of CO₂ was studied for MIX-P, MIX-S, S-0 and S-100. Growth experiments were carried out at 0°C ± 1°C in CO₂/N₂ containing atmospheres with 0, 25, 50, 75 and 100% CO₂. Growth medium was incubated in Erlenmeyer flasks in anaerobic jars as previously described (Dalgaard, 1995). Temperature was controlled by placing the anaerobic jars in a Refritherm 6E (Struers Ltd., Rødovre, Denmark). Growth of MIX-P, MIX-S, S-0 and S-100 in FJ was determined by viable counts (VC) (4 × 5 growth curves) and growth of MIX-P in GMB (2 × 5 growth curves) was determined by VC and by absorbance measurements. Growth rates were estimated from absorbance measurements as previously described (Dalgaard et al., 1994). All cultures were inoculated with 10²–10⁴ cfu/ml.

2.4. Analyses

Viable counts. One ml of medium was appropriately diluted in physiological saline containing 0.9% NaCl and 0.1% Bacto-peptone (Difco). Counts of P. phosphoreum were determined on spread plates of IA supplemented with 0.5% NaCl (10°C, 7 days) and counts of S. putrefaciens in IA (25°C, 3 days). Absorbance (ABS) was measured at 540 nm (Spectronic 20, Milton Roy Compagny, USA).

2.5. Modelling of growth curves

The 3-and 4-parameter Logistic models (see Ratkowsky, 1983; Seber and Wild, 1989 for reviews of growth models) were fitted to viable count growth curves. As in previous studies a log-transformation was found appropriate for stabilizing the
variance of VC growth data (Dalgaard et al., 1994). Eqs. 1 and 2 show the integrated and log-transformed form of the 3-and 4-parameter Logistic models.

\[
\log(N(t)) = \log\left(\frac{N_{\text{max}}}{1 + \exp(-\mu_{\text{max}}(t - t_0))}\right)
\]

(1)

\[
\log(N(t)) = \log\left(N_{\text{min}} + \frac{N_{\text{max}} - N_{\text{min}}}{1 + \exp(-\mu_{\text{max}}(t - t_0))}\right)
\]

(2)

\( t \) is the time (hours), \( N(t) \) the cell concentration at time \( t \), \( N_{\text{min}} \) and \( N_{\text{max}} \) the minimum and maximum asymptotic cell concentration (cfu/ml or cfu/g), \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)) and \( t_0 \) the time (hours) when half the maximum cell concentration is reached. The parameter \( N_{\text{min}} \) allow the 4-parameter Logistic model to have an asymptotic concentration of bacteria different from zero.

The lag time of microbial cultures has been defined as the time a growth curve is delayed relative to exponential growth from the same initial cell concentration (Fig. 1, Lodge and Hinshelwood, 1943; Monod, 1949). In agreement with this definition, an expression for lag time estimation (Eq. 3), has been obtained by setting the cell concentration at time zero, \( N(0) \), expressed by the 4-parameter Logistic model equal to \( N \) (lag time) expressed by the 3-parameter model.

\[
\text{Lag time} = t_i - \frac{1}{\mu_{\text{max}}} \times \ln\left(\frac{N_{\text{max}} + N_{\text{max}} \times \exp(\mu_{\text{max}} \times t_i)}{N_{\text{max}} + N_{\text{min}} \times \exp(\mu_{\text{max}} \times t_i)} - 1\right)
\]

(3)

For a given growth curve the presence of a significant lag phase can be tested by an \( F \)-test for comparison of the 3-and 4-parameter Logistic models.

\( \mu_{\text{max}} \) was determined for absorbance growth curves as previously described (Dalgaard et al., 1994).
2.6. Comparisons of growth models

The 'modified Gompertz' models of Gibson et al. (1987) and of Zwietering et al. (1990), the model of Baranyi et al. (1993) and the log-transformed 4-parameter logistic model (Eq. 2) were compared. The difference of estimated values of lag time, $\mu_{\text{max}}$ and of the residual mean squares (rms) were used for comparison of the models. Rms were calculated as the residual sum of squares divided by the degrees of freedom.

Models were fitted by Fig. P (Anonymous, 1991) or by special software (DMDEMO.EXE) written by József Baranyi.

2.7. Shelf life prediction and model validation

For exponentially growing cultures, without a lag phase, shelf life can easily be calculated as the time SSO requires for multiplication from $N(0)$ to a minimal spoilage level (MSL) (Eq. 4).

\[
\text{Shelf life (days)} = \frac{[\log(\text{MSL}) - \log(N(0))] \times \ln(10)}{\mu_{\text{max}}(\text{hours}^{-1}) \times 24} \tag{4}
\]

To use Eq. 4, however, requires that a generally accepted MSL can be identified. Very variable levels of *S. putrefaciens* have been reported at the time of sensory rejection of packed cod but a level of about 30 mg-N trimethylamine (TMA)/100 g were found in several studies (Jensen et al., 1980; Cann et al., 1983; Jørgensen et al., 1988; Dalgaard et al., 1993). Therefore, shelf life was predicted as the time *S. putrefaciens* and *P. phosphoreum* required for production of 30 mg-N TMA/100g (Eq. 5). This equation was obtained from the exponential growth model combined with the yield factor for TMA production ($Y_{\text{TMA/CFU}}$). Values of $\mu_{\text{max}}$ were determined in the present study. $Y_{\text{TMA/CFU}}$ for *P. phosphoreum* and *S. putrefaciens* were recently estimated to be $10^{-8.8}$ mg–N TMA/cfu and $10^{-9.5}$ mg-N TMA/cfu, respectively (Dalgaard, 1995).

\[
\text{Shelf life (days)} = \frac{[\log\left(\frac{30 \text{mg-N TMA/100 g}}{Y_{\text{TMA/CFU}} \times 100} + N(0)\right) - \log(N(0))] \times \ln(10)}{\mu_{\text{max}}(\text{hours}^{-1}) \times 24} \tag{5}
\]

Two methods were tested for validation of the predictive models of *S. putrefaciens* and *P. phosphoreum*, developed in model substrates. Firstly, shelf life and TMA production observed in product experiments were compared to microbial growth and TMA production. Secondly, $\mu_{\text{max}}$ of *S. putrefaciens* determined in product experiments was compared to $\mu_{\text{max}}$ values from model system experiments. No selective techniques for enumeration of *P. phosphoreum* are available and $\mu_{\text{max}}$ in the product studies therefore has not been determined.
Shelf life, TMA production and growth of *S. putrefaciens* were determined in nine replicated product experiments with packed cod fillets stored at 0°C. Fillets were stored in different concentrations of CO₂ as previously described (Dalgaard et al., 1993). These results were used for validation of the kinetic models of the effect of CO₂ on the activity of *S. putrefaciens* and on *P. phosphoreum*.

3. Results

3.1. Modelling of growth curves

In Fig. 2 typical examples of growth curves from model system and product experiments are shown. From these growth curves almost identical estimates of kinetic parameters were obtained from the 4-parameter Logistic function and from the model of Baranyi et al. (1993) (Fig. 3). On average estimates of $\mu_{\text{max}}$ from the ‘modified Gompertz’ models of Gibson et al. (1987) and of Zwietering et al. (1990) were respectively, 19 and 14% higher than $\mu_{\text{max}}$ estimated from the Logistic model. Opposed to the Logistic and to the Baranyi et al. (1993) model, several large negative lag times estimates were obtained from the ‘modified Gompertz’ model of Gibson et al. (1987) (Fig. 3). The Zwietering et al. (1990) model gave some negative lag time estimates but of smaller magnitude than the other ‘modified Gompertz’ model (results not shown).

*F*-tests for comparison of the log-transformed 3-and 4-parameter Logistic models showed that no significant lag phases were found in any of the 18 product experiments. A significant lag phase was only estimated in two of the 20 VC growth curves from model system experiments with *P. phosphoreum* and *S. putrefaciens*. Consequently, $\mu_{\text{max}}$ is the most important parameter for prediction of shelf life.
3.2. Modelling the effect of CO$_2$

Similar values of $\mu_{\text{max}}$ were obtained for *P. phosphoreum* grown in FJ and in GMB and similar values were also obtained for MIX-S, S-0 and S-100 grown in FJ. Average values ± standard deviations are indicated in Fig. 4. A simple square root type model (Eq. 6) was found appropriate for describing the effect of CO$_2$ on $\mu_{\text{max}}$:

\[ \sqrt{\mu_{\text{max}}} = b(\%\text{CO}_2,_{\text{max}} - \%\text{CO}_2) \]  

(6)
Fig. 4. Effect of CO\textsubscript{2} on the maximum specific growth rates ($\mu_{\text{max}}$) of *Shewanella putrefaciens* (squares) and of *Photobacterium phosphoreum* (circles). Experiments were carried out at 0°C under anaerobic conditions.

In this two parameter model $\%CO\text{\textsubscript{2}}_{\text{max}}$ is the CO\textsubscript{2} concentration where $\mu_{\text{max}}$ theoretical becomes zero. The parameter in this way corresponds to $T_{\text{min}}$ in the Ratkowsky square root model (McMeekin et al., 1993). 100% CO\textsubscript{2} reduces $\mu_{\text{max}}$ of *P. phosphoreum* by about 40%, $\%CO\text{\textsubscript{2}}_{\text{max}}$ was 400% (4.0 atm). 100% CO\textsubscript{2} reduces $\mu_{\text{max}}$ of *S. putrefaciens* by about 90%, $\%CO\text{\textsubscript{2}}_{\text{max}}$ was 150% (1.5 atm).

Fig. 5. Observed and predicted maximum specific growth rates ($\mu_{\text{max}}$) for *Shewanella putrefaciens* grown at 0°C. Observed values were determined in product studies with packed cod and predicted values were from model system experiments incubated with the same CO\textsubscript{2} level as used in the product studies (remaining gas being N\textsubscript{2}).
3.3. Prediction of shelf life and model validation

Table 1 shows the effect of CO₂ on predicted and observed shelf lives of packed cod. The values of \( \mu_{\text{max}} \) used for prediction of shelf life were obtained from Eq. 6 as shown in Fig. 4.

The kinetic model of \( P. \) phosphoreum was found valid from 0% to between 50 and 100% CO₂. The model predicted microbial spoilage accurately for CO₂ concentrations from 0% to 100% as the effect of CO₂ on the TMA production in packed cod corresponded to the predicted TMA production for \( P. \) phosphoreum grown in model substrates. The keepability of products stored in 100% CO₂ was limited by non-microbial spoilage reactions giving fillets a soft texture (Dalgaard et al., 1993) and shelf life of these products were therefore overestimated (Table 1).

At high CO₂ concentration shelf life predicted from the activity of \( S. \) putrefaciens was considerably overestimated (Table 1). Fig. 5 shows a comparison of \( \mu_{\text{max}} \) for \( S. \) putrefaciens determined from product studies and from model system experiments. In general \( \mu_{\text{max}} \) determined in model system experiments was higher than \( \mu_{\text{max}} \) found in product experiments stored in the same concentration of CO₂.

4. Discussion

It has been shown that microbial spoilage of packed fish can be predicted from the activity of the specific spoilage organism, \( P. \) phosphoreum, grown in model substrates (Table 1). The results, however, clearly show that a kinetic model for \( S. \) putrefaciens, an organism without importance for spoilage of packed cod, can result in very misleading shelf life predictions. Also, when non-microbial spoilage reactions influence the keepability of a product, shelf life predicted from kinetic microbial models naturally will be overestimated.

The predicted shelf lives confirm that \( P. \) phosphoreum is the organism primarily responsible for spoilage of packed cod. The spoilage domain of this organism, defined as the range of conditions where a given SSO is causing spoilage, include CO₂ concentrations from 0% to somewhere between 50 and 100% CO₂.

\( \mu_{\text{max}} \) for \( S. \) putrefaciens were somewhat higher in model substrates than in product experiments (Fig. 5). This, however, does not assure that shelf life prediction from growth in model substrates are fail-safe. The results show that comparison of observed and predicted values of growth rates should not be used for validation of kinetic shelf life models or at least such comparisions should only be used within the spoilage domain of a given SSO. For the few products where the SSO have been identified little is known about the spoilage domains of these organisms. We therefore suggest kinetic spoilage models should be validated against shelf life data determined by sensory evaluations and not by comparison of growth rates.

That the 'modified Gompertz' models overestimate values of \( \mu_{\text{max}} \) corresponds to results from other studies (Whiting and Cygnarowicz-Provost, 1992; Baranyi et al., 1993; McClure et al., 1993; Dalgaard et al., 1994). The 10–20% overestimation of \( \mu_{\text{max}} \) obtained by the 'modified Gompertz' models, however, is of relatively
moderate importance compared to the possible errors that can be introduced when shelf life predictions are based on kinetic models used outside the spoilage domain of a given SSO (Table 1). The 'modified Gompertz' models, however, can provide negative lag time estimates, indicating that these models are inappropriate for estimation of kinetic parameters from growth curves of the studied fish spoilage bacteria. For these growth curves the log-transformed Logistic models were preferred as they are simple, easy to use together with non-linear regression programmes and give the same estimates of kinetic parameters as the more complex model of Baranyi et al. (1993).

MAP with high CO\textsubscript{2} concentrations have caused the dominating microflora of both meat and fish products to change from Gram negative to Gram positive organisms. CO\textsubscript{2} increases lag times and reduces growth rates and Gram positive bacteria in general were more resistant to CO\textsubscript{2} than Gram negative (see Dixon and Kell, 1989; Stammen et al., 1990; Farber, 1991; Dainty and Mackey, 1992 for reviews). Precultures used in the present study assured that media were inoculated with actively growing cells. This may explain that no lag times were detected even for strains grown in 100% CO\textsubscript{2}. The CO\textsubscript{2} resistance of \textit{P. phosphoreum} explains why shelf life of MAP cod is extended by less than one week compared to aerobic and VP storage. When taking into account that the solubility of CO\textsubscript{2} is reduced with increasing temperatures (Ogrydziak and Brown, 1982) the CO\textsubscript{2} resistance of \textit{P. phosphoreum}. 100% CO\textsubscript{2} reduces \( \mu_{\text{max}} \) by 40% at 0°C, is comparable to the most CO\textsubscript{2} resistant Gram positive organisms (Molin, 1983). The shelf life extension of MAP cod, however, is shorter than found for MAP products where a Gram positive microflora becomes dominating indicating that \textit{P. phosphoreum} grows faster at chilled temperatures than these Gram positive organisms. We have found no data for the CO\textsubscript{2} resistance of \textit{P. phosphoreum} to compare our results to.

At 25°C, the growth rate of \textit{S. putrefaciens} was reduced by 12–35% with 20% CO\textsubscript{2} and by 60–90% with 60% CO\textsubscript{2} (Lee, 1981). At 3°C, 60% CO\textsubscript{2} reduced \( \mu_{\text{max}} \) by 50% (Gill and Tan, 1980). Fig. 4 shows that 20% CO\textsubscript{2} and 60% CO\textsubscript{2} reduced

### Table 1

<table>
<thead>
<tr>
<th>Observed shelf life a</th>
<th>0% CO\textsubscript{2}</th>
<th>25% CO\textsubscript{2}</th>
<th>50% CO\textsubscript{2}</th>
<th>75% CO\textsubscript{2}</th>
<th>100% CO\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed TMA production during spoilage of packed cod fillets</td>
<td>13–14</td>
<td>16</td>
<td>20</td>
<td>–</td>
<td>15–16</td>
</tr>
<tr>
<td>Predicted TMA production by \textit{P. phosphoreum} (1–10 cfu/ml b)</td>
<td>12–14</td>
<td>14–16</td>
<td>16–19</td>
<td>19–22</td>
<td>21–24</td>
</tr>
<tr>
<td>Predicted TMA production by \textit{S. putrefaciens} (100 cfu/ml c)</td>
<td>13</td>
<td>21</td>
<td>31</td>
<td>48</td>
<td>80</td>
</tr>
</tbody>
</table>

a Shelf life determined by sensory methods from product experiment.
b Assumed initial level of bacteria.
c Initial level of bacteria measured in product studies.
\[ \mu_{\text{max}} \text{ of } S. \text{ putrefaciens by } 35 \] and 65\%, respectively and the \( CO_2 \) sensitivity determined in the present study correspond to other previous results.

The model predictions (Table 1) clearly show that an organism as \( CO_2 \) sensitive as \( S. \text{ putrefaciens} \) can not possibly be responsible for spoilage of product with the short shelf life extensions found for fresh fish products. Formulating the \( CO_2 \) sensitivity of this organism as a kinetic model (Eq. 4), however, has facilitated a comparison of the microbial response determined in model substrates with data from product experiments.

Spoilage reactions (SR) and the spoilage microflora of fresh and lightly preserved fish products are most likely to change when storage conditions are changed over a wide range (Gram et al., 1987; Liston, 1992; Dalgaard et al., 1993). This dynamic nature of the spoilage process indicate that empirical product orientated models rather than kinetic models may be the most useful for predicting the effect of different storage conditions on shelf life. Kinetic modelling, however, seems to be valuable for evaluation of microbial fish spoilage and when sufficient knowledge of SR, SSO and the spoilage domain have been obtained the kinetic models can provide shelf life predictions.

Several predictive models for pathogenic organisms have been developed by a batch-wise approach, where growth was quantified in large factorial model systems experiments and later validated by comparison with results from product studies. The number of microorganisms potentially causing spoilage of a product is likely to be very high and many of these are not known. For example was \( P. \text{ phosphoreum} \) only recently found to be responsible for spoilage of packed cod (Dalgaard, 1995). In this situation a batch-wise modelling approach may result in modelling growth under conditions where the organism studied is of no importance for spoilage. In relation to fish spoilage an evaluation of the spoilage process will most often be needed before kinetic spoilage models can be developed. We find that an iterative approach in this situation is most likely to be successful for modelling of spoilage. Factors influencing shelf life could be identified by step-wise comparison of results from model system and product systems experiments. In situations where a given SSO is found to have an extended spoilage domain a kinetic spoilage model can be developed from model system experiments.

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References

Anonymous, (1991) Fig. P user’s Manual version 6.0 Fig. P. Fig. P Software Corporation, Durham, North Carolina, USA.


