Advances in the predictive modelling of fungal growth in food

Angela M. Gibson and Ailsa D. Hocking

Mathematical modelling of fungal growth, and the ability to predict whether a particular fungus will grow in a food, and if so at what rate, has not received a similar degree of interest as modelling of bacterial growth. One of the main problems is the difficulty of acquiring sufficient, reproducible data that are suitable for modelling. In this review, we aim to introduce the principles of modelling of fungal growth and summarize some of the recent literature that describes the application of modelling and predictive techniques to yeasts and moulds. Particular attention has been paid to the use of automated methods for assessing growth.

There is a need for improved understanding of the factors controlling the growth of fungi in foods, particularly those factors that are associated with new manufacturing processes and packaging techniques. Customer demand for 'more natural' and 'fresher' foods has also led to the reduction or removal of preservatives and reduced levels of salt and sugar; this has resulted in increased concern about the microbiological safety of foods, and changes in the types of spoilage patterns encountered – particularly an increase in mould spoilage problems[1]. Some moulds are capable of producing toxic metabolites that may be carcinogenic and therefore constitute a public health risk, whereas the growth of spoilage fungi results in poor appearance and off-flavour development, leading to customer rejection. Economic losses can be considerable, especially in the stored grains industry.

Assuming sufficient nutrients are available (as is usually the case with foods), microbial growth is controlled primarily by temperature, water activity (a_w) and pH; additional factors such as the presence of preservatives, heat treatment or modified-atmosphere packaging also contribute. In principle, provided sufficient information is known about the factors controlling microbial growth, the growth responses of microorganisms of concern can be predicted.

Predictive food microbiology and the principles of modelling bacterial growth have already been thoroughly reviewed in this journal by Buchanan[2]. Many models describing the growth of a variety of bacteria, particularly food pathogens, have been produced, and a commercially available software package, Food Micromodel (Food Micromodel Ltd, Randalls Road, Leatherhead, UK KT22 7RY), derived from a huge database on microbial growth relevant to foods, is now available. To date, this software package contains models that are mainly restricted to bacterial food pathogens and some spoilage bacteria, although it also includes a model for the growth of the spoilage yeast *Zygosaccharomyces bailii*.

Angela M. Gibson and **Ailsa D. Hocking** are with the Food Safety and Quality Program, CSIRO Food Science and Technology, Sydney Laboratory, PO Box 52, North Ryde, New South Wales 2113, Australia (fax: +61-2-9490-8581; e-mail: angela.gibson@dfst.csiro.au).

Predictive modelling of filamentous fungal growth has not received the same level of attention as that of bacterial growth. This may well be because of the inherent complexities associated with the quantification of fungal growth. Measurement of hyphal extension rate, usually reported as radial growth rate in μm/h, is probably the simplest and most direct measure, but it does not necessarily represent the true nature of fungal growth. Whereas bacteria reproduce by fission, and growth normally takes place only at surfaces or homogeneously through a liquid medium, fungal hyphae can penetrate the physical three-dimensional matrix of foods. A prerequisite for producing a useful and reliable model must be a database containing large amounts of relevant data, preferably in the form of growth or survivor curves. The problems associated with the acquisition of good growth curves for bacteria have been discussed by Bratchell *et al.*[3], but the difficulties of obtaining reproducible and similar quality growth curves for fungi are much more complicated.

Types of models

The two most widely used models for describing bacterial growth in foods are the 'square-root' or 'Belehradek-type' model, as used by Ratkowsky *et al.*[4], and the polynomial model, as used by Gibson *et al.*[5] A square-root model[6], for suboptimal levels of temperature (T), a_w and pH combined, was described as:

$$k = C \times (T - T_{min})^2 \times (pH - pH_{min}) \times (a_w - a_{wmin})$$

where k is the specific growth rate, C is a constant and T_{min}, pH_{min} and a_{wmin} are theoretical growth minima below which the predicted growth rate is zero.

Regression analysis and the subsequent formation of a polynomial model is the main alternative to the above approach, and a wide range of computer programs is available to perform such analyses. When sufficient data are available and relatively small numbers of variables are being studied, significant factors and interactions can be determined simultaneously with step-wise regression analysis, as used by Gibson *et al.*[5] When a wider range of factors at several levels is studied, an analysis of variance can be used initially to determine the relative effects of individual factors and their interactions. A step-wise regression analysis that includes

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only significant factors and interactions is then used to produce a first- or second-order polynomial design (see Smith *et al.*[7]). Once produced, and validated, the model can be used to predict the effect of changing several factors simultaneously. The choice of model is partly subjective but also depends on the number and type of variables (environmental conditions) that are to be analysed and what is actually being measured (growth rate, or time to a particular event such as growth or spoilage).

Models can be categorized into probability or kinetic models (measuring growth rate or generation time) according to the mathematical approach used. Models are also described as mechanistic or empirical, although the difference between the two is not always clear cut[8]. Some empirical models include an aspect of mechanistic modelling (e.g. the square-root model, because it originates in part from a more fundamental base) and vice versa. Mechanistic or semi-mechanistic models usually include parameters from differential equations of applicable known theories (growth kinetics, substrate utilization and depletion rates, substrate or end-product diffusion rates, etc.) and attempt to describe what is actually occurring during growth. Such models can give more insight into the behaviour of a biological system[9] than empirical models can. Mechanistic models have long been used in the areas of biotechnology and chemical engineering where there is a need to optimize growth rather than control it. Experience from modelling bacterial growth in foods has shown that the necessary mathematical models cannot simply be copied from those that have been long established in biotechnology and chemical engineering[8,10]. Additionally, in the case of filamentous fungi, estimation of growth is more complicated owing to the formation of surface colonies and also of hyphae throughout the food; a cell count is not appropriate.

Empirical models simply describe the conditions under which an experiment was performed, that is, the effect on microbial growth of the physical and chemical components of the food. Such models are usually polynomials. Although many other factors in foods affect microbial growth (as defined by mechanistic models), these account for a small proportion of the total variation in growth when compared with the main factors such as temperature, a_w, pH and presence of preservatives. Because empirical models are descriptions of the experimental conditions, they should not be used to make predictions outside the limits of the original experiments.

Probability models

Probability models allow the prediction of whether a particular event, such as growth or toxin production, might occur under various conditions, but provide no information about the rate at which that growth occurs.

Such a model was used to predict the probability of the growth of *Z. bailii* in a model fruit drink system as affected by pH, °Brix and three preservatives, sorbate (S), benzoate (B) and sulphite (SO_2), all at three levels[11]. The end point was growth or no growth after 3 weeks at 23°C. The probability (P) of growth (η) was related to a logistic regression model:

$$P = \frac{1}{1 + e^{(-\eta)}}$$

The value of η was modelled by a polynomial equation involving the various factors and their levels plus significant interactions:

$$\eta = a + b(°\text{Brix}) + c[SO_2] + d[H^+] + e[S] + f[B] + g[S]\cdot[B] + h[S]\cdot[SO_2] \ldots \text{etc.}$$

where a, b, c, etc., are constants generated during the regression analysis.

The model can be used to predict the probability of growth for any combination of factors, at any level, within the limits of the original experimentation.

Mechanistic or semi-mechanistic models

A relatively simple mechanistic model[12] was used to study the effects of sorbic acid and pH on the growth of *Penicillium chrysogenum*, *Cladosporium cladosporioides* and *Ulocladium atrum*. Minimum inhibitory concentrations (MICs) were modelled as a function of pH, which was determined by the ratio of undissociated and total sorbic acid. Nonlinear regression analysis produced a formula to predict the MIC by substituting values for the amount (moles) of total and undissociated acid and calculating a constant derived from testing the MIC at only two pH levels, one low and one high.

$$MIC_1 = \frac{1}{[(1-a)/k_1] + (a/k_2)}$$

where MIC_1 is the MIC determined for total acid, a is the ratio between undissociated and total acid, k_1 is the MIC for dissociated acid and k_2 is the MIC for undissociated acid. Although this model is useful for explaining the preservative effect of sorbic acid at varying pH levels, it does not take into account the effects of other factors that are present in foods that may also have an effect on mould growth.

A semi-mechanistic model was used to describe the effects of temperature, pH, a_w and colony size on mould growth and aflatoxin development[9]. The model took into account growth rate changes (affected by a_w, pH and temperature, including temperature cycling), mould mass (which imposes a growth limit owing to substrate depletion or diffusion limitations), aflatoxin production (assumed to be proportional to growth rate, cell mass and a yield coefficient), toxin degradation, and the decline in growth rate with respect to increase in mould concentration. The resultant model comprised a series of 20 differential equations and was validated using data from the literature, although each data set usually related only to one or two portions of the model. The author stated that the model correctly predicted the decrease in the optimum temperature for aflatoxin production with increasing time, but did not accurately represent the effects of spore load on

the maximum toxin concentration, and proposed that an additional factor needed to be taken into account. Good qualitative agreement, with literature data, for the effects of temperature cycling was also claimed for model predictions. This type of semi-mechanistic model may be considered superior to an empirical model because it describes rates of growth that are governed by assumptions about mould biology and physical laws of diffusion.

The model was further modified[13] to study the implications of storage conditions (O_2 and CO_2 levels) on the risk of aflatoxin development in seeds, forage and foods. Toxigenesis was more sensitive than growth to decreased O_2 and increased CO_2 levels. Higher rates of O_2 depletion therefore lowered the rates of aflatoxin production in the initial phase of storage, but also resulted in higher aflatoxin levels once O_2 was again present after opening the containers at the end of storage.

Empirical models

An empirical approach to modelling the effects of a_w on the surface growth of four closely related species of *Aspergillus* was used by Gibson *et al.*[5] They investigated the appropriateness of models that were previously used to predict bacterial growth for the interpretation of mould growth data[14] comprising growth curves (colony diameter versus time) of *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus parasiticus* and *Aspergillus nomius*. Growth at 30°C and ten different a_w levels (0.810–0.995) was modelled. The modelling was carried out in two stages: first, a flexible model[15] describing the change in colony diameter (mm) with respect to time was fitted to the growth curve data. Following curve-fitting, the maximum colony growth rate (g), expressed as the increase in diameter/h, was estimated for each a_w level. These values were then fitted with respect to a_w using a linear regression model. A full description of the modelling is given in Gibson *et al.*[5]

A natural logarithm transformation was introduced to stabilize the variance of the fitted values for growth rate; additionally, a novel transformation of a_w (to a term designated b_w) was carried out to fit the data to a simpler curve shape. Transformation of a_w to b_w was according to the formula:

$$b_w = \sqrt{(1 - a_w)}$$

The use of ln g versus b_w curves was more suitable for parabolic fitting and the following model was fitted separately for each isolate:

$$\ln g = C_0 + C_1 b_w + C_2 b_w^2$$

where the coefficients C_0, C_1 and C_2 were calculated by regression. Optimum a_w values and colony growth rate at optimum a_w were also calculated for each isolate.

The time (t_3) for a mould colony to reach a 3 mm diameter colony was also calculated, because a colony of that size would be visible and the product considered spoiled. The model for t_3 is:

$$\ln t_3 = D_0 + D_1 b_w + D_2 b_w^2$$

where the coefficients D_0, D_1 and D_2 were also calculated by regression.

The models were validated, where possible, by comparisons with literature data, although such comparisons were often difficult because different authors used a range of storage temperatures and humectants. When comparable data were available, for example for *A. flavus* and *A. parasiticus*, model predictions generally agreed well with literature information. Predicted growth rates (converted to radial growth rates) fell well within the range of published radial growth rates except at extremes of the model, that is, at high a_w (0.99) and low a_w (0.85). Model predictions close to the limits of a model are always subject to greater errors than those within the range of the model, and those at high a_w values will be particularly so because of the inherent problems in accurately measuring a_w at such levels. Furthermore, model predictions can be related only to the growth of moulds on the food surface and cannot take into account hyphal development within a product. Most of the mould spoilage of foods occurs at the surface, for a variety of reasons, including O_2 restriction within the product; the complex structure of foods makes the measurement and therefore prediction of hyphal growth within a product impossible.

The original data of Pitt and Miscamble[14] mentioned above comprised three isolates of four species, resulting in 12 different models, one for each isolate. In a separate study[16], data from the three isolates of each species were merged and remodelled, thus reducing the 12 isolate-dependent models to four species-dependent models.

The combined effects of chitosan and sugar concentration (°Brix) on the growth of *Aspergillus niger* and *A. parasiticus* on low-sugar candied kumquat were modelled by Fang *et al.*[17] The number of 'days to visible growth' was monitored turbidometrically and significant factors and their interactions were identified. A second-order polynomial model predicting shelf life, y (days to visible growth), was generated:

$$y = a + b[\text{chitosan}] + c(°\text{Brix}) + d[\text{chitosan}]^2 + e[\text{chitosan}](°\text{Brix}) + f(°\text{Brix})^2$$

where a, b...f are constants generated by regression.

An interesting application of modelling techniques was used to study the spoilage of feta cheese[18]. The effects of temperature, pH, a_w and NaCl and sorbitol concentrations on the growth of spoilage yeasts were modelled. Growth was monitored using optical density measurements and growth rates calculated using linear regression. Two different models were fitted to their data: a nonlinear equation derived from the Arrhenius equation, as used by Schoolfield *et al.*[19]; and a Belehradek-type model, as used by Ratkowsky *et al.*[4,20] The Arrhenius equation was found to fit the data better, and plots of growth rate against temperature were compared for the two models. Their results showed that the pH (range 3.5–4.5) had no significant effect on growth rate. The NaCl concentration of the whey was the most

important controlling factor and growth rate decreased linearly with increasing NaCl concentration (0–16%, w/w). However, fairly large changes in NaCl concentration were required to affect the growth rate significantly; for example, at 5.5% NaCl (a_w 0.966), growth rate was reduced to approximately half that at 0% NaCl. Increasing the sorbitol concentration (from 0% to 50%) caused a linear decrease in the growth rate until an a_w of approximately 0.94, when the growth rate decreased dramatically. Minimum a_w for growth was unaffected by humectants (NaCl or sorbitol). The overall conclusion was that control of spoilage yeasts in feta cheese could not be achieved by manipulating pH, temperature or NaCl concentration/a_w; instead the authors suggested removal of fermentable substrate from whey by a 'suitable yeast', before packaging. Although the effect of these physicochemical parameters on the growth of spoilage yeasts will be of interest to the feta cheese industry, the usefulness of the models *per se* was not discussed.

Thermal death models

Linear regression analysis has also been used to estimate the parameters of kinetic models (first-order polynomials) in the study of the combined effects of environmental factors – temperature, pH, a_w and E_h (redox potential) – on the heat resistance of seven foodborne microorganisms[21]. *Lactobacillus plantarum*, *Lactobacillus brevis*, *Saccharomyces cerevisiae*, *Z. bailii*, *Yarrowia lipolytica*, *Paecilomyces varioti* and *Neosartorya fischeri* were heated in a synthetic medium, pH range of 3.0–4.5, a_w range of 0.850–0.98 and E_h of 110/150, 240 and 460 at three temperatures within a range of 48–90°C. The best-fitting model for defining the thermal death rate coefficient (k) took the form:

$$\log_{10}k = a + (b \times \text{pH}) + (c \times E_h) + (d \times a_w) + (e \times T)$$

where the coefficients of the model parameters (a, b, c, etc.) were calculated by linear regression. Correlation coefficients for the models were best for the lactobacilli (R^2 of 0.96–0.99) and worst for the yeasts (R^2 of 0.81–0.88).

For all microorganisms, the heat destruction rate increased with decreasing pH and increasing a_w. Increasing the E_h of the heating medium increased the heat destruction rate of lactobacilli, whereas the heat resistance of yeasts and moulds increased with increasing E_h.

Automated methods

Automated methods, where available, allow the accumulation of large amounts of data quickly and are potentially an ideal source of data for modelling. Two such techniques that have been used include indirect conductimetry and flow cytometry, although they have been applied only to yeast growth. There is also potential for the metabolic activity of moulds to be detected impedimetrically[22].

Indirect conductimetry (monitoring CO_2 evolution) was investigated as a novel means of achieving a large database suitable for modelling[23]. The effects of pH, a_w, temperature (T) and potassium sorbate concentration [KS] on the growth responses of *Z. bailii* were studied. Technical difficulties prevent the direct measurement of yeast growth by conductance, so CO_2 evolution was used as an indirect method of growth assessment. Both detection times and maximum rate of change (MR) in CO_2 evolution were measured and modelled using a second-order polynomial. Significant variables were selected using step-wise regression analysis. All single factors, T^2, and pH × [KS], pH × T and [KS] × T interactions were significant. The model for MR took the form:

$$\text{MR} = a + b(a_w) + c(\text{pH}) + d[\text{KS}] + e(T) + f(T^2) + g(\text{pH})[\text{KS}] + h(\text{pH}\cdot T) + i[\text{KS}](T)$$

where a, b, c, etc., are constants calculated by regression.

The use of indirect conductimetry (in this case to monitor the evolution of CO_2) avoids the problems of interference experienced with conductivity measurements from food components, although growing the microorganism directly in one product may not allow information to be related to other products, even if they have a similar pH, a_w and storage temperature, because of their many differing structures and properties (e.g. grains, fruit, cakes, sauces). Without large numbers of comparative studies between detection times or MRs and traditional counting procedures, it would be difficult to predict how much a yeast would grow in a food of a given composition; however, the ability to collect such large amounts of information quickly would allow a relative assessment of the effects of varying the levels of the factors being investigated, and thus aid product formulation.

Another automated technique, namely flow cytometry, was investigated by Sørenson and Jakobsen[24] to study the combined effects of temperature (10–30°C), pH (4.7–6.0) and NaCl (1–12%, w/v) on the growth of the yeast *Debaryomyces hansenii*. Flow cytometric determinations correlated well with viable yeast populations determined as colony-forming units by traditional plating techniques. Growth curves were fitted using the modified Gompertz equation[8]. Lag phase and maximum specific growth rate were then derived. A second-order polynomial model was used to describe the effects of the environmental conditions on the growth parameters. The model was validated by repeating the experiment using different laboratory media. Good agreement was claimed between observed results from the second experiment and the predicted values based on the first experiment. Ideally, independent data should have been sought, but were unavailable.

The process of model building used above is directly comparable with that of Gibson *et al.*[5] and Baranyi *et al.*[16] A two-stage process was used: first, growth curve data were modelled; then, the curve parameters were modelled as a function of the environmental conditions. The methodologies vary only in the choice of curve-fitting procedure, either the modified Gompertz curve or the Baranyi model being used. The Baranyi model

might be considered superior because it is able to cope with data with and without an upper asymptote and, providing the initial inoculum level is always in the same state at time zero, the lag phase does not need to be modelled separately.

Current situation and future trends

The ability to predict mould growth ideally requires some measure of growth with respect to time. In the case of bacterial growth, this is a relatively simple, if time-consuming, process, but in the case of moulds, this is more difficult because moulds are not unicellular. Theoretically, yeasts can be enumerated using methods that are similar to those used for bacteria and it is interesting to note that the majority of fungal modelling applications described to date have been for yeasts. In such cases, some kind of automated technique has been applied to speed up data gathering. Methods involving optical density and conductimetry avoid the need to carry out plate counts; however, the optical density change over time may not always be directly related to viable counts, and predictions may not therefore be directly related to the rate or amount of growth that might occur in a product. A simple relationship between optical density 'growth rates' and growth rates derived from viable counts has been determined for bacteria[6] but has yet to be established for yeasts. However, the techniques are simple and provide rapid means of assessing the relative effects of different levels of inhibitors, or of adding or removing inhibitors from a food product. Indirect conductimetry has the added advantage that the yeast can be grown directly in the food product of concern, although this would result in a 'product-specific' model.

In the case of moulds, there is no rapid or simple method of gaining estimates of growth with respect to time. Indirect methods of measuring fungal growth, such as chitin and ergosterol analyses, cannot be related directly to fungal mass. The ergosterol content of hyphae can vary with substrate, growth conditions, and the age of the mycelium; in addition, there are interspecies differences. Mould spoilage is often visible in the form of surface colonies; consequently, mould growth has been studied by measuring colony diameters, on agar plates, over time. A growth rate function (usually expressed as radial growth rate in μm/h) can be derived by plotting colony diameter (or radius) against time, and measuring the slope of the straight part of the line. The colony or radial growth rate obtained under various conditions can then be modelled.

The study of Gibson *et al.*[5] was the first application of empirical modelling to mould growth and the authors considered their model an 'initial model' and acknowledged its limitations. The modelling was seen as a numerical exercise because the data used had not been acquired specifically for the purpose of modelling, and because additional factors such as pH should have been taken into account. In the studies of Pitt and Miscamble[14], a_w was adjusted using sugars, thus these models should be interpreted only for foods incorporating sugars as a humectant. Models for a_w using other humectants need to be produced. Although data at 25°C, 30°C and 37°C were available[14], the model was derived from data at one temperature, 30°C. Models that incorporate temperature are currently under investigation, but ideally a broader range of temperatures is required to produce a reliable model incorporating temperature. This initial modelling process provided valuable guidance on experimental design for future work that could further improve the models, but also highlighted the technical problems associated with the large amount of tedious and labour-intensive work necessary to provide the raw data.

Although all mould growth does not occur at the surface and the models of Gibson *et al.*[5] cannot take into account proliferation of hyphae within a product, the relevance of more mechanistic models that describe hyphal extension rate[9] to foods is questionable both from the mathematical point of view[8,10] and because of the physical problems of measuring hyphal growth in a food matrix. The usefulness to food processors of models describing colony formation on a product needs to be evaluated, and collaborative efforts by several groups of workers, using standardized methodology, need to be established to produce sufficiently large databases that are suitable for modelling. The relevance of approaches measuring the 'number of days to visible growth' turbidometrically in microtitre plates[17] needs to be further investigated. Is the growth of moulds in liquid culture comparable to that in a structured food product? In the case of yeasts, automated techniques such as indirect conductimetry and flow cytometry provide large amounts of data extremely quickly, and flow cytometry has the added advantage of providing viable counts over time, allowing the modelling of rate and amount of growth over time.

Studying mould colony growth over time is a labour-intensive task and the luxury of producing the type of data of Pitt and Miscamble[14] is rarely feasible. Computer-linked automated laser colony counters are already available. Further modifications to this technology could possibly provide an automated method for measuring large numbers of colony diameters and perhaps provide sufficient data for modelling. The possibility of using impedence[22] to monitor mould growth also needs further investigation. Modelling of mould growth has not developed as rapidly as bacterial modelling because of the difficulties of acquiring sufficient suitable data and the lack of support from food manufacturers and processors.

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Review

Functional foods for athletes

F. Brouns

There is an increasing awareness of the fact that nutritional factors can influence the physical and mental performance capacity of individuals who are involved in intense exercise. In addition, specific nutritional substances are thought to influence physiological functions or metabolism in the body in such a way that performance enhancement may be achieved and recovery from exhaustive exercise may be improved. In this review, I discuss some of these interactions and emphasize the need for health- and performance-related claims on sports foods to be supported by scientific evidence.

F. Brouns is at Novartis Nutrition Research Unit and Maastricht University, Nutrition Research Center, Department of Human Biology, PO Box 1350, NL-6201 BJ Maastricht, The Netherlands (fax: +31-43-3670676; e-mail: f.brouns@hb.unimaas.nl).

One of the most important nutritional concerns for athletes is the increased need for energy. Training or competition will increase the daily energy expenditure by 2090 to >4180 kJ (500 to >1000 kcal) per h of exercise, depending on body weight and exercise intensity. Athletes must therefore adapt their food consumption to meet their energy needs. This increased food intake should be well balanced with respect to macro- and micronutrients. However, this is not always as simple as it is thought to be by many nutritionists.

Many specific athletic events may be characterized by extremely high exercise intensities. Running a marathon, for example, expends ~10450–12540 kJ (2500–3000 kcal). Depending on the time needed to finish, this may induce an energy expenditure of ~3135 kJ (750 kcal) per h in a recreational athlete and 6270 kJ (1500 kcal) per h in the elite athlete who finishes the race in ~2–2.5 h. A professional cycling race, such as the 'Tour de France', costs ~27170 kJ (6500 kcal) per d, a figure that will increase to ~37620 kJ (9000 kcal) per d when the athletes are cycling over a mountain pass[1]. Compensating for such high energy expenditures by ingesting normal, solid meals will be difficult for any athlete who is involved in such competitions, because their capacity to digest and absorb will be impaired during intensive physical activity, largely as a result of decreased splanchnic blood flow and changes in peristaltic activity[2,3].

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