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Time to turbidity measurement as a tool for modeling spoilage by *Lactobacillus*

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SUMMARY

A method has been proposed to obtain growth rate estimates from simple time-to-visible-growth measurements by means of inoculum variation. In case the data are censored an algorithm using a maximum likelihood estimation method is given. Growth rates for *Lactobacillus plantarum* obtained by this method have been used to develop a model for the prediction of the growth rate as a function of temperature and pH. The model was validated by plate counts. It can be applied in a pH range of 3.2 to 8 and a temperature range of 6 to 21 °C.

INTRODUCTION

In the past 'absolute' keepability of mayonnaise and dressings was required, and products were formulated in such a way that spoilage organisms were prevented from growing. Today, however, there is a tendency towards milder products and consequently such products are more vulnerable to microbial spoilage. Moreover, a limited shelf-life is often acceptable and achievable with modern logistic systems. Therefore, more detailed knowledge of the effect of various factors on growth kinetics of spoilage organisms is required. Ideally, the influence of various factors on lag times and growth rates in the food itself should be known. However, very often growth in laboratory medium is an excellent reflection of growth in the actual food. This is illustrated in Fig. 1, which shows the growth of *Lactobacillus plantarum* in MRS broth [2] and a mayonnaise water phase.

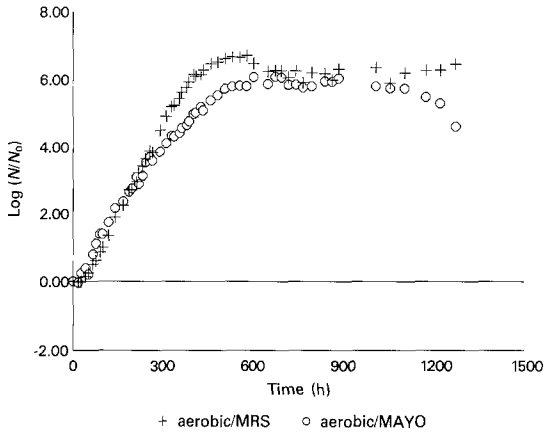

Fig. 1. Growth of *L. plantarum* at pH 4.1, temperature 12 °C. Mayo = mayonnaise water phase.

MODELING GROWTH IN A LABORATORY MEDIUM

For our experiments we have chosen MRS from which acetic acid and citrate were omitted because of possible inhibitory action of these acids at low pH. *Lactobacillus plantarum* was chosen as a model organism.

There are several methods to determine lag times and growth rates. Plate count is by far the most reliable method to establish growth curves, but it is extremely laborious. An alternative is the measurement of optical density. However, this method is not reliable enough because only the last part of the growth curve is measured.

GROWTH PARAMETERS FROM TIME TO TURBIDITY

An alternative method of obtaining information on lag time and growth rate is by measurement of the time at which growth becomes visible combined with inoculum size variation. The time to reach visible growth can be measured in bioscreen, microtiter plates or tubes. We chose tubes because of the versatility of the method.

There is no reason to assume that the growth rate is dependent on the size of the inoculum, because it is almost totally determined by the genotypic properties of the organism and the culture conditions. On the other hand, duration of the lag is influenced by the previous history of

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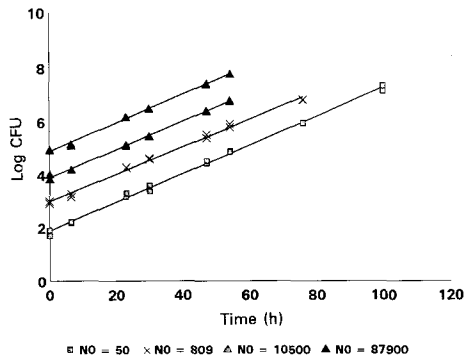

Fig. 2. *L. plantarum* at pH 4.1; 15 °C exponentially growing inocula; (pH 5.7, 30 °C). N_0 = inoculum size (cells ml^{-1}).

the cell: damaged cells usually show very long lag times whereas the lag time of a cell inoculated from an exponential phase culture may be absent or negligible (Fig. 2). When the inoculum is in the stationary phase a clear lag is observed, which is independent of inoculum size (Fig. 3). Only when the inoculum is subjected to extreme stress conditions might the inoculum size have an influence on the duration of the lag. The development of a general model for lag is therefore extremely complicated. In our experiments we have avoided extreme conditions to ensure that lag is independent of inoculum size.

If the growth curve is simplified by the tangent in the point of maximum growth, the growth rate can be directly derived from the linear relation between time to turbidity, t_{turb}, and log[inoculum size]:

$$t_{turb} = t_{lag} + \{\log[N_{turb}] - \log[N_i]\}/r, \quad (1)$$

where

t_{lag} = the duration of the phase,
N_{turb} = the number of organisms ml^{-1} at which turbidity is reached (for *L. plantarum* in MRS this number was established as $10^{6.4}$,
N_i = the number of organisms ml^{-1} of the inoculum, and
r = the growth rate constant (the slope of the growth curve).

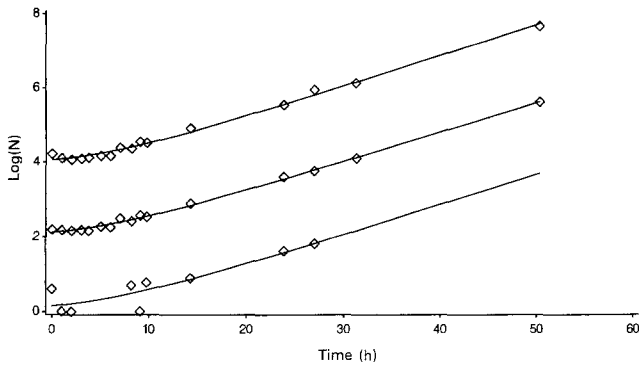

Fig. 3. Growth of *L. plantarum* in MRS at 15 °C and pH 5.7 inoculum overnight culture.

By varying the inoculum size N_i, a plot of t_{turb} vs 6.4 − log[N_i] shows a linear relationship, from which the lag time and the growth rate constant can be derived as the intercept and the reciprocal slope of the regression line, respectively.

Estimation of growth rate from time to turbidity

Although the model is simply linear it should be borne in mind that the data is censored, because observation of turbidity was only carried out at intervals. The exact time to turbidity of a particular tube lies therefore somewhere between the first moment that turbidity was observed and the previous observation time. Hence, most observations were interval-censored and for conditions where no growth occurred during the experiment right-censored values were obtained.

The SAS-package [3] provides a procedure LIFEREG which fits linear models to censored data. It uses a maximum likelihood estimation method.

A second problem was, that as time to turbidity increases its variance increases also. This problem is usually overcome by analyzing the logarithm or the square root of the response, whichever of the two gives the best stabilization of the variance. In our case, however, this would ruin the linearity of the model and make the use of LIFEREG impossible. The only way to maintain a linear model and account for the increasing variance is to use weighted regression. Weighting each observation with its reciprocal variance is equivalent to applying the above mentioned transformation. If we know that the variance increases with the square of time to turbidity, the weights used should be the reciprocal square of time to turbidity. However, as the data for time to turbidity is censored, we have no unique values for it. Therefore we have to use the *predicted* values of the time to turbidity for weighting. Theoretically this is a better method anyway, but not often used because it requires iterative calculations, even with simple linear regression. The latter is obvious, because initially, we have no predicted values available. So, the estimation procedure we used is as follows:

(1) Initially, weights are calculated from the observations (the mean value of the lower and upper censoring value for interval-censored observations and the lower one for right-censored observations).
(2) The model is applied using these weights in LIFEREG, and predicted values are calculated.
(3) Using the predicted values, the weights are recalculated.
(4) Stages (2) and (3) are repeated until the model parameters converge (usually 5 to 6 iterations).

Application of this method to time to turbidity data showed that lag time estimates were rather poor, but growth rates could be estimated quite well. In view of the above considerations on lag time we concentrated on modeling the growth rate. A typical result with some right-censored values is given in Fig. 4.

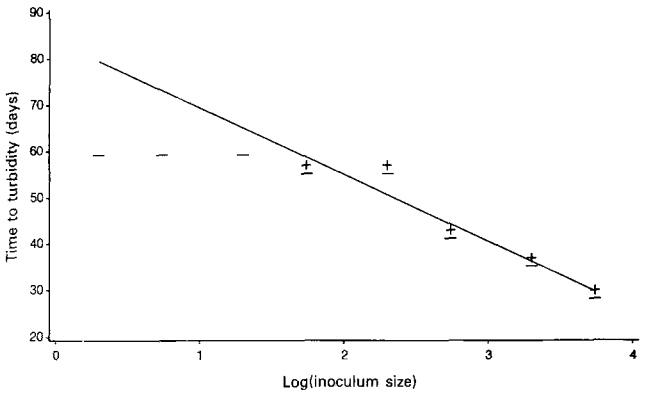

Fig. 4. Estimation of growth rate from time to turbidity data. – represents an observation where growth was not visible; + represents an observation where growth was visible.

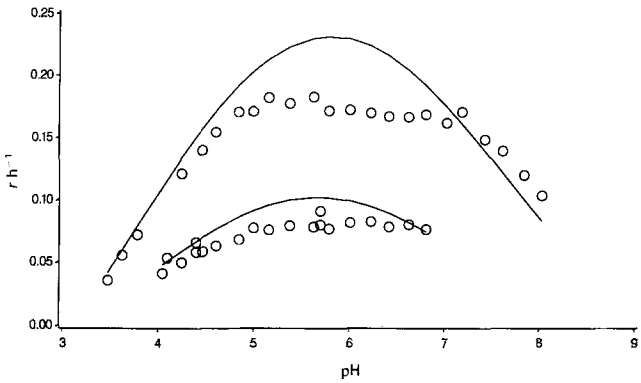

Fig. 5. Growth rate of *L. plantarum*. Extrapolated predictions by expanded growth model at 15 (lower curve) and 21 °C (upper curve).

pH/TEMPERATURE MODEL

From time to turbidity studies we calculated growth rates at pH 3.8, 4.1 and 4.4 (pH was adjusted with HCl) each at 8, 10, 12 and 15 °C.

In order to fit the data the model built by Adams et al. [1] was used in the squared form:

$$r = b(\mathrm{pH} - \mathrm{pH}_{\min})\,(T - T_{\min})^2 \qquad (2)$$

where

r = growth rate constant,
pH = the lower pH limit for growth,
$T_{\min}$ = the lower temperature limit for growth, and
b = constant.

As this model is not linear in the parameters the non-linear regression procedure NLIN in SAS [3] was used. In order to stabilize the variance, weighted regression was applied. The reciprocal squared predicted rate constants were used as weights.

The fit was satisfactory but extrapolation towards higher pH failed. For example, at pH 6.7 the predicted rate was far too high, because the model assumes that the growth rate increases linearly with pH. For physiological reasons, however, it is obvious that there must be a maximum pH above which growth is no longer possible. Moreover it was felt that the minimum growth temperature should not necessarily be independent of pH, especially when covering a wider pH range, say to pH 7 or 8.

To achieve a maximum in the growth rate as a function of pH we introduced a term ($\mathrm{pH}_{\max}$ – pH) into the model, analogous to the lower pH limit. As regards the minimum growth temperature it was assumed that it should be higher near $\mathrm{pH}_{\min}$ and $\mathrm{pH}_{\max}$ and at a minimum in the neighborhood of the optimum pH. Therefore the model was expanded to the following form:

$$r = b(\mathrm{pH} - \mathrm{pH}_{\min})(\mathrm{pH}_{\max} - \mathrm{pH})(T - T_{\min})^2 \qquad (3)$$
$$T_{\min} = T_0 + p(\mathrm{pH} - \mathrm{pH}_0)^2$$

where

r = growth rate constant,
$\mathrm{pH}_{\min}$ = the lower pH limit for growth,
$\mathrm{pH}_{\max}$ = upper pH limit,
pH_0 = the pH at which $T_{\min}$ has its lowest value,
T_0 = the lowest value of $T_{\min}$,
T = the temperature, and
b,p = constants.

The expanded model fitted the pH 6.7 data much better.

Model validation

To test the predictive power of the model we determined the growth rate of *L. plantarum* at 15 and 21 °C within a pH range of 3.5–8 by means of plate counts. Figure 5 shows the observations and the model predictions. Bearing in mind that these predictions were far extrapolations, especially the observations at 21 °C, the result was not too bad. After recalculation of the model parameters using all data together, the fit of the plate count data was improved without affecting the fit of the original time to turbidity data (Fig. 6). Figure 7 shows observed vs calculated rates for all observations. The parameter estimates of the updated model were as follows:

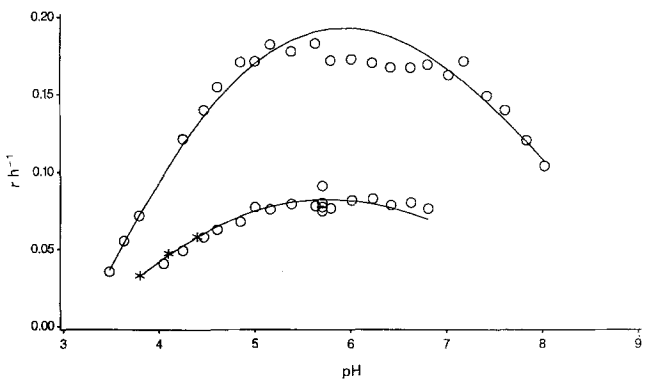

Fig. 6. Growth rate of *L. plantarum*; predictions by updated expanded growth model at 15 and 21 °C.

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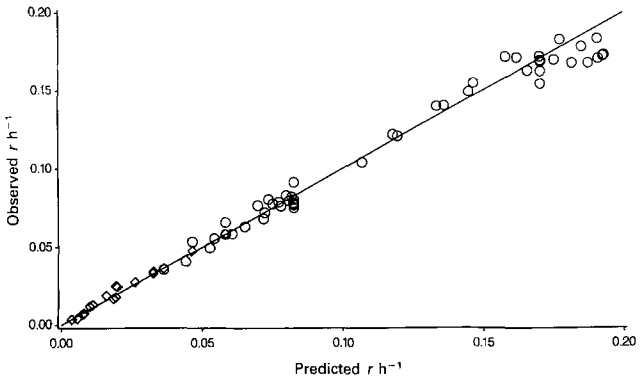

Fig. 7. Growth rate of *L. plantarum*; expanded growth model. Diamonds represent time to turbidity data and circles plate count data.

pH_{min} = 3.19,
pH_{max} = 9.8,
T_0 = 2.94 °C,
pH_0 = 4.3,
b = 6.1×10^{-5}, and
p = 0.24.

The model with a pH-dependent T_{min} gave a significantly smaller residual weighed Sum of Squares than the model with constant T_{min}. Also, the parameter p appears to be significantly positive at the 95% level. Both observations support our opinion that T_{min} is dependent on pH.

CONCLUSION

Time to turbidity measurements provide a simple means of establishing reasonable estimates of growth rate under various conditions. Use of an exponentially growing inoculum would appear to be the method of choice. A model for the growth rate of *L. plantarum* has been developed which can be applied between pH 3.2 and 8 and temperatures between 6 and 21 °C.

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