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The use of automated tubidimetric data for the construction of kinetic models

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SUMMARY

An automated tubidimetric instrument (Bioscreen) was used to observe the growth response of *Listeria monocytogenes* to combinations of temperature (15–30 °C), hydrogen-ion (0.1–21.9 μm) (equivalent pH 4.66–7.0) and NaCl concentration (0.5–9.5% w/v). Compared to traditional plate count techniques, the technique allowed many more data points to be captured and replicates to be used, with less expenditure of effort. Optical density curves were filtered (smoothed) to minimize the effect of signal noise and the mean signal from uninoculated wells was subtracted to minimize the effect of signal draft. A novel procedure for fitting growth curves to optical density data has been developed. The procedure involves the use of the logistic function and a calibration equation for fitting, in a single step, in the dimension of optical density. This approach allowed the four parameters of the logistic equation to be derived at each set of experimental conditions. A quadratic response surface was then fitted to the curve parameters using temperature, NaCl and hydrogen-ion concentration as three independent variables. Predicted time to 1000-fold increase in cell numbers compared well to predictions from predictive microbial growth equations generated in other laboratories using traditional plate counting. We propose that this technique should be further evaluated as a method for generating data for modeling the kinetics of microbial growth.

INTRODUCTION

Optical density or absorbance has been used for many years to measure the concentration, expressed as mass, number or mean cell length, of bacterial suspensions [24]. The advent of automated turbidimetric instruments (e.g. Bioscreen) has recently allowed microbiologists to acquire optical density measurement much more efficiently. This approach has been used for example, to enumerate bacteria in food samples [17,19,20,21]. Bioscreen has also been used to observe the effects of different organic acids (and mixtures thereof) on the growth rates of *Salmonella enteritidis* and *Escherichia coli* in a laboratory medium [1] and has been shown to be a useful tool for screening the effects of combinations of antimicrobial factors on growth of microorganisms [22].

Predictive microbiology, where the responses of microorganisms can be modeled with respect to the main controlling factors, such as pH, temperature and a_w offers many advantages over traditional challenge testing [30]. Kinetic models are perhaps the most useful, as they can be used to predict changes in microbial numbers in time even if one (or more) of the controlling factors affecting growth is changing e.g., during a chilled distribution chain. Development of such kinetic models [2,3,4,14] however, usually requires a large number of viable count growth curves to be generated which is time consuming and involves large quantities of laboratory media. If some of this information could be generated in a more cost-effective manner, it would greatly facilitate generation of models predicting microbial growth and drastically cut the cost of effort and materials required to generate the data necessary for the construction of such models.

Turbidimetric data have been used to generate equations which describe the growth responses of different microorganisms [5,6,7,15,23,27,28,29], but relatively few studies have attempted to derive mathematical equations predicting kinetic responses (such as doubling times) from optical density measurements. In this work, a novel method of analyzing these types of data is described. The technique involves incorporating a calibration equation converting optical density to viable cell numbers into a sigmoid growth curve in one step in the dimension of optical density. We propose that this technique should be further evaluated as a method for generating data for modeling the kinetics of microbial growth.

MATERIALS AND METHODS

Organism and media

Listeria monocytogenes (ATCC 19115) was maintained on Heart Infusion Agar (Difco) at 4 °C and grown in Tryptic Phosphate Broth (TPB) [10] at 30 °C. The inoculum for the

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microwells was taken from an early stationary phase (16 h) culture.

Viable cell numbers for estimating the inoculum size and generating the calibration curve were determined by making decimal dilutions in TPB and directly plating 20 μl onto Tryptic Soy Agar (TSA). The plates were incubated for 24 h at 30 °C, colonies counted and viable numbers calculated according to the method of Farmiloe et al. [13].

Growth in microwell plates

Solutions of different NaCl concentrations (2–18% w/v) in 9.5 ml volumes were autoclaved (121 °C for 10 min) and then combined aseptically with 9.5 ml of double strength TPB, acidified with either 1 M or 0.1 HCl to give pH values between 7.0 and 4.66, and then made up to a final volume of 20 ml with distilled water. The final concentrations (after inoculation) of NaCl were 0.5–9.5% (w/v). The media were dispensed aseptically in 350 μl volumes into Honeycomb microplates (Labsystems, Uxbridge, Middlesex) and inoculated with 50 μl of a dilute suspension of *L. monocytogenes* to give an initial concentration of 10^3 cells ml^{-1} in each well. An automated turbidimetric instrument (Bioscreen C, Labsystems, Basingstoke, Hants, UK) was used to assess the effect of NaCl concentration and hydrogen-ion concentration on the increase in turbidity of *L. monocytogenes*. Microplates were incubated at 15 °C, 20 °C and 30 °C for 150 h and the absorbance at 600 nm of each microwell was automatically recorded every 30 min. Prior to reading, the plates were shaken for 2 min.

EXPERIMENTAL PLAN

NaCl	Hydrogen-ion concentration (μm) (and equivalent pH) 0.1 (7.00)	7.41 (5.13)	14.8 (4.83)	21.9 (4.66)
0.5	*	*	*	*
1.5	*	*	*	*
2.5	*	*	*	*
3.5	*	*	*	*
4.5	*	*	*	*
5.5	*	*	*	*
6.5	*	*	*	
7.5	*	*	*	
8.5	*	*		
9.5	*	*		

Calibration

The relationship between plate counts and optical density (Fig. 1) of *L. monocytogenes* was determined by measuring the optical density of dilutions of a culture growth at 0.5% NaCl, pH 7.0 and 30 °C. A simple quadratic equation was used to describe this relationship.

Optical density data were transferred to a VAX minicomputer in ASCII format and were adjusted for drift associated with electrical components of the system by subtracting the mean drift associated with uninoculated wells at each temperature.

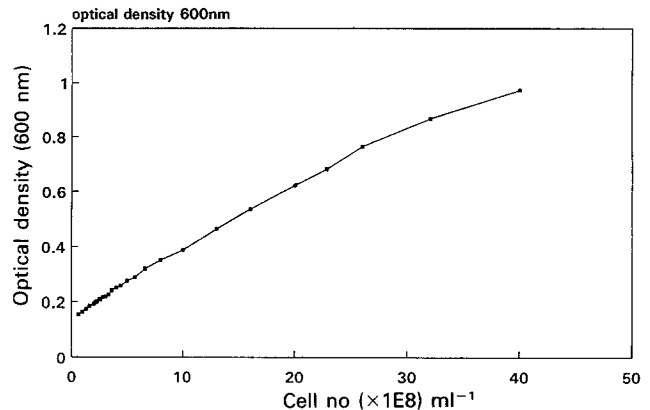

Fig. 1. Calibration curve comparing the optical density of *L. monocytogenes* to viable cell count, grown in TPB containing 0.5% NaCl, pH 7.0 at 30 °C.

Electrical signal noise was filtered out using a spline function provided in the SAS statistical package (SAS Software Ltd, Cary, NC, USA).

Statistical approach

The following assumptions were made before analyzing data. The logarithm of cell number was assumed to increase with a logaristic function (alternative sigmoid functions may also be used such as Gompertz, Richards or Morgan–Mercer–Flodin, [29]), and the resulting curves were assumed to pass through the initial cell number derived from plate counts.

The following mathematical approach was used:

Logistic function

$$z = \alpha + \frac{\beta}{1 + e^{\beta - \delta t}} \quad (1)$$

and can be rewritten as:

$$y = \alpha + \frac{(\omega - \alpha)}{1 + e^{4\sigma(\tau - t)/(\omega - \alpha)}} \quad (2)$$

where: α = lower asymptote
ω = upper asymptote
τ = position of maximum slope
σ = maximum slope

For optical density, let

$$y = a + b \cdot x + c \cdot x^2 \quad (3)$$

and

$$\ln(x) = f(t) \quad (4)$$

where: y = optical density
x = cell count
t = time
f = a suitable function e.g. logistic

then

$$y = a + b \cdot e^{f} + c \cdot e^{2f} \tag{5}$$

Thus if we take our function $f(t)$ as

$$f(t) = \alpha + \frac{\beta}{1 + e^{\gamma - \delta t}} \tag{6}$$

we can write

$$y = a + b \cdot \exp\left(\alpha + \frac{\beta}{1 + e^{\gamma - \delta t}}\right) + c.\exp\left(2\alpha + 2\frac{\beta}{1 + e^{\gamma - \delta t}}\right) \tag{7}$$

and we need parameters constrained so that the initial cell count, I, occurs at time $t = 0$. i.e.

$$\ln(I) = \alpha + \frac{\beta}{1 + e^{\gamma}} \tag{8}$$

Thus we can replace α in the earlier expression by

$$\ln(I) - \frac{\beta}{1 + e^{\gamma}} \tag{9}$$

The expression

$$\alpha + \frac{\beta}{1 + e^{\gamma - \delta t}} \tag{10}$$

will therefore be replaced by

$$\ln(I) + \frac{\beta(1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})} \tag{11}$$

We therefore want to fit

$$y = a + b \cdot \exp\left(\ln(I) + \frac{\beta(1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})}\right) + c \cdot \exp(2\ln(I) + \frac{2\beta(1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})} \tag{12}$$

Since I, b and c are known quantities, we can set $B = b \cdot I$ and $C = c \cdot I^2$ and achieve

$$y = a + B \cdot \exp\left(\frac{\beta(1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})}\right) + C \cdot \exp\left(\frac{2\beta(1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})}\right) \tag{13}$$

This curve has the lower asymptote of

$$a + B \cdot \exp\left(\frac{-\beta}{1 + e^{\gamma}}\right) + C \cdot \exp\left(\frac{-2\beta}{1 + e^{\gamma}}\right) (= a + b \cdot e^{\alpha} + c \cdot e^{2\alpha}) \tag{14}$$

and an upper asymptote of

$$a + B \cdot \exp\left(\frac{\beta}{1 + e^{-\gamma}}\right) + C \cdot \exp\left(\frac{2\beta}{1 + e^{-\gamma}}\right) \tag{15}$$

We need to estimate α, β, γ, δ after which we can plot the curve of log cell number versus time from

$$\ln(x) = \ln(I) + \frac{(\beta 1 - e^{-\delta t})}{(1 + e^{-\gamma})(1 + e^{\gamma - \delta t})} \tag{16}$$

The plot of $\ln(x)$ versus time has a maximum slope of $\beta\gamma/4$ at the point $t = \gamma/\delta$, which is the maximum specific growth rate.

RESULT

The calibration equation took the form:

$$y = 0.1375 + 2.799 \cdot 10^{-10} x - 1.732 \cdot 10^{-20} x^2 \tag{17}$$

where: y = optical density
x = cell numbers

A correction is needed to this equation to account for the differences between optical densities from different media. Therefore

$$y = a + 2.799 \cdot 10^{-10} x - 1.732 \cdot 10^{-20} x^2 \tag{18}$$

where a is the intercept determined from initial optical density measurements.

Figures 2a and 3a are examples of curves fitted to increases in optical density of *L. monocytogenes* at 30 °C, pH 7.0 with 0.5 and 8.5% NaCl respectively using the combined Eqn (13). Figures 3b and 4b are examples of the same data, but plotted using a log cell number as the y-axis. Figure 4 shows the effect of temperature pH and NaCl on growth (expressed in log cell number) of *L. monocytogenes*.

From the optical density data (+), converted to equivalent cell numbers in Figs 2b and 3b it can be seen that any attempt to fit a growth curve through these points would result in poor fitting. Small increases in optical density reflect relatively large increases in cell numbers, hence the scatter in the lower half of the curve. Therefore, the errors associated with fitting to transformed data are larger

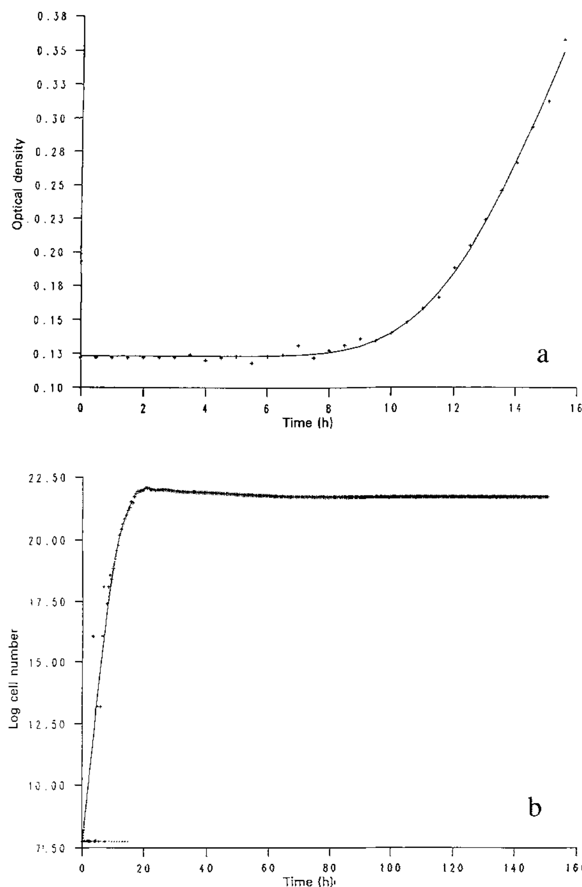

Fig. 2. The increase in optical density (a) (absorbance units) and fitted growth curve (b) (after transformation using the calibration curve and logistic function) of *L. monocytogenes* grown in TPB with 0% NaCl at pH 7.0 and 30 °C in the Bioscreen. +'s represent the optical density data points.

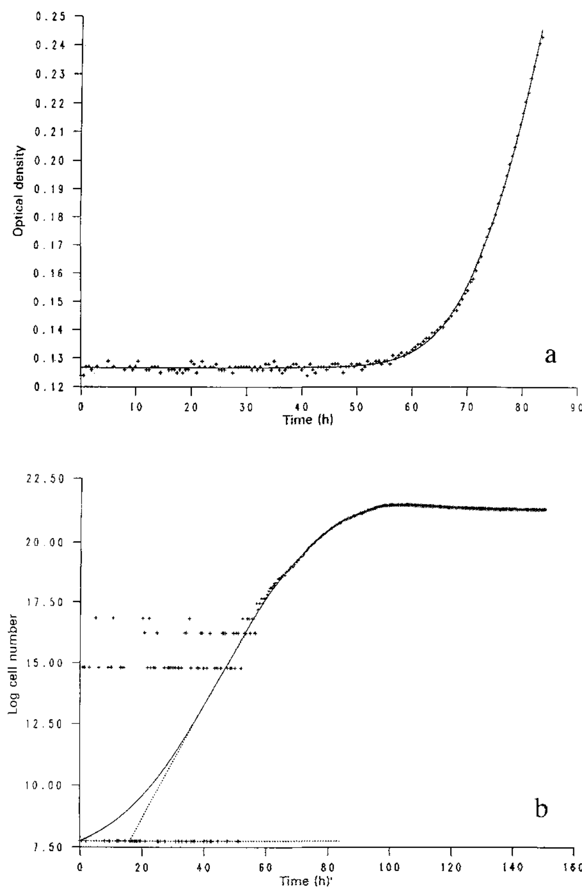

Fig. 3. The increase in optical density (a) (absorbance units) and fitted growth curve (b) (after transformation using the calibration curve and logistic function) of *L. monocytogenes* grown in TPB with 8% NaCl at pH 7.0 and 30 °C in the Bioscreen. +'s represent the optical density data points.

(unpublished data) than if the growth curve is fitted in the dimension of optical density (Figs 2a and 3a).

There was good agreement between the numbers estimated from viable counts taken from the Bioscreen plate, and numbers estimated from optical density measurements (using Eqn 13).

Analysis of parameters from curve fitting

Estimates of α, ω, $\log(\sigma)$, and $\log(\tau)$ were plotted against temperature, NaCl and hydrogen-ion concentration in order to describe the effect of these variables on growth of *L. monocytogenes*. A general response surface was fitted to each of the terms α, ω, $\log(\sigma)$, and $\log(\tau)$ using temperature, NaCl and hydrogen-ion concentration as three independent variables. Coefficients from these equations are shown in Table 1.

Figure 5 shows a series of predicted growth curves from the response surface equations, showing the combined effects of temperature, hydrogen-ion and NaCl concentration on growth of *L. monocytogenes*.

DISCUSSION

Sigmoid curves have been fitted to optical density data previously [11], but there have been no attempts to derive kinetic data such as generation times. Previous studies [5,6, 28,29] have derived 'growth rates' from optical density measurements but these rates are a measure of the changes in optical density with time, and are not directly correlated (by calibration) with viable cell numbers. One approach to using this type of data has been to convert the optical densities to viable counts, using a calibration, to obtain estimates of lag time and specific growth rate [8,9]. This approach is reasonable for yeasts, which have a relatively large cell size and hence can be detected at concentrations of 10^3–10^4 cells ml^{-1}. An alternative approach has been taken for bacteria which, because of their smaller size, can only be detected at higher concentrations e.g., 10^6–10^7 cells ml^{-1}. By including a calibration curve and a curve fitting function in the transformation of optical density data, it is possible to fit a sigmoid growth curve to optical density data. Use of this aproach has enabled us to use optical density measurements to derive specific growth rates directly. A response surface was then fitted to these data.

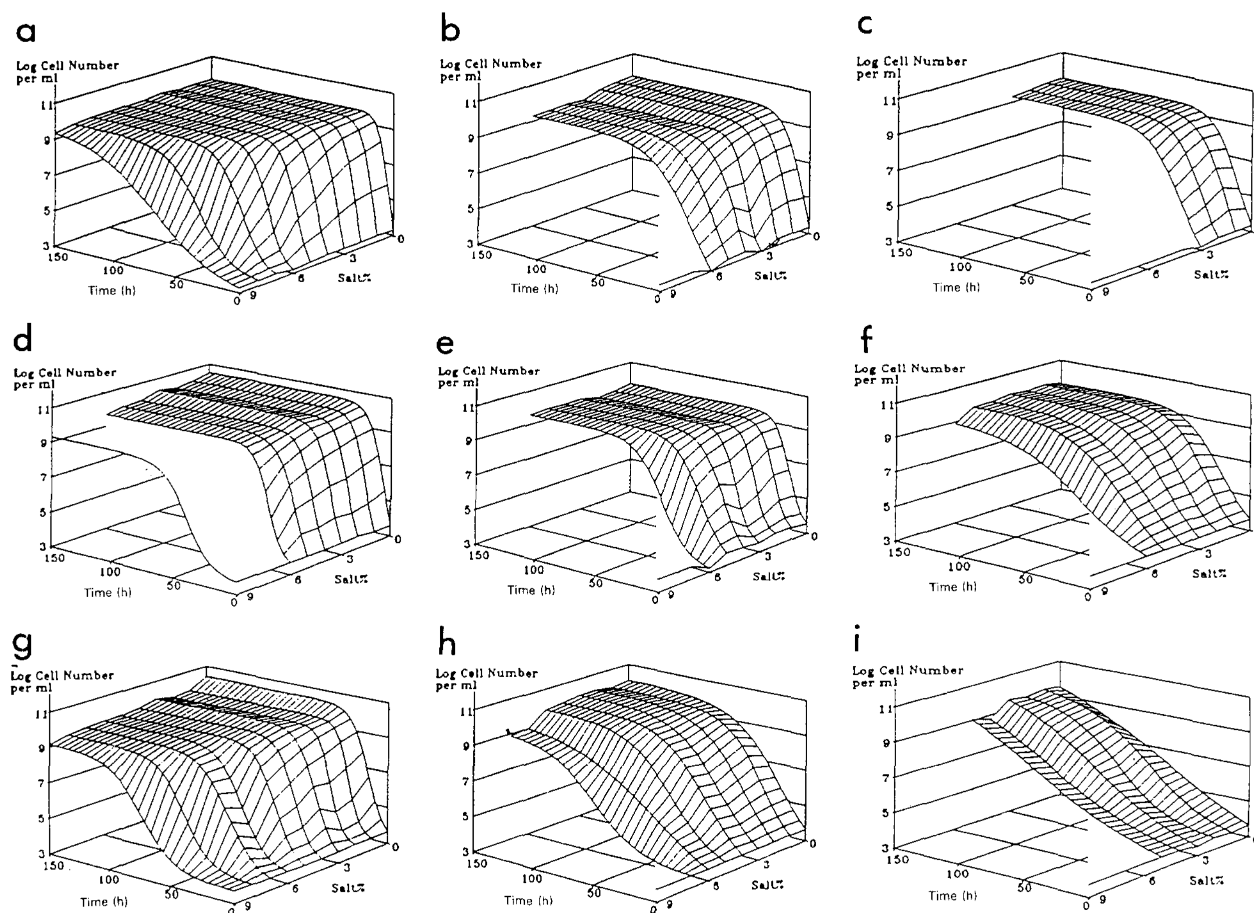

Fig. 4. The effect of temperature, pH and NaCl on growth of *L. monocytogenes*: a, b, and c at 30 °C; d, e, and f at 20 °C; g, h, and i at 15 °C; a, d, and h at pH 7.0; b, e, and h at pH 5.13; c, f, and i at pH 4.83.

TABLE 1

Coefficients for the quadratic response surface equations describing the effects of NaCl, temperature and hydrogen-ion concentration on the parameters from the logistic function

	α	ω	$\log(\sigma)$	$\log(\tau)$
Intercept	−1145.422419	662.018346	−285.288571[c]	289.2532883[c]
NaCl	−8.461423[a]	−2.520718	0.82679[c]	−1.326091[c]
NaCl*Temp	0.030637[a]	0.007731	−0.0028[c]	0.004565[c]
$NaCl^2$	−0.014162	0.001453	−0.007453[c]	0.009172[c]
Temp	8.110524	−4.283591	1.909442[c]	−1.909849[c]
$Temp^2$	−0.01428	0.00718	−0.003193[c]	0.003159[c]
[Hyd-ion]	−1.713318	2.591013[a]	−0.426904[c]	0.365209[b]
[Hyd-ion]*Temp	0.005553	−0.009557[a]	0.001309[c]	−0.001171[b]
$[Hyd\text{-}ion]^2$	0.00546	0.012111[a]	−0.000281	0.00086
[Hyd-ion]*NaCl	−0.011653	0.017268	−0.000577	−0.000157

[a] 5% significance.
[b] 1% significance.
[c] 0.1% significance.

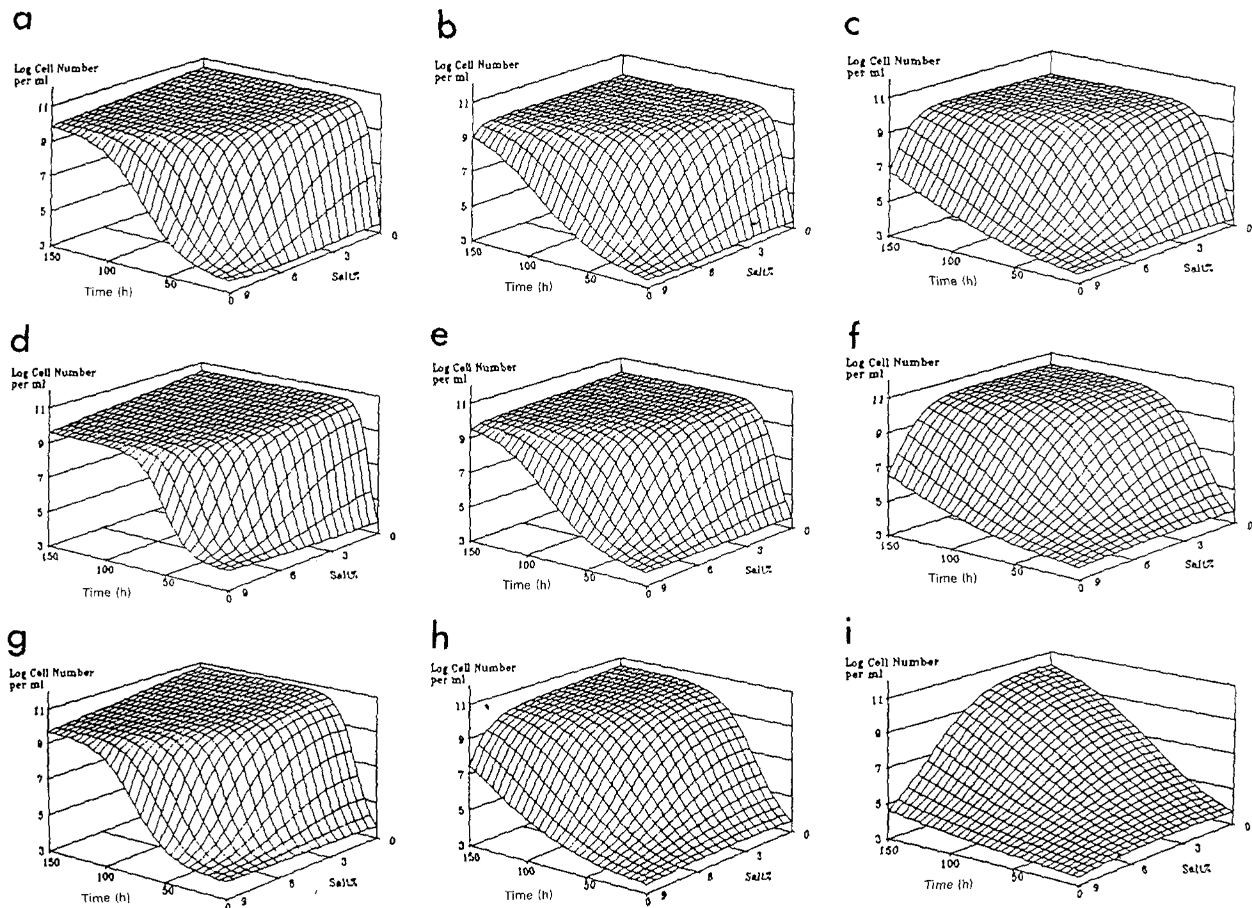

Fig. 5. The predicted effect of temperature, pH and NaCl on growth of *L. monocytogenes* from the response surface equations: a, b, and c at 30 °C; d, e, and f at 20 °C; g, h, and i at 15 °C; a, d, and h at pH 7.0; b, e, and h at pH 5.13; c, f, and i at pH 4.83.

Response Surface Methodology (RSM) [18] is commonly used to describe the response of microorganisms to a number of environmental or controlling factors, enabling predictions of that response to be made. Responses such as time to increase in optical density [7] Anderson and Cole, unpublished] (Figs 6a and 6b), growth curve parameters [4,14,25, 26], shelf-life of crumpets [31], and CO_2 production [32] have been modeled using this technique. Using RSM in combination with data described in this paper, it is also possible to generate equations predicting the generation times of microorganisms in response to a number of controlling factors, such as pH, NaCl concentration and temperature from optical density data.

To compare results using this method with other data, the predictive equations have been used to estimate generation times which are shown in Fig. 7, together with predictions from the Pathogen Modeling Program (version 3.1) (based on plate counts) distributed by the Microbial Food Safety Research Unit (USDA, Philadelphia, USA). These comparisons show that the estimates from Bioscreen data are generally lower (showing faster growth rates, except at 30 °C) than those from the USDA model, and that NaCl is much less inhibitory, in the Bioscreen system, at 4.5%. Bioscreen data also show that NaCl has a less inhibitory effect on the growth rate of *L. monocytogenes* at 20 °C than at 30 °C.

We have also compared time to 1000-fold increase in cell numbers to data predicted from the USDA model and data from MAFF (UK) (Fig. 8). The estimates derived using our method are slightly less than those predicted from the two other sources, suggesting that the lag times and/or doubling times are shorter in the system we have used. There are several possible explanations for this, there may be strain to strain variation in growth responses (although we would anticipate these differences to be small), but a more likely explanation is that the environment within the microwell plate is more aerobic, and therefore more conducive to the growth of *L. monocytogenes*. It has been shown that *L. monocytogenes* shows faster growth in aerated systems as opposed to anaerobic systems [4]. Three plate counts (plotted with •) were done simultaneously with the Bioscreen experiments, and these agreed with estimates from the predictive equation.

Alternatively, in this work we have used a single calibration for cell numbers vs optical density obtained at under near optimum conditions for growth. Recent observations in our laboratory (P. Stephens, unpublished) and by (R.L. Buchanan, unpublished), have indicated that

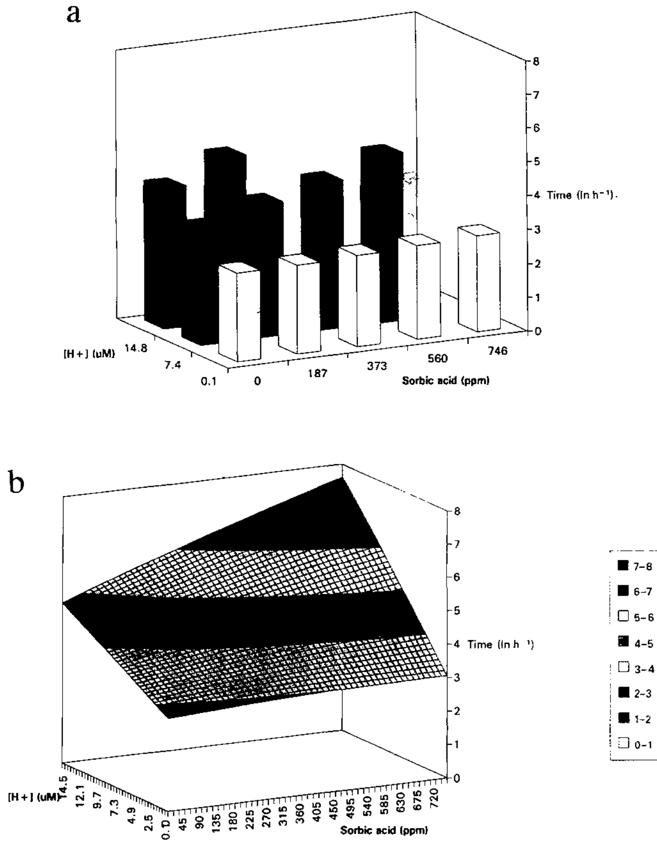

Fig. 6. (a) The effect of hydrogen-ion concentration and sorbic acid concentration on the time to reach a population density of 1E8 cells ml^{-1} for *L. monocytogenes* at 30 °C in 1% NaCl [Anderson and Cole 1991, unpublished]. (b) Predictions from a quadratic response surface model describing the effect of hydrogen-ion concentration and sorbic acid concentration on the time to a population density of 1E8 cells ml^{-1} for *L. monocytogenes* at 30 °C in 1% NaCl [Anderson and Cole 1991, unpublished].

the morphology of *L. monocytogenes* changes significantly in high concentrations of NaCl, with cells becoming elongated. This would probably affect the relationship between optical density and cell number, resulting in a higher optical density than expected. In other words, we would predict faster than actual growth. However, the use of a calibration under near optimum conditions, in this work, will give 'fail-safe' predictions of growth.

Further studies will be carried out to investigate the change in optical density associated with increased solute concentration, and a range of inoculum sizes may be used at every condition. This would avoid errors due to changes in the relationship between optical density and cell number and may also allow a more direct measurement of growth rate. Assuming the lag time to be constant for a given set of conditions, the difference in time to reach a set optical density should be directly proportional to the inoculum size.

Rapid methods for estimating microbial biomass [12, 16,33], based on biophysical (e.g., direct and indirect photometric detection, direct epifluorescent filter technique, direct electronic counting, impedance and conductance changes), biochemical (measurement of ATP, NADH, organic/inorganic compounds through bio- or chemiluminescence) or bioelectrical methods (biosensors) offer considerable savings in time and effort for the practising microbiologist, particularly if the method is automated, enabling much data to be generated with comparatively little

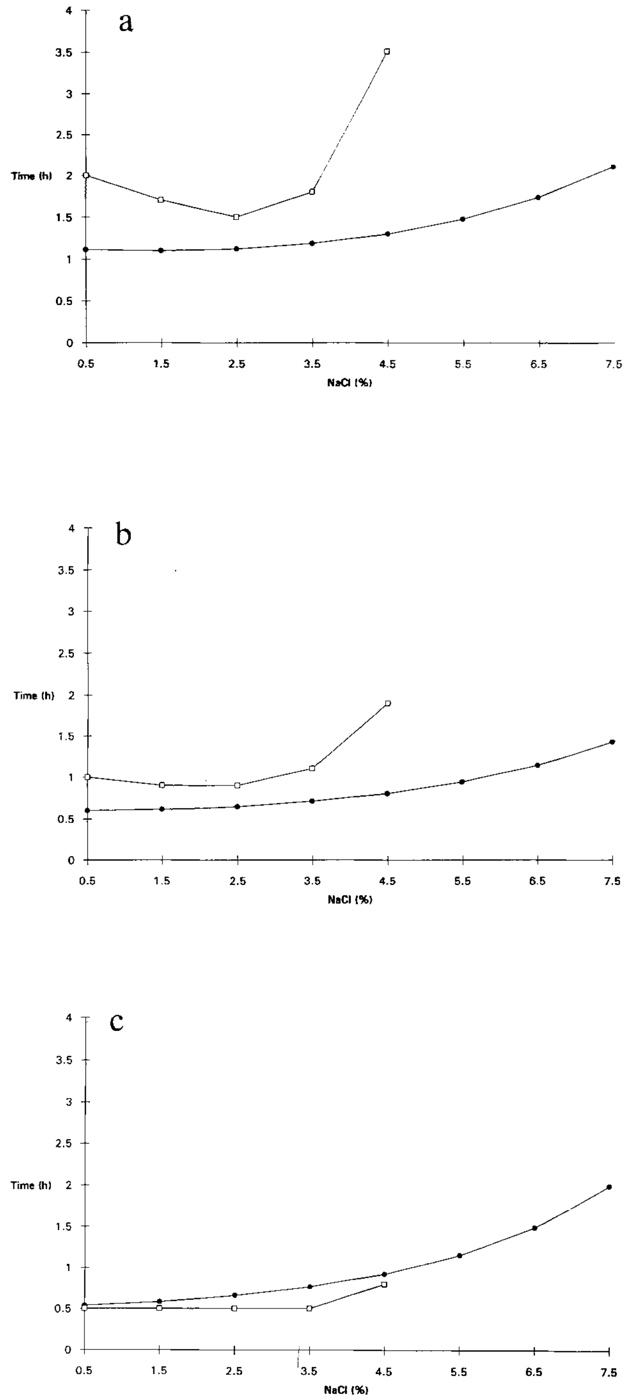

Fig. 7. The effect of increasing NaCl concentration on the predicted doubling times of *L. monocytogenes* at 15 °C (a), 20 °C (b), and 30 °C (c) at pH 7.0 from the Pathogen Modeling Program (USDA (□)) and the response surface equation fitted to Bioscreen data (●).

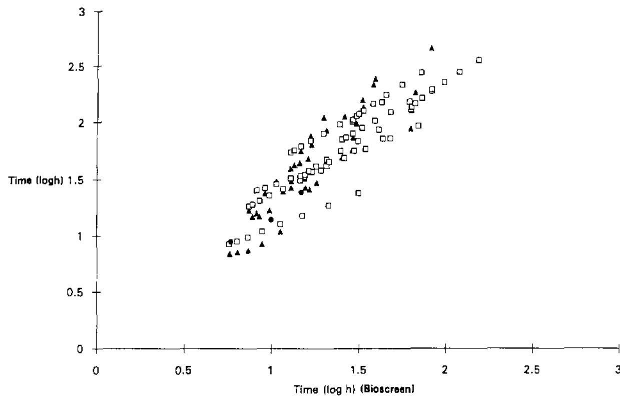

Fig. 8. A comparison of predicted time of 1000-fold increase in cell numbers from the Bioscreen response surface equation, Pathogen Modeling Program (USDA) and data from MAFF (UK) over a range of environmental conditions. (▲), USDA; (□), MAFF; (●), viable counts.

cost. It is important that microbiologists continue to examine alternative methods for measuring the growth responses of microorganisms, not only because of the tremendous savings in effort but also because of the inherent errors associated with traditional viable plate counts.

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