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Preparation and validation of a growth model for *Bacillus cereus*: the effects of temperature, pH, sodium chloride and carbon dioxide

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Abstract

The growth responses of a vegetative inoculum of *Bacillus cereus* as influenced by varying conditions of temperature, pH value and sodium chloride concentration (% w/v) and carbon dioxide concentration (% v/v) were determined in laboratory medium. Growth curves in concentrations of NaCl in the range 0.5-10.5% (w/v), pH values in the range 4.5–7.0, CO_2 concentrations in the range 10–80% (v/v) and storage temperatures from 10°C to 30°C were fitted using the regime of Baranyi et al. (1993). A response surface model was prepared and predictions of doubling time, growth rate, lag time and time to 1000-fold increase could be obtained for any set of conditions within the matrix studied. This model is included in Food MicroModel Version 1. Predicted doubling times from the model were compared to observed doubling times in the literature and the model was found to give realistic estimates of doubling time for a range of foods including milk, meat and poultry and carbohydrate-based products.

Keywords: Predictive model; Growth; *Bacillus cereus*; Food safety; pH; Sodium chloride; Temperature; Carbon dioxide

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1. Introduction

Bacillus cereus is a Gram-positive aerobic endospore-forming rod-shaped organism, frequently recovered from the environment and food, including liquid milk and milk products (Ahmed et al., 1983), dried products such as soup and milk, in which spores survive (Kim and Goepfert, 1971), pulses and cereals (Blakey and Priest, 1980) and spices (Powers et al., 1976). It causes food spoilage, notably of dairy products (Billing and Cuthbert, 1958; Overcast and Atmaram, 1974) and food poisoning (Hauge, 1955; Kramer and Gilbert, 1989; Lund, 1990).

B. cereus can be divided on a basis of growth temperatures into psychrotrophic and mesophilic strains. Psychrotrophic strains grow at 5°C and relatively rapidly at 10°C, while mesophilic strains fail to grow below 8°C and grow slowly at 10°C. Both types have been implicated in food poisoning.

B. cereus can cause illness by one of two types of enterotoxin both of which can be produced in foods (Varnam and Evans, 1991). Symptoms of illness caused by the emetic toxin are nausea and vomiting, but rarely diarrhoea, commencing 1–6 h after consumption. The illness can be confused with that caused by staphylococcal enterotoxin (Johnson, 1984). Foods most commonly associated with illness caused by the emetic toxin include cooked rice dishes and other farinaceous foods (Kramer and Gilbert, 1989) where rice is cooked in bulk and allowed to cool slowly, permitting outgrowth of surviving spores (Lund, 1990), and also milk and ice cream (Van Netten et al., 1990; Granum et al., 1993). The emetic toxin specifically affects primates and although thought to be produced during sporulation, a clear relationship between sporulation and toxin production has not been demonstrated (Turnbull, 1986). It has a molecular weight of less than 5000 kg/mol and is relatively pH stable and heat stable, being still active after heating to 126°C for 90 min (Johnson, 1984).

The diarrhoeal toxin appears to be produced during exponential growth either in the food or in the small intestine and has a molecular weight of 38 000–46 000 kg/mol (Kramer and Gilbert, 1989). It is heat-labile, being inactivated by heating at 56° for 5 min (Turnbull, 1976) and is more pH-labile than the emetic toxin, since it is inactivated by pH values of > 11.0 and < 4.0. It causes diarrhoea 8–16 h after consumption of the food and is accompanied by abdominal cramps and occasionally nausea, but seldom vomiting, and can be confused with illness caused by *Clostridium perfringens* (Johnson, 1984). Foods implicated include puddings and soups (Mossel et al., 1967), green vegetables (Portnoy et al., 1976; Schmitt et al., 1976), mashed potatoes (Anon, 1975), sauces (Hauge, 1955) and proteinaceous foods such as cooked meats (Luby et al., 1993; Kramer and Gilbert, 1989; Lund, 1990).

Recent research tentatively suggests that there may be a third, more serious, type of illness caused by *B. cereus* in which the incubation time is 1–3 days and illness is of longer duration (3–21 days). It is postulated that the cells may colonise the intestinal mucosa and produce an enterotoxin in vivo, which would explain the nature of the symptoms (Granum, 1994).

Other *Bacillus* spp. have also been responsible for illness including *B. licheniformis*, generally causing diarrhoea (Varnam and Evans, 1991), *B. subtilis*, generally causing emesis, (Kramer et al., 1982) and *B. pumilis* (Kramer and Gilbert, 1989; Lund, 1990).

Raevuori and Genigeorgis (1975) investigated the combined effect of pH and NaCl on the reduction of numbers of *B. cereus* in laboratory media and developed equations relating the decimal reduction of the population to the values of pH and NaCl concentration at 30°C. The values obtained for specific conditions were verified in cooked rice and cooked meat. Garcia-Arribas and Kramer (1990) studied the effect of pH on the growth of *B. cereus* and observed that although pH 8.8 extended the lag phase, this was followed by rapid growth. The minimum pH value supporting growth in broth is 4.9 and the maximum is 9.3 (Goepfert et al., 1972). In food, the minimum pH for initiation of growth is 4.35 and the upper limit is greater than 8.8 (Raevuori and Genigeorgis, 1975). Garcia-Arribas and Kramer (1990) reported that all the strains examined were able to increase the pH of an acid environment (pH 5.0) to a value which was more supportive of growth and toxin production.

There are inconsistent reports of the limiting effect of NaCl concentration on growth of *B. cereus*. Raevuori and Genigeorgis (1975) and Claus and Berkeley (1986) report that 11–89% of strains of *B. cereus* grew in 7% NaCl. Mossel et al. (1967) suggested 5% NaCl included in isolation agars to be a useful selective agent for *B. cereus* but 10% NaCl was too inhibitory. Peters et al. (1991) identified the temperature ranges over which *B. cereus* could grow at differing NaCl concentrations. These ranged from growth at all temperatures examined (14–41°C) at NaCl concentration of 0.5% (w/v) with pH 4.7 the minimum permitting growth. At 5% (w/v) NaCl, the temperature range supporting growth decreased to 21–39°C and the minimum pH was 5.5, while at 7% (w/v) NaCl no growth was recorded at any temperature.

Modelling has become an established technique for obtaining predictions for the growth and death or survival of microorganisms within a specific range of conditions (McMeekin et al., 1993). Predictive models exist for a number of food-borne pathogens including salmonellae (Gibson et al., 1988), *Listeria monocytogenes* (Buchanan et al., 1989; Buchanan and Phillips, 1990; Cole et al., 1990), *Yersinia enterocolitica* (Adams et al., 1991; Hudson, 1993; Sutherland and Bayliss, 1994) and *Staphylococcus aureus* (Broughall and Brown, 1984; Ross and McMeekin, 1991; Sutherland et al., 1994). Predictive models for *Bacillus* spp. include that of Baker and Griffiths (1993) for *B. cereus*, where temperature, pH, a_w, and starch and glucose concentrations were varied. Quintavalla and Parolari (1993) used turbidimetric techniques to model time to visible growth for *Bacillus* strains and Benedict et al. (1993) have developed a four-factor model for *B. cereus* encompassing temperature, pH and NaCl and nitrite concentrations.

The aim of this work was to produce, for inclusion in Food MicroModel Version 1, a four-factor growth model with controlling factors of temperature, pH, NaCl and carbon dioxide (CO_2), using an inoculum of vegetative mesophilic strains of *B. cereus* and to compare predictions from this model with published data from the literature.

2. Materials and methods

2.1. Strains

Six mesophilic strains of *B. cereus* were used as a mixed inoculum: F2797/87, F3351/87, F3752A/86, F196/73 (all producing emetic toxin), F3748/75 (diarrhoeal toxin), supplied by Dr. R.J. Gilbert of the Food Hygiene Laboratory, Colindale and B-6/Ac (emetic toxin) originally supplied by Prof. D.A.A. Mossel, Eijkmann Foundation, University of Utrecht. The cultures were maintained on Plate Count Agar slopes (Unipath CM 325) and subcultured every 3 months.

2.2. Media

Nutrient Broth No. 2 (Unipath CM 67) was used for the growth of *B. cereus* strains with NaCl added to give final concentrations up to 10.5% (w/v). The pH values of the broths were adjusted to between 4.75 and 7.0 inclusive using 2M HCl. Tryptone soya agar (Unipath CM 132) incubated at 30°C was used for determination of changes in viable numbers (cfu/ml) with time.

2.3. Inoculum

Strains were grown separately in Nutrient Broth No. 2 (Unipath CM 67) at pH 7.5 without added NaCl and subcultured daily for 2 successive days at 30°C. A third subculture was grown for 6 h at 30°C, to the exponential phase. Ten ml aliquots of broth containing similar numbers of each strain were mixed and diluted in Nutrient Broth No. 2 to approximately 10^5 cfu/ml.

2.4. Experimental procedure

2.4.1. Data generation with respect to pH, temperature and NaCl

Broths were dispensed in 300 ml amounts into a sterile batch culture system and autoclaved at 121°C for 15 min.

The batch culture system used was as follows: a 500-ml conical flask containing glass beads, to prevent clumping of germinated cells and spores, was stoppered by a rubber bung with two holes, to allow insertion of glass tubing. The shorter length (10 cm) of glass tubing was fitted externally with a sterile disposable filter (0.45 μm pore size, with Luer fitting; Sigma F1387) by 4cm of silicone tubing, to sterilise air entering the flask during sample removal. The external end of the longer length (22 cm) of glass tubing was joined to one of two ports in a Teflon screw cap 2 cm in diameter, via 11 cm of silicone tubing. A sterile disposable 5-ml syringe (Terumo, UK) joined to a second sterile disposable filter unit was attached to the second port on the Teflon cap. A sterile 5-ml bottle was screwed into the Teflon cap and a sample of inoculated broth withdrawn from the flask into the 5-ml bottle using the syringe. The bottle was aseptically detached and replaced by another sterile bottle. The unit, excluding the disposable filters and syringe, was autoclaved (121°C for 15 min) before addition of the broth and again after adding the broth.

The broths were equilibrated overnight at the appropriate incubation temperature and, prior to inoculation, 10 ml aliquots were removed aseptically and the pH values measured, but not adjusted. The remaining 290 ml of broth were inoculated with 1 ml of the mixed strain inoculum to give a final concentration of ca.10^3cfu/ml. The sterile disposable syringe and filters were aseptically attached and a sample of broth withdrawn to determine accurately the initial number. Each broth was then incubated, with shaking, at the appropriate temperature.

During subsequent sampling, broth was withdrawn sequentially into two 5-ml bottles. The first sample of each pair was discarded due to the presence of residual broth in the tubing which caused anomalies in recovery. The second sample was used to determine the numbers present by preparation of decimal dilutions in quarter strength Ringers solution (Unipath BR 52) and plating 20 μl onto duplicate plates of TSA. The plates were incubated at 30°C for 24 h, the mean of the duplicated plate counts determined and the number of colony forming units per ml calculated.

2.4.2. Data generation with respect to pH, temperature, NaCl and CO_2

The media were dispensed in 100-ml quantities, from which 10ml aliquots were aseptically withdrawn after autoclaving, and the pH value measured, but not adjusted. The remaining 90 ml of broth were inoculated with 1 ml of mixed strain inoculum prediluted to ca. 10^5 cfu/ml.

Sterile membrane pads (Sartorius Ltd., Epsom, UK) of 47 mm diameter were aseptically placed in 60-mm diameter sterile plastic vented petri dishes (Bibby Sterilin Ltd, Stone, UK). To each pad was added 2 ml of the inoculated broth containing approximately 10^3/ml *B. cereus*. Each petri dish containing an inoculated filter pad was individually packaged in a Dyno HPDE 150 ml tray (Dynopack Ltd., Reading, UK) with a top web of polyamide laminate (Suprovac 90; Kempner, Witham, UK) of water vapour permeability ca. 1.1 g/m^2/d at 23°C, 85% RH and gas permeability: oxygen ca. 25, CO_2 ca. 90 and nitrogen ca. 6 cm^3/m^2/d at 20°C at 50% RH. Packs were flushed with an atmosphere of 10, 20, 40, 60 or 80% CO_2, with a balance of nitrogen (N_2), using a Mecapac M500 modified atmosphere packaging machine (ECM Mecapac, Bagonet, France). Immediately after gas packaging, the atmosphere was checked using a Gowmac Gas Chromatograph Model 5292/202 (Gowmac, Gillingham, UK). These packs are representative of commercial modified atmosphere packaging of foods in CO_2 and N_2, where no attempt is made to limit effusion of CO_2 or influx of atmospheric air. The gas flushed filter pads were stored at 4°C until transport to the laboratory in insulated boxes, a journey of just over 1 h. At the laboratory, one pack from each set of conditions was sampled initially and the remainder were incubated at the appropriate temperatures. At intervals during incubation, inoculated pads were sampled by opening the gas flushed pack, aseptically removing and macerating the entire pad in 90 ml 1/4 strength Ringer's solution, preparing decimal dilutions and plating 20-μl volumes on duplicate TSA plates. Plates were incubated for 24 h at 30°C. The mean of the count on duplicate plate counts

was determined and the number of colony-forming units per ml (of suspension saturating the pad) was calculated.

2.4.3. Experimental design

Combinations of conditions from the following matrix, representative of conditions encountered in a wide range of foods, were selected, the conditions being distributed evenly through the following matrix:

Incubation temperature (°C)	10, 15, 20, 25, 30
pH	4.5, 5.0, 5.5, 6.0, 6.5, 7.0
NaCl (%w/v)	0.5, 2.5, 4.5, 6.5, 8.5, 10.5
Carbon dioxide	(%v/v) 0, 10, 20, 40, 60, 80

Growth responses from 177 conditions from this matrix were used to produce the model.

2.4.4. Statistical modelling

The data were modelled in two stages. The first stage involved fitting growth curves with the function of Baranyi et al. (1993) using Program 2 of McClure et al. (1993) to estimate the main growth parameters, i.e. the specific growth rate (μ), lag time (λ) and $\log_{10}$ maximum population density (γ). For each combination of conditions, the bacterial numbers were modelled in this manner as a function of time.

The second stage of modelling described the the variation of growth parameters μ, λ and γ as a function of growth conditions (pH, NaCl, temperature and CO_2). To stabilise the variance of the response parameter, the natural logarithm was taken and expressed by a quadratic function of the controlling factors as shown below:

$$\ln g = p_1 + p_2x_1 + p_3x_2 + p_4x_3 + p_5x_4 + p_6x_1x_2 + p_7x_1x_3 + p_8x_1x_4 + p_9x_2x_3 + p_{10}x_2x_4 + p_{11}x_3x_4 + p_{12}x_1^2 + p_{13}x_2^2 + p_{14}x_3^2 + p_{15}x_4^2 + \epsilon$$

where g is the modelled growth parameter

p_i ($i = 1, 2 \ldots 15$) are the coefficients to be estimated

x_1 = pH value

x_2 = NaCl concentration

x_3 = temperature

x_4 = CO_2 concentration

ϵ is a random error with assumed zero mean and constant variance.

3. Results and discussion

Table 1 shows some examples of published compared with predicted doubling times, using the commercially available Food MicroModel Version 1, for *B. cereus* in liquid media and representative foods. Although a four-factor model was produced, the paucity of literature data relating to growth of *B. cereus* in carbon dioxide meant that the carbon dioxide concentration for predictions always had to be entered as 0%. Consequently, it was not possible to validate the CO_2 aspect of the model.

Figs. 1–4 depict the comparisons made between predicted and published (observed) doubling times in different food categories. Ideally, the points should lie along the line of equivalence, i.e. the $\log_{10}$ predicted value should equal the $\log_{10}$ observed value. In the case of points below the line, the observed doubling time is longer than the predicted and so the prediction can be regarded as "safe". Fig. 1 indicates that apart from one (discussed below), the points lay close to or below the line, indicating that predictions for all foods were realistic and safe.

Some authors did not provide information about the conditions of pH and NaCl in the food and assumptions were necessary. In some cases for the literature data, spores were used to inoculate the food, whereas our model is based on a vegetative inoculum. Although use of spores may affect the duration of the lag phase of growth of *B. cereus*, since the spores need to be activated, germinate and outgrow, a spore inoculum is unlikely to affect growth rate (or doubling time) once exponential growth has been initiated, and our results show good agreement between doubling times predicted by the (vegetative) model and those observed by other authors using spore inocula.

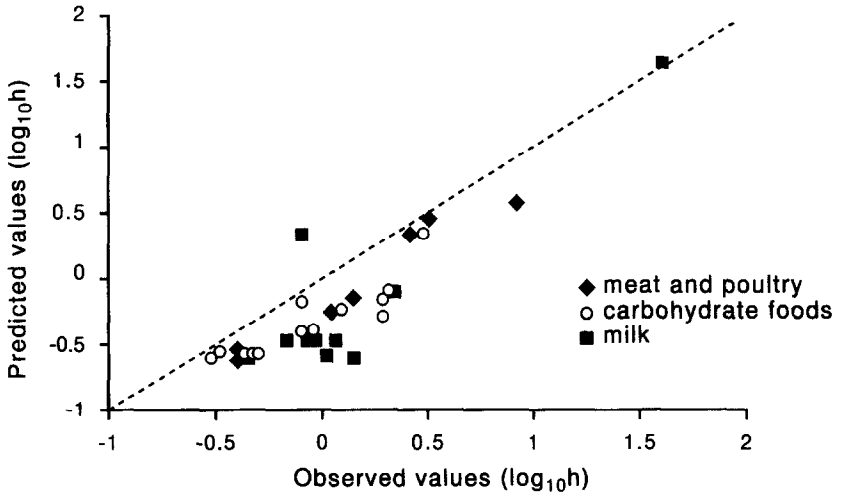

Fig. 1. Comparison of observed and predicted doubling times for *B. cereus* in all foods.

Table 1
Comparison of observed and predicted doubling times for *B. cereus* in foods

Temp (°C)	pH	NaCl (%w/w)	Substrate	DT publ (h)	DT pred (h)	Inoculum (log no/ml)	Assumptions	Source	Comments	Ref.
30	5	0.5	Brain heart infusion	0.43e, 0.8d	1.17	4.0-5.0	NaClC		Vegetative cells used, cultures aerated by shaking	1
30	6.8	0.5	Non-fat milk medium	0.34-0.6	0.26	4.00	NaCl	C	Spores used	2
22	6	0.5	Cooked poultry breast	1.4-1.5	0.72	3.00	NaCl	A	DT varies with strain,	3
15	6.5	0.5	Cooked poultry leg	2.6-3.7	2.17	3.00	NaCl	A	Vegetative cells used	
15	6	1.7	Liver sausage	8.34	3.77	2.00		C	Spores used, substrate contained NO2 (120ppm)and PO4	4
20	6.4	0.5	Reconstituted dried milk	2.2	0.80	1.00	NaCl, pH	C	Natural inoculum of	5
26	6.5	0.5	Infant formula (C)	0.93	0.34	0.50	NaCl, pH		*Bacillus* spp. (spores)	6
30	6.7	0.5	Pasteurised milk	0.45	0.25	4.00	NaCl	C	Vegetative cells	7
15	7	0.5	Cooked rice	3.0-4.3	2.21	5.00	NaCl, pH	C	Spores used	8
30	7	0.5	Cooked rice	0.43-0.52	0.27	1.00	NaCl, pH	A	Spores used	9
22	6.1	0.5	Pinto beans	0.8-1.2	0.67	4.00	NaCl	C	Spores used	10

Temp., temperature; DT publ., published doubling time; DT pred., predicted doubling time using Food MicroModel Version 1; Assumptions, assumptions made about conditions of growth in published papers; Source, source of information about published generation times; A, authors' own calculation; B, calculated from authors' graphs; C, calculated from authors' graphs; e, emetic; d, diarrhoeal strains; Ref., reference.

References: 1. Garcia-Arribas and Kramer, 1990; 2. Wong and Chen, 1988; 3. Sooltan et al., 1987; 4. Asplund et al., 1988; Rodriquez and Barrett, 1986;

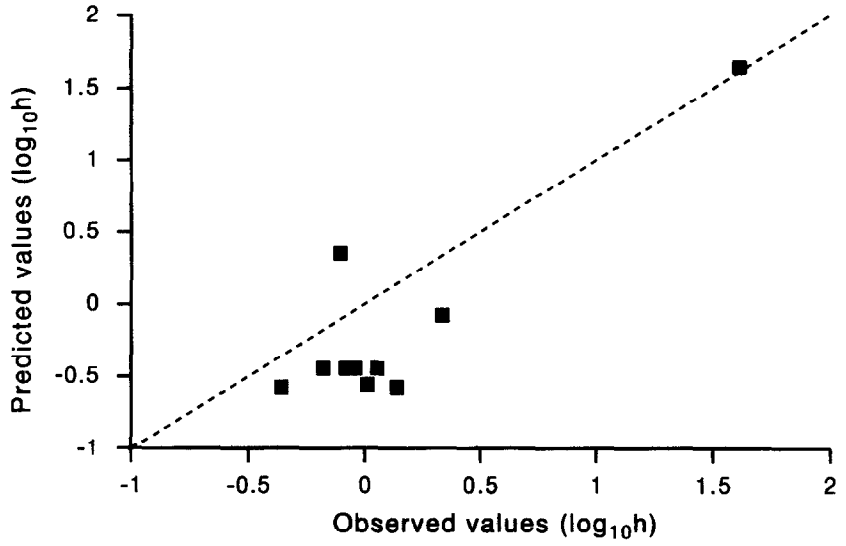

Fig. 2. Comparison of observed and predicted doubling times for *B. cereus* in milk.

Almost all the data available in the literature reporting growth responses in media (Shehata and Collins, 1971, Chung and Sun, 1986, Rajkowski and Mikolajcik, 1987) record growth rates and doubling times of *B. cereus* determined by optical techniques. Although Rajkowski and Mikolajcik (1987) prepared a standard curve of optical density vs. cell number, and used cell numbers derived from this

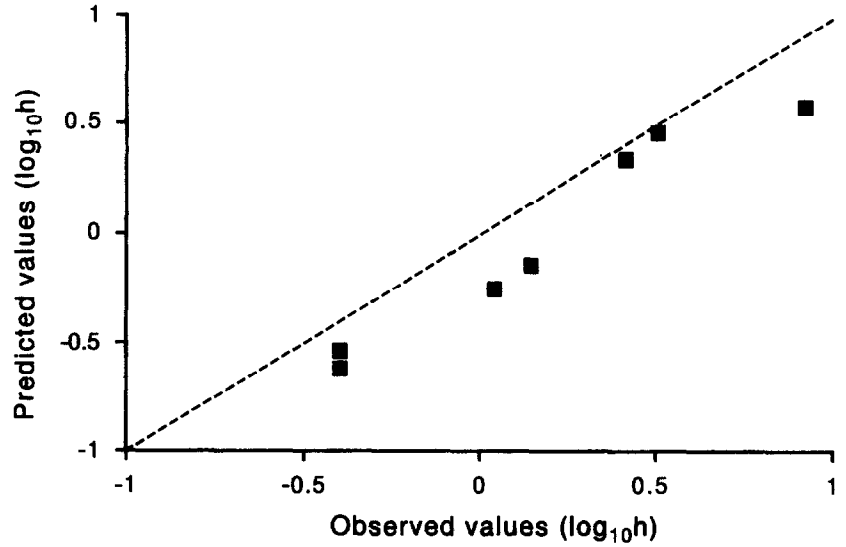

Fig. 3. Comparison of observed and predicted doubling times for *B. cereus* in meat and poultry.

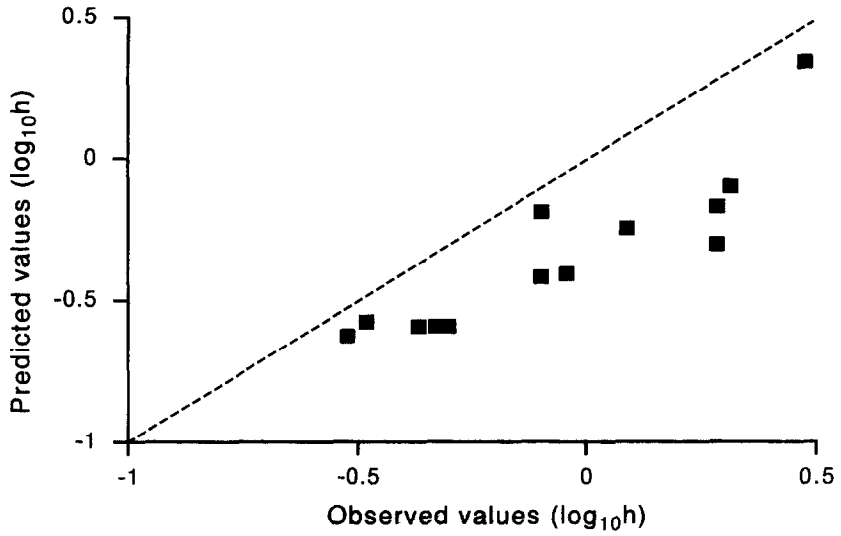

Fig. 4. Comparison of observed and predicted doubling times for *B. cereus* in carbohydrate foods.

calibration to determine doubling time, there are nevertheless, serious limitations associated with such techniques for measuring growth responses (McMeekin et al., 1993). The most notable is that the relationship betweeen cell number and turbidity is linear over only a limited range, corresponding to about a ten-fold increase in numbers. Hence, comparisons derved from optical data should be treated with some caution and for this reason have not been used in validation of the model. Furthermore, among the growth responses available in the literature where data were generated in liquid media using conventional microbiological techniques, very few indeed are within the temperature range of Food MicroModel; most are at temperatures greater than 30°C.

Comparison of predicted and observed doubling times of *B. cereus* in poultry and meat products (Fig. 2) agree relatively well for poultry (Sooltan et al., 1987, Table 1, Ref. 3), although the NaCl concentration in poultry flesh was estimated (0.5%). Agreement between predicted and observed doubling times in liver sauage (Asplund et al., 1988, Table 1, Ref. 4) was less satisfactory, but this product contained 120μg/l nitrite which would inhibit growth of *B. cereus* (Asplund et al., 1988, Table 1, Ref. 4) and phosphate.

In the comparison of predicted and observed doubling times in milk (Fig. 3), some data relate to dried milk powders, since the concern is that spores surviving the drying process will germinate and outgrow when the milk is reconstituted. Some experiments used a naturally-occurring (spore) inoculum, which was not specifically *B. cereus* (Rodriquez and Barrett, 1986, Table 1, Ref. 5; and Harmon and Kautter, 1991, Table 1, Ref. 6). The NaCl content of milk was assumed to be 0.5% (w/v). The pH value was not indicated in the papers by Rodriquez and Barrett (1986) (Table 1, Ref. 5) and Harmon and Kautter (1991) (Table 1, Ref. 6) and was

assumed to be 6.4–6.5. Fig. 3 shows that one point is significantly above the line of equivalence, indicating that the predicted doubling time is appreciably longer than the observed. This point relates to the growth of *B. cereus* in fruit-flavoured pasteurised milk (Wong et al., 1988) and no obvious reason can be identified for the discrepancy between the observed and predicted value for doubling time. However, the pH value (4.7) is close to the lower limit for growth of *B. cereus* and it is recognised that close to the boundary of growth/no growth conditions, "edge effects" can occur with response surface models.

A model developed using mesophilic *B. cereus* strains can produce misleading predictions if used for determining growth responses of psychrotolerant strains, particularly at temperatures of 15°C and below; i.e. Meer et al. (1991) determined that the doubling time of psychrotolerant *B. cereus* at 10°C in milk (pH assumed to be 6.8 and NaCl content 0.5%w/v) was 4 h while Food MicroModel (based on mesophilic strains) predicted a doubling time of 7.8 h. Thus, predictive models should be used only in the relevant context of conditions and strains.

Predicted and observed doubling times for *B. cereus* in carbohydrate foods are compared in Table 1 and Fig. 4 and include cooked rice, which has been responsible for a number of outbreaks of the emetic illness (Kramer and Gilbert, 1989). Where the substrate is plain boiled rice or pinto beans, the published and predicted doubling times are in close agreement (Johnson et al., 1983, Table 1, Ref. 8; Gilbert and Stringer, 1974, Table 1, Ref. 9; Nester and Woodburn, 1982, Table 1, Ref. 10).

The data presented indicate that the Food MicroModel Version 1 four-factor model for mesophilic strains of *B. cereus* gives predictions for growth that generally demonstrate good representation of growth responses in food. It gives a rapid estimate of the growth responses to be expected under a variety of environmental conditions and is therefore useful in providing a quick response to the "what if—?" questions that accompany development, or proposed changes in formulation, of foods and should thereby eliminate much repetitive and wasteful challenge testing.

Other models exist which predict the growth responses of *B. cereus* (Baker and Griffiths, 1993, Quintavalla and Parolari, 1993) but only in this model have predictions been compared extensively with data available from the literature. Furthermore, the model of Quintavalla and Parolari (1993) is based on measurements of optical density which have considerable limitations as discussed above. In addition, the main findings of that paper are the identification of the locations of certain optimum points of the respective response surfaces and the estimations of these locations can be more erroneous than the prediction for a particular set of environmental factor values.

The predictions in this paper are derived from the *B. cereus* model for mesophilic strains, prepared using a vegetative inoculum, in Food MicroModel Version 1, which is available as a disc for personal computers from Food MicroModel, Randalls Road, Leatherhead KT22 7RY, UK. Food MicroModel is covered by agreements on the Intellectual Property Rights involving the MAFF and the institutes and associations participating in the MAFF-funded programme.

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