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Modelling *Bacillus cereus* growth

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Abstract

The aim of this study was to model the growth, in a model system, of toxigenic strains of *Bacillus cereus* as a function of temperature, pH and water activity. Optical density (OD) values were transformed into numbers of colony-forming units (CFU) by the use of a 'calibrating' relation. The growth curves were then fitted to the Gompertz function which allowed the estimation of the lag-time (λ) and the growth rate (μ). These two parameters were then modelled according to the controlling factors which were pH and water activity at 20 and 30°C. © 1997 Elsevier Science B.V.

Keywords: Bacillus cereus; Bacterial growth; Turbidimetric data; Modelling

1. Introduction

Bacillus cereus is a spore-forming bacteria, Gram positive, aerobic or facultatively anaerobic. This microorganism occurs in different foods such as milk, vegetables, spices, rice and sauces (Masson and Vanos, 1982). Its pathogenicity is linked to the production of two toxins; a thermostable emetic enterotoxin (Johnson, 1984), and a thermosensitive diarrhoegenic enterotoxin (Goepfert et al., 1972). It is generally admitted that a population of more than 10^5 bacteria per gramme is required for intoxication to occur (Johnson, 1984). Contamination of a food product by *Bacillus cereus* does not necessarily cause a modification of the organoleptic properties, which explains possible consumption without suspicion on the part of the consumer (Christiansson, 1992). Its ability to sporulate makes it quite resistant to heat treatments. The occurrence of viable spores in food products is a common phenomenon: it is therefore important to be able to predict the germination and the growth of the microorganism in the product while taking into account food characteristics and environmental conditions.

A few studies have already been carried out aiming at modelling the growth of *Bacillus cereus* (Quintavalla and Parolari, 1993; Baker and Griffiths, 1993; Langeveld and Cuperus, 1993; Benedict et al., 1993). In some of these studies, growth was monitored by plate counting but some others used turbidimetric data. Our work aimed at modelling the

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growth parameters of *Bacillus cereus* as function of temperature, pH and a_w from turbidimetric data.

2. Materials and methods

2.1. Selection of bacterial strains and production of spores

The bacterial strains of *Bacillus cereus* used in this study were isolated on PEMBA medium (CM 617, OXOID, Basingstoke, UK) from different food products (Table 1) and identified using the API 50 CHB test kit (50 430, BIOMERIEUX S.A, Marcy-l'Etoile, France). The strains were also examined for diarrhoegenic enterotoxin production using BCET-RPLA kits (TD 950, OXOID, Basingstoke, UK). Only toxigenic strains able to grow at a temperature of 7°C and above were used. The vegetative cells of the different strains were stored at −80°C in Eugon medium (BK 068, BIOKAR, Beauvais, France) containing 15% (w/v) Glycerol (104 091, MERCK, Nogent-sur-Marne, France). Cells were spread on milk agar (CM 21, OXOID, Basingstoke, UK) and incubated at 40°C for five days. Spores and vegetative cells were then harvested and added to distilled water before a first centrifugation (11 000 rpm, 15 min). The supernatant was eliminated. Vegetative cells were killed by addition of a water/ethanol solution (50% v/50% v) and storage at 4°C for two hours. Spores were then cleaned by three centrifugations in distilled water (11 000 rpm, 15 min). Spore suspensions (adjusted to 10^8 spores per ml of distilled water) were preserved at −80°C. At the begining of each experiment, the pool of spores, a mixture of the twelve spore suspensions, was realised by mixing 0.5 ml of each spore suspension.

2.2. Experimental design

From literature data describing growth conditions of *Bacillus cereus* (Rajkowski and Mikolajcik, 1987; Kramer and Gilbert, 1989; Van Netten et al., 1990) and results of tests we obtained using the gradient plate technique (Wimpenny and Waters, 1984), the experimental field was defined as follows: temperature ranging from 7°C to 30°C, pH ranging from 5 to 7.5 adjusted with HCl or NaOH, water activity ranging from 0.95 to 0.99 and adjusted with glycerol because, with this solute, bacterial growth generally occurs at lower a_w values than when adjusted with NaCl or sugars (Jakobsen et al., 1972; Jakobsen and Murrell, 1977).

Data were collected according to a factorial design (two tests carried out for each set of conditions) built with the following levels:

temperature: 7, 10, 20 and 30°C (and an additional study at 4°C)

pH: 5, 5.5, 6, 6.5, 7, 7.5

a_w: 0.991, 0.987, 0.975 and 0.95 respectively corresponding to 0, 7, 14 and 21% (w/v) added glycerol.

2.3. Medium and culture conditions

The microbial growth was monitored in Brain Heart Infusion (CM 225, OXOID, Basingstoke,UK). The medium was buffered with M.E.S (2-(N-mor-

Table 1
Origin of the *Bacillus cereus* strains used in the pool

Strain number	Origin
1	Liquid whole egg
8	Cooked pasta dishes
14.2	Floating island (dessert)
14.3	Floating island (dessert)
17	Rice pudding
20	Carrot-chicken sauce
21	Mixed salad with rice and curry
22	Wheat flour
26	Whole fresh cow milk
29	Ionised poultry mechanically deboned meat
63	Prepared food
110	Collection du C.N.R.Z., 78 350 Jouy-en-Josas, France

pholino)ethane-sulfonic acid, M2850, SIGMA, Saint Quentin Fallavier, France) for pH ranging from 5 to 6.7 and with M.O.P.S (3-(N-morpholino)propane-sulfonic acid, M1254, SIGMA, Saint Quentin Fallavier, France) from 6.5 to 7.9. pH was adjusted using HCl or NaOH. The water activity was modified by adding glycerol (104091, MERCK, Nogent-sur-Marne, France) and measured with the Thermoconstanter Humidat TH1 apparatus (NOVACINA). The medium was sterilized by filtration on 2 μm porosity filters (SM 16534, SARTORIUS, Palaiseau, France). Before inoculation, in order to activate the spores, the spore pool was heated for 15 minutes at 75°C. 20 ml of the medium were then inoculated at a level of 5×10^3 spores per ml. Once inoculated, the medium was distributed into multiwell plates (each well containing 350 μl of medium) suitable for automatic turbidimetry with a Bioscreen apparatus (Labsystems, Helsinki, Finland).

For the lower temperatures (7°C and 10°C), the use of the Bioscreen apparatus proved difficult because of erratic results due to water condensation and perhaps cells agregation phenomena. Nevertheless, in order that the experimental conditions remain unchanged on the whole factorial design, the growth of *Bacillus cereus* was monitored in multiwell plates placed in refrigerated incubators; 25 wells were filled with the inoculated medium and, periodically, using Tryptone Salt diluent (1 g/l Tryptone, L42, OXOID, 8.5 g/l NaCl, L5, OXOID, distilled water) counting of the bacterial concentration in one well was carried out on P.C.A. medium which gave directly the number of CFU yielding growth curves expressing CFU versus time.

At 20 and 30°C, data were collected with the Bioscreen spectrophotometer (Labsystems, Helsinki, Finland) which can simultaneously monitor 200 cultures. In these conditions, growth curves were expressed as $OD = f(t)$. To model the growth parameters, these data had to be converted into $CFU = f(t)$ using a 'calibrating' relation expressing CFU as a function of OD. To calculate this relation between numbers of bacteria (CFU) and Optical Density (OD 660nm) values, growth was monitored in multiwell plates incubated in the Bioscreen spectrophotometer for any condition of a reduced factorial design (pH: 5, 6.5, 7.5, a_w: 0, 7, 21% of glycerol, temperature: 20 and 30°C, two retests). During the exponential growth phase of each culture, at regular time intervals, 12 samples of the medium were taken. For each sample, after having recorded the OD, enumeration of the bacteria was carried out on P.C.A. Consequently, for any condition of the reduced factorial design, two regression lines (each made of twelve points) of the following form were obtained:

$$\mathrm{Ln(CFU)} = a + b\mathrm{Ln(OD)}$$

Using a covariancy analysis and a simple regression, a and b were calculated as functions of temperature, pH and water activity. This 'calibrating' relation was then used to convert OD data to CFU data.

2.4. Calculation of the growth parameters and statistical analysis

In order to obtain a reliable estimation of the growth parameters, all the growth curves were fitted to the Gompertz function (Zwietering et al., 1990) using STATGRAPHICS Plus version 6.0 software (STSC Inc., Rockville, Maryland 20852, U.S.A).

The growth-rate and lag-time models were calculated using Stepwise Regressions with Forward selection procedure (STATGRAPHICS Plus version 6.0 software). The system begins with no variable in the model and adds one variable at a time, as long as the new variable adds significance to the model. At each stage, the significance of variables is checked and the system removes variables that become insignificant.

3. Results

The additional study carried out at 4°C indicated that our strains were unable to grow at that temperature for any condition of pH and water activity. At 7 and 10°C, no growth was observed for the whole range of glycerol concentrations (0 to 21%), at pH 5.0. At pH 5.5, growth was only observed at high water activity. From pH 6 to 7.5, growth at 7–10°C occurred except in the presence of 21% glycerol (results not shown). At 20 and 30°C, no growth except at high water activity was observed at pH 5.0. From pH 5.5 to 7.5, growth was observed for all glycerol concentrations tested (results not shown). Because of the difficulties and the poor repeatability

encountered at 7 and 10°C, modelling was only done with data obtained at 20 and 30°C.

The slopes of all the regression lines (CFU versus OD) were submitted to a covariancy analysis. This analysis showed that the only significant factor acting on the slope was the water activity modulated by means of glycerol concentration. Using a simple regression, a and b have thus been expressed as functions of glycerol concentration.

The equation is:

$$\mathrm{Ln(CFU)} = (10.3 - 0.04G) + (1.21 + 0.010G)\mathrm{Ln}(1000(\mathrm{testOD} - \mathrm{blankOD}))$$

where G is the percentage of glycerol.

This equation was then submitted to validation. To do that, several cultures were monitored in 4 different conditions of the reduced factorial design. During the exponential growth phase and after each OD measurement, a 300 μl sample of medium was taken and submitted to bacterial counting on PCA medium. Thus, a series of values (OD/CFU) was obtained. The OD value read and the level of glycerol were introduced into the 'calibrating' relation which gave a predicted number of CFU. The predicted number of CFU was then compared with the experimental number (which was the count on PCA medium)(Fig. 1). The correlation coefficient, R, between the predicted and the experimental values was 0.9, which was considered satisfactory. This relation was then used to transform the OD data obtained at 20 and 30°C into CFU data.

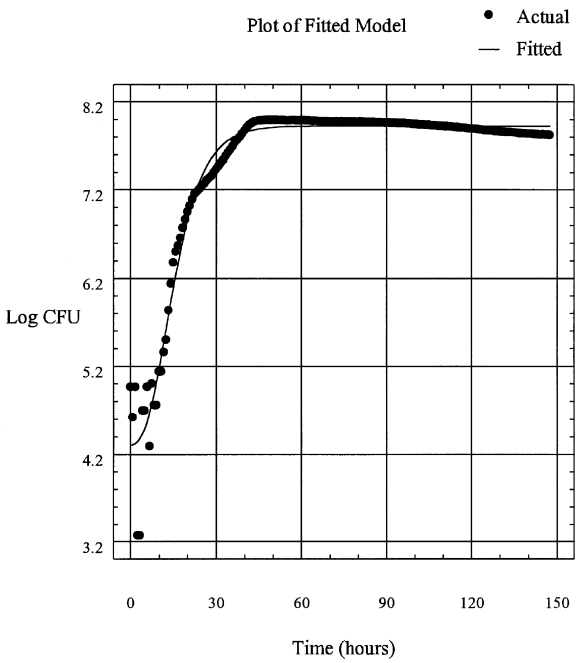

Fig. 2. Example of good fitting to the Gompertz function for data obtained at 20°C, pH 7, 0% glycerol.

At these temperatures, growth curves obtained at 20 and 30°C fitted well to the Gompertz function (Fig. 2). The R^2 values were all higher than 0.95. Therefore, the growth parameters were calculated.

Excluding conditions in which no growth occured

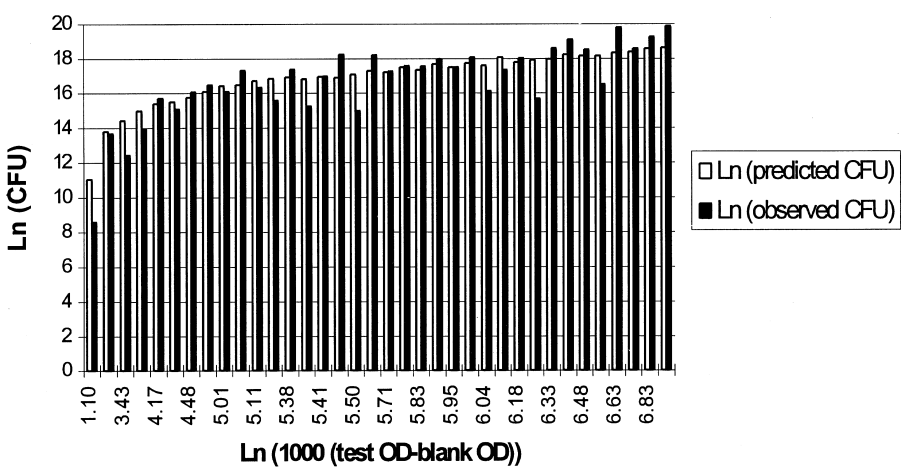

Fig. 1. Graph showing the relationship between observed and predicted values for the validation of the calibrating relation.

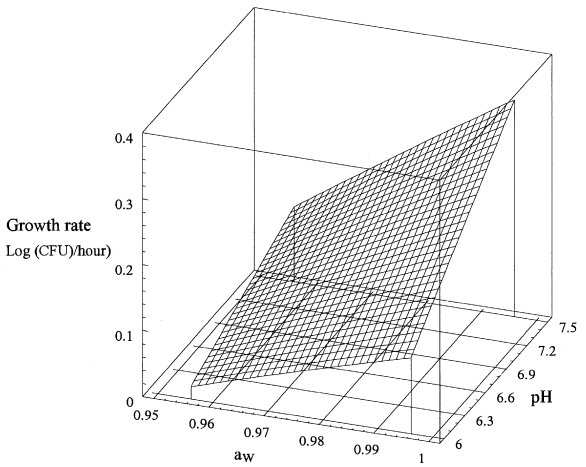

Fig. 3. Response surface showing the predicted growth rate at 20°C.

(Buchanan and Phillips, 1990), models were finally established in the following experimental range: pH: 6 to 7.5, a_w: 0.99 to 0.95 corresponding to 0 to 21%(w/v) of added glycerol and temperature, 20 to 30°C.

The growth rate model obtained (Fig. 3) can be written as follows:

$$\mu = 4.010 - 0.090\text{pH}.T + 0.098a_w.\text{pH}.T - 4.788a_w^2$$

μ in Log (CFU) per h, ($R = 0.95$)

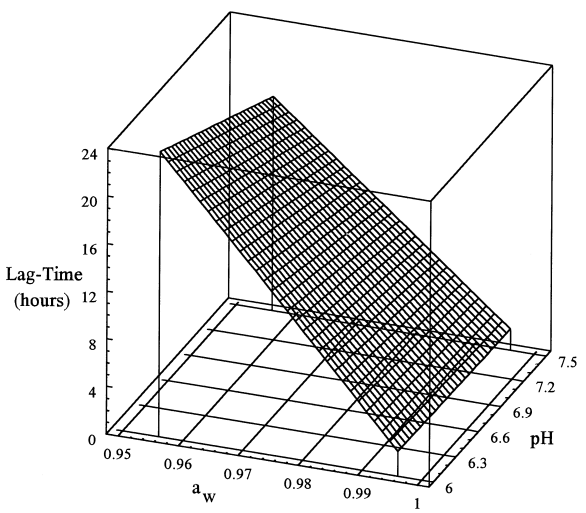

Fig. 4. Response surface showing the predicted lag-time at 20°C.

For the lag-time model (Fig. 4), a poor repeatability was noticed for the two retests in each condition. In the interests of safety, it was decided to keep only the lowest lag-time value for each condition. The following model was obtained:

$$\lambda = 1108.6 - 1109.6a_w - 4.6\text{pH}.T + 4.6a_w.\text{pH}.T$$

λ in h, ($R = 0.91$)

As for any polynomial model, these models cannot be extrapolated outside the experimental range.

4. Discussion

The choice of inoculating with a pool of spores offers the advantage of taking into account the very large strain variability in the various physiological properties of *Bacillus cereus* (Kramer and Gilbert, 1989) but provides, especially in borderline growth conditions, growth curves which are difficult to fit to the Gompertz function.

Collecting data from turbidimetric measurements led to the building of a calibrating model expressing CFU versus OD as a function of a_w. This kind of model is original as it converts OD in CFU taking into account the effect of the medium's characteristics on the calibrating relation. An hypothesis concerning the water activity effect on the calibration relation could be the following; when microbial cells are exposed to various a_w, their adaptative responses involve both morphological and physiological phenomenons: plasmolysis (Troller, 1980), accumulation of compatible solutes like potassium, amino acids (e.g. proline) and polyols (e.g. glycerol) (Beuchat, 1981). We can imagine that such changes have an effect on the results of turbidimetric measurements and, consequently, on the calibration relation.

At low temperatures, it proved impossible to use the turbidimetric system especially because of the condensation phenomena and perhaps also of the agregation phenomena observed by Fermanian et al. (1994) at low temperature till 20°C. So below this temperature, growth was monitored by plate counting. Our results indicate that all these strains are unable to grow at 4°C in 42 days. It was also noticed that at pH 5 or water activity inferior or equal to 0.95

(equivalent to 21% or more added glycerol) no growth occurred. These results are in agreement with those of Raevuori and Genigeorgis (1975) who gave 0.95 as a_w limit for growth. As for the effect of pH, these authors observed that the inhibitory effect of an extreme pH was less dramatic in foods than in laboratory media, which is liable to give a 'fail-dangerous' prediction.

As recommended by Buchanan and Phillips (1990), in order to obtain a better fit of the model with the experimental data, 'no-growth' data were eliminated from consideration.

The response surfaces derived from the models clearly show an important effect of a_w and interactions between the three controlling factors. When pH and a_w decrease, the growth rate is reduced and the lag-time is prolonged. It may be noticed that, for the lag-time model, the influence of pH increases as water activity decreases.

Another point which has to be stressed is the lack of repeatability for the growth parameters between the two retests, particularly in borderline growth conditions. It is known that this dispersing effect of borderline conditions is even more important when considering the behaviour of spore inocula (McClure et al., 1994). When modelling the lag-time from the lowest value for each condition, the model's robustness is reduced but not at the expense of safety.

All these models are still to be validated in foods.

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References

Baker, J.M., Griffiths, M.W., 1993. Predictive modeling of psychotrophic *Bacillus cereus*. J. Food Protect. 56, 684–688.

Benedict, R.C., Partridge, T., Wells, D., Buchanan, R.L., 1993. *Bacillus cereus*: aerobic growth kinetics. J. Food Protect 56, 211–214.

Beuchat, L.R., 1981. Microbial stability as affected by water activity. Cereal Food World 26, 345–349.

Buchanan, R.L., Phillips, J.G., 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. J. Food Protect. 53, 370–376.

Christiansson, A., 1992. The toxicology of *Bacillus cereus*. Bull. Int. Dairy Fed. 275, 30–35.

Fermanian, C., Fremy, J.M., Claisse, M., 1994. Effect of temperature on the vegetative growth of type and field strains of *Bacillus cereus*. Lett. Appl. Microbiol. 19, 414–418.

Goepfert, J.M., Spira, W.M., Kim, H.U., 1972. *Bacillus cereus*: food poisoning organism. A review. J. Milk Food 35, 213–227.

Jakobsen, M., Filtenborg, O., Bramsnaes, F., 1972. Germination and outgrowth of the bacterial spore in the presence of different solutes. Lebensm. Wiss. Technol. 5, 159–162.

Jakobsen, M., Murrell, W., 1977. The effect of water activity and a_w-controlling solute on sporulation of *Bacillus cereus* T. J. Appl. Bacteriol. 43, 239–245.

Johnson, K.M., 1984. *Bacillus cereus* foodborne illness—An update. J. Food Protect. 47, 145–153.

Kramer, J.M., Gilbert, R.J., 1989. *Bacillus cereus* and other *Bacillus* species. In: Doyle, M.P. (Ed.), Foodborne Bacterial Pathogens, Marcel Dekker, New York and Basel, pp. 22–64.

Langeveld, L.P.M., Cuperus, F., 1993. Aspects regarding the application of a predictive model for growth of *Bacillus cereus* in pasteurized milk. Bull. Int. Dairy Fed. 287, 6–10.

McClure, P.J., De W. Blackburn, C., Cole, M.B., Curtis, P.S., Jones, J.E., Legan, J.D., Ogden, I.D., Peck, M.W., Roberts, T.A., Sutherland, J.P., Walker, S.J., 1994. Modelling the growth, survival and death of microorganisms in foods: the U.K Food Micromodel Approach. Int. J. Food Microbiol. 23, 265–276.

Masson, A., Vanos, B., 1982. Recherche d'un milieu sélectif pour l'isolement de *Bacillus cereus* et recherche qualitative de la contamination par *Bacillus* sp. dans divers épices, arômates et condiments. Ind. Aliment. Agric. 99, 238–251.

Quintavalla, S., Parolari, G., 1993. Effects of temperature, a_w and pH on the growth of *Bacillus* cells and spores: a Response Surface Methodology study. Int. J. Food Microbiol. 19, 207–216.

Raevuori, M., Genigeorgis, C., 1975. Effect of pH and sodium chloride on growth of *Bacillus cereus* in laboratory media and certain foods. Appl. Microb. 29, 68–73.

Rajkowski, K., Mikolajcik, E.M., 1987. Characteristics of selected strains of *Bacillus cereus*. J. Food Protect. 40, 199–205.

Troller, J.A., 1980. Influence of water activity on microorganisms in foods. Food Technol. 5, 76–82.

Van Netten, P., De Moosdijk, A., Van Hoensel, P., Mossel, D.A.A., Perales, I., 1990. Psychotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bact. 69, 73–79.

Wimpenny, J.W.T., Waters, P., 1984. Growth of microorganisms in gel stabilized two-dimensional diffusion gradient systems. J. Gen. Microbiol. 130, 2921–2926.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van't Riet, K., 1990. Modeling of the bacterial growth curve. Appl. Environ. Microb. 56, 1875–1881.