J Ind Microbiol Biotechnol (2003) 30: 421–426
DOI 10.1007/s10295-003-0066-9

ORIGINAL PAPER

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Development of a structured model for batch cultures of lactic acid bacteria

Received: 21 January 2003 / Accepted: 22 April 2003 / Published online: 9 July 2003

Abstract A combined stochastic-deterministic model able to predict the growth curve of microorganisms, from inoculation to death, is presented. The proposed model is based on the assumption that microorganisms can experience two different physiological states: non-proliferating and proliferating. The former being the physiological state of the cells right after their inoculation into the new extracellular environment; the latter the state of microorganisms after adaptation to the new medium. To validate the model, a *Lactobacillus bulgaricus* strain was tested in a medium at pH 4.6 at two different temperatures (42°C and 35°C). Curves representing the bacterial growth cycle were satisfactorily fitted by means of the proposed model. Moreover, due to the mechanistic structure of the proposed model, valuable quantitative information on the following was obtained: rate of conversion of non-proliferating cells into proliferating cells, growth and death rate of proliferating cells, and rate of nutrient consumption.

Keywords Predictive microbiology · Modeling · Lactic acid bacteria

Introduction

The growth curve of a typical bacterium in a batch cultivation consists of four consecutive phases: the lag phase, during which there is no appreciable change in cell number; the exponential growth phase, where the number of living cells increases exponentially; the stationary phase, during which period the number of microorganisms does not change appreciably; the death phase, characterized by an exponential decrease in the number of living cells. Generally, microbiologists are interested in minimizing or preventing microbial growth. Therefore, they have focused their attention on developing mathematical models able to describe the first two phases of the entire growth curve, i.e., models able to predict the microbiological consequence of food storage. However, because of a number of health benefits deriving from probiotic bacteria (such as the inhibition of pathogens, reduction of colon cancer risk, stimulation of immune function, and reduction of serum cholesterol levels [10,16,19]), the stationary and death phases of the growth curve are also of great interest to food microbiologists. In fact, the ultimate intent in the probiotic use of foods is to provide the human gastrointestinal tract with a viable population of probiotic bacteria. The number of viable cells in food is of great importance, as demonstrated by other authors in studies on increasing numbers of colonization and dose-response, thus defining the required doses [9,14,15].

A number of models have been developed to predict bacterial growth in foods [4,13,17]. Many of these models may be classified as empirical [20,21], describing sigmoid functions that approximate bacterial growth curves (cell concentration versus time). While empirical models are useful for correlating a wide range of batch growth data and have predictive value, they fail to provide any real insight into the underlying mechanisms controlling cell growth. In contrast, mechanistic models, which are more complex from a mathematical point of view, give a detailed description of all phenomena involved during cell growth [3,22], providing valuable quantitative information that can be advantageously used to control (either promoting or inhibiting) microorganism growth.

In the present paper, a mechanistic model describing the phenomena behind the dynamic changes observed in all phases of the investigated experimental system is developed. A combined stochastic-deterministic approach was used to describe the entire cell growth curve.

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The ability to resolve the growth curve of a typical bacterial batch cultivation into the three basic phenomena involved—namely, cell adaptation to the new extracellular environment, cell growth, and cell death—is the main feature distinguishing the proposed model from others reported in the literature [5,6].

Materials and methods

Bacterial strains

Yogurt from retail food stores was decimally diluted in sterile saline and 1 ml of each dilution was transferred into Petri dishes. Modified (pH 5.4) melted MRS agar (Oxoid, Milan, Italy) (10–15 ml) was poured over the inoculum and allowed to solidify, after which the plates were incubated under anaerobic conditions at 37°C for 72 h. Typical colonies were picked up, purified, Gram stained, and biochemically tested with the API System 50 CH (bioMérieux, Marcy L'Etoile, France).

Microbiological analysis

Lactobacillus delbruekii subsp. *bulgaricus*, isolated from yogurt was tested in MRS broth (Oxoid), at pH 4.6 and at two different temperatures: 35 and 42°C. Working cultures were grown in MRS plus 7‰ agar and incubated at 37°C for 48 h.

Aliquots of MRS broth (250 ml) were inoculated with the working cultures (0.5% v/v). Bacterial counts were carried out at intervals, using the pour plate method on MRS agar plus covering layer, and incubating the plates at 37°C for 48 h. The cell counts were performed until colony-forming units (cfu) were detected (about 180 h at 35°C and about 200 h at 42°C). Every trial was carried out in triplicate. During experimentation, the pH was monitored to ensure that no inhibitory levels of acidity were reached; the pH never dropped below 3.5 during cell growth at the two temperatures investigated.

Modeling

The proposed model (see Fig. 1) is based on the assumption that microorganisms can experience only two different physiological state: non-proliferating and proliferating, the former being the physiological state of cells right after their inoculation into the new extracellular environment, and the latter the state of microorganisms fully adapted to the new medium. While cells are in the non-proliferating state, they try only to adapt themselves to the new medium. In this physiological state the proliferation and death rates of microorganisms are negligible, they can only convert to the other state: the proliferating state. In contrast to non-proliferating microorganisms, proliferating cells can proliferate and die. The above picture is similar to that of Srivastava and Volesky [17], who assumed that there is a bottleneck-substance that must reach a certain level to induce the growth of the cells. In fact, there is a continuous exchange between the above two hypothesized physiological states, which represent the extremes of the whole spectrum of physiological states experienced by the microorganisms. However, as also reported by Srivastava and Volesky [18], the above assumption is reasonable if one wants to evaluate the growth rate. Two additional assumptions used to derive the model are: (1) the limiting nutrient concentration was considered one of the main factors influencing cell growth rate (see p. 375 in [1]); (2) the amount of nutrient released by the dead cells, which contributes to the increase in the limiting nutrient concentration, is neglected (see p. 397 in [1]).

The rate at which non-proliferating microorganisms convert themselves to the proliferating state depends on their previous history (i.e., on the state of cells prior to inoculation) as well as on conditions in the new extracellular environment [5,6]. To describe the rate of conversion of non-proliferating cells, a stochastic approach, similar to that proposed in 1998 by Baranyi [2] to describe the lag phase, was adopted. Thus, the amount of non-proliferating cells converted into proliferating cells at a given time was evaluated by integrating the product of Q_0 (see Appendix) and the probability density function (see p. 30 in [11] of non-proliferating cell conversion. In particular, the Normal probability density function (see p. 49 in [11]) was used in this paper to predict the probability that non-proliferating cells convert to proliferating ones in the time interval included between time t and time $t + \mathrm{d}t$:

$$R_1(t) = Q_0 \cdot \left\{ \frac{1}{\sigma \cdot \sqrt{2 \cdot \pi}} \cdot \exp\left[-\frac{1}{2} \cdot \left(\frac{t - m}{\sigma} \right)^2 \right] \right\} \quad (1)$$

where m is the mean of the probability density function (it can be envisaged as the time at which half of the non-proliferating cells convert into proliferating cells), and σ is the standard deviation of the probability density function, i.e., a measure of the heterogeneous cell situation. In fact, according to Eq. 1, the microorganisms do not behave in the same manner; i.e., some of them are able to adapt to the new extracellular environment faster than others. This aspect, which is close to the real physical situation, is generally neglected in many of the models reported in the literature [17], which assume that during growth all cells behave in the same manner.

As reported above, proliferating cells are fully adapted to the new medium, hence they can proliferate. The proliferation rate of adapted microorganisms depends on both the cell concentration and the physicochemical state of the extracellular environment. As

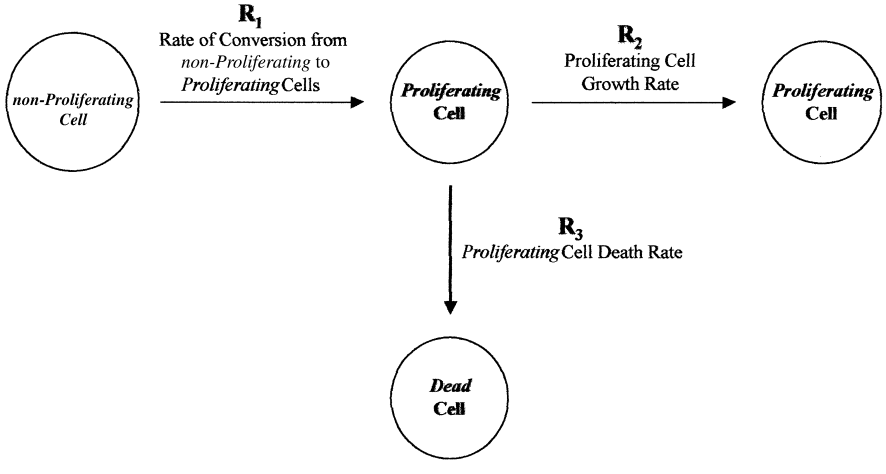

Fig. 1 Schematic representation of the proposed microbial growth model

reported above, the latter depends only on the limiting nutrient concentration. To describe the proliferation rate of adapted microorganisms, a first order kinetic was used:

$$R_2(t) = \{\exp[F(t) - k_1] - 1\} \cdot k_2 \cdot P(t) \quad (2)$$

The term $\{\exp[F(t)–k_1]–1\}\cdot k_2$ is the "inhibition function" [3], which accounts for the dependence of the proliferation rate on the limiting nutrient concentration. The parameter k_1 is the threshold value for limiting nutrient concentration. In fact, for values of limiting nutrient concentration equal to or lower than k_1, $R_2(t)$ is equal to zero. The parameter k_2 is the kinetic constant of the cell proliferation phenomenon; for a given value of concentration of both proliferating cells and nutrient, the higher k_2, the higher the rate of cell proliferation.

As reported in the literature, the rate at which proliferating cells die follows a first order kinetic [7]:

$$R_3(t) = k_3 \cdot \exp[k_4 - F(t)] \cdot P(t) \quad (3)$$

The term $k_3\cdot\exp[k_4–F(t)]$ accounts for the dependence of $R_3(t)$ on limiting nutrient concentration. The parameter k_4 is the threshold value for limiting nutrient concentration. In fact, for limiting nutrient concentration equal to or higher than k_4, the value of $R_3(t)$ can be considered negligible. On the contrary, for values of $F(t)$ lower than k_4, $R_3(t)$ becomes increasingly large, leading to a decrease in cell population. The parameter k_3 is the kinetic constant of the cell dying phenomenon: for a given concentration of both proliferating cells and nutrient, the higher k_3, the higher the cell death rate.

To evaluate the change in the extracellular environment over time, it was assumed that the rate of nutrient consumption depends on the proliferating cell concentration through a power law type expression:

$$\frac{dF(t)}{dt} = -k_5 \cdot [P(t)]^{0,1} \quad (4)$$

The parameter k_5 is the kinetic constant of the phenomenon describing nutrient consumption: for a given concentration of proliferating cells, the higher the value of k_5, the higher the rate of substrate consumption.

According to the above description of the evolution of the microbial population of a batch cultivation during storage, the concentration of microorganisms is given by the following expression:

$$N(t) = Q(t) + P(t) \quad (5)$$

To predict the entire growth curve it is necessary to predict the evolution of both $Q(t)$ and $P(t)$ during storage. According to the expression reported above, the rates at which both $Q(t)$ and $P(t)$ change during storage are:

$$\frac{dQ(t)}{dt} = -R_1(t) \quad (6)$$

$$\frac{dP(t)}{dt} = R_1(t) + R_2(t) - R_3(t) \quad (7)$$

Equations 4, 6 and 7 form a set of three ordinary differential equations, whose unknowns are $Q(t)$, $P(t)$ and $F(t)$. The above system was numerically solved using a fourth-order Runge-Kutta formula [12] with the following initial conditions: $Q(0) = Q_0$, $P(0) = 0$, $F(0) = F_0$. The numerical solution was used to fit the experimental data, and to predict the time course of microbial population on storage.

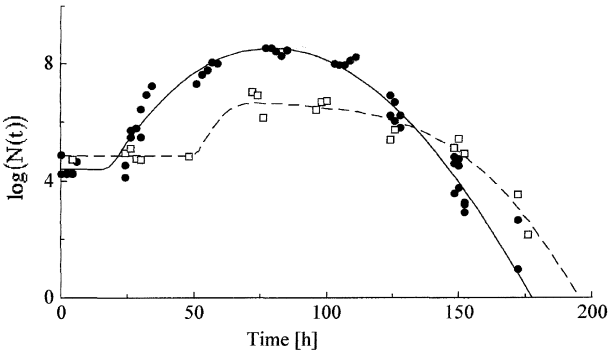

Fig. 2 log[$N(t)$] plotted as a function of time for tests conducted at 35°C (●) and 42°C (□). *Solid line* best fit of the model to the experimental data obtained at 35°C, *dashed line* best fit of the model to the experimental data obtained at 42°C

Results and discussion

To validate the proposed model, the growth curve of *L. delbruekii* subsp. *bulgaricus* was experimentally determined at 42°C and 35°C. Figure 2 shows log[$N(t)$] plotted as a function of time. In the same figure the best fit of the proposed model to the experimental data is also shown. The values of the model's parameters obtained by fitting the experimental data are listed in Table 1. The criterion used to evaluate the goodness of fit was the relative percent difference between experimental and predicted values or mean relative deviation modulus, defined by the following equation [8]:

$$\bar{E}\% = \frac{100}{n_{exp}} \cdot \sum_{i=1}^{n_{exp}} \frac{\left| Y^i_{exp} - Y^i_{pred} \right|}{Y^i_{exp}} \quad (8)$$

An E% value less than or equal to 5% indicates a very good fit, while a value more than 5% but less than 10% indicates a reasonably good fit [8]. The values obtained in the present investigation for E% were 4.76% and 2.44% for the data obtained at 35°C and 42°C, respectively. The results obtained prove that the proposed model satisfactorily fits the data, thus corroborating the validity of the approach and the hypothesis used to derive it.

Table 1 Values of model parameters obtained by fitting the experimental data. Q_0 Initial concentration of non-proliferating cells, m mean value of the probability density function, σ standard deviation of the probability density function, $k_{1–5}$ constant(s), to be regarded as fitting parameters

	Q_0 $\left[\frac{cfu}{ml}\right]$	m [h]	σ [h]	k_1 $\left[\frac{g}{l}\right]$	k_2 $\left[\frac{l}{h}\right]$	k_3 $\left[\frac{l}{h}\right]$	k_4 $\left[\frac{g}{l}\right]$	k_5 $\left[\frac{g/l}{h} \times \frac{1}{(cfu/ml)^{0.1}}\right]$
35°C	2.63×10^4	18.6	1.55	51.5	2.46×10^2	1.86×10^2	53.6	4.71×10^3
42°C	7.43×10^4	52.3	2.01	53.3	0.351	0.397	49.2	9.49×10^3

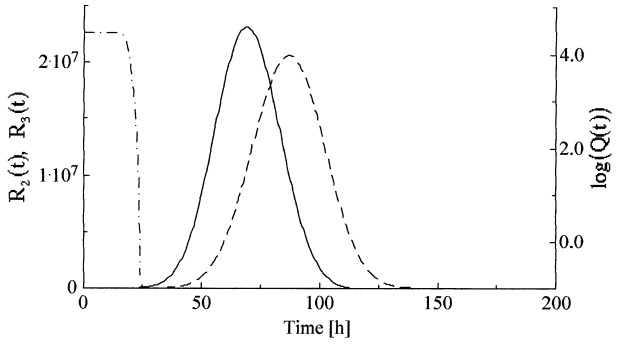

Fig. 3 $R_2(t)$, $R_3(t)$ and $\log[Q(t)]$ plotted as a function of time for a test conducted at 35°C. *Solid line* $R_2(t)$, *dashed line* $R_3(t)$, *dash-dot line* $\log[Q(t)]$

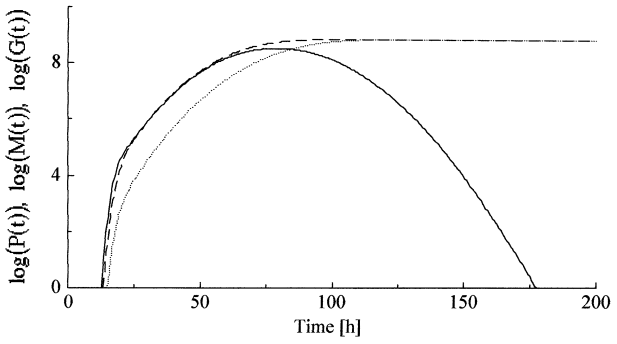

Fig. 4 $\log[P(t)]$, $\log[G(t)]$ and $\log[M(t)]$ plotted as a function of time for a test conducted at 35°C. *Solid line* $\log[P(t)]$, *dashed line* $\log[G(t)]$, *dotted line* $\log[M(t)]$

As reported in the literature [17], the strength of a mechanistic model, besides its ability to predict the experimental data, is to provide valuable information on all the phenomena involved during the cell growth-cycle. In the following, the proposed model is used to evaluate the evolution of the following during the cells' growth-cycle: (1) rate of conversion of non-proliferating cells into proliferating cells, (2) growth and death rate of proliferating cells, (3) rate of nutrient consumption. All curves shown were obtained using the data listed in Table 1.

Figure 3 shows $R_2(t)$, $R_3(t)$ and the $\log[Q(t)]$ plotted as a function of time for a test conducted at 35°C. As expected, both $R_2(t)$ and $R_3(t)$ are bell-shaped functions. In particular, $R_2(t)$ remains equal to zero until the cells have become adapted to the new extracellular environment, whereupon it starts to increase. Afterwards, due to the reduction of limiting nutrient concentration, and probably to the increase of inhibitory substances, $R_2(t)$ decreases until it falls to zero. $R_3(t)$ follows a trend similar to that of $R_2(t)$, but is delayed with respect to the latter. In fact, the rate at which living cells die is initially low because of both high limiting nutrient concentration, and the low concentration of toxic catabolites. As the proliferating cells grow and the limiting nutrient concentration decreases, $R_3(t)$ increases, passes through a maximum and then decreases until it falls to zero. The decrease of $R_3(t)$ is caused by the reduction in $P(t)$.

Figure 4 shows $\log[P(t)]$, $\log[G(t)]$ and $\log[(M(t)]$ plotted as a function of time for a test conducted at 35°C. The values of $P(t)$, $G(t)$ and $M(t)$ were evaluated according to the following expressions:

$$P(t^*) = \int_0^{t^*} [R_1(t) + R_2(t) - R_3(t)] \cdot \mathrm{d}t \tag{9}$$

$$G(t^*) = \int_0^{t^*} [R_2(t)] \cdot \mathrm{d}t \tag{10}$$

$$M(t^*) = \int_0^{t^*} [R_3(t)] \cdot \mathrm{d}t \tag{11}$$

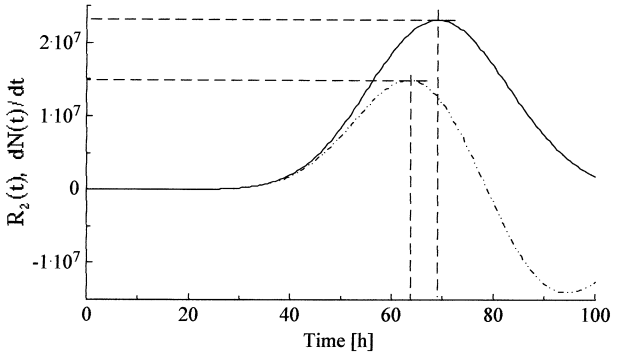

Fig. 5 $R_2(t)$ and $\frac{\mathrm{d}N(t)}{\mathrm{d}t}$ plotted as a function of time for a test conducted at 35°C. *Solid line* $R_2(t)$, *dash-dot line* $\frac{\mathrm{d}N(t)}{\mathrm{d}t}$

The data shown in Fig. 4 are in agreement with the data shown in Fig. 3: after the cells become adapted to the new environment, they start to grow and then die. Right after the lag phase, the value of $P(t)$ is slightly higher than $G(t)$ due to the conversion of non-proliferating cells into proliferating cells, while $G(t)$ is higher than $M(t)$ due to the high concentration of limiting nutrients. As time passes, the limiting nutrient concentration decreases, leading to an increase in the number of dead cells, which in turn causes the decrease in $P(t)$. As the time further increases, $G(t)$ and $M(t)$ level off to constant values, while $P(t)$ falls to zero.

Figure 5 shows $R_2(t)$ and $\frac{\mathrm{d}N(t)}{\mathrm{d}t}$ plotted as a function of time for a test conducted at 35°C. The same figure also indicates the maximum values that the above functions reach during the cell growth-cycle. The maximum of $\frac{\mathrm{d}N(t)}{\mathrm{d}t}$ can be experimentally determined and it is generally referred to as the maximum growth rate. However, as is evident from the data in Fig. 5, the maximum of $R_2(t)$, which is the "real" maximum growth rate, is higher than $\left.\frac{\mathrm{d}N(t)}{\mathrm{d}t}\right|^{\max}$, and delayed in time with respect to $\left.\frac{\mathrm{d}N(t)}{\mathrm{d}t}\right|^{\max}$. The results obtained in the specific case under investigation suggest two considerations: (1) the number of cells that die during the exponential growth phase cannot be neglected, as has been generally accepted; (2) the

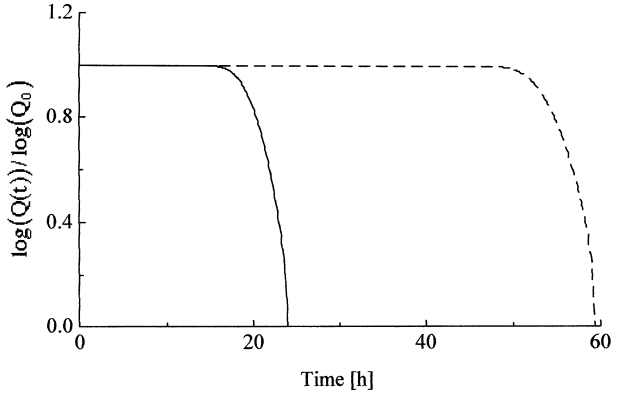

Fig. 6 $\frac{\log(Q(t))}{\log(Q_0)}$ plotted as a function of time for the two investigated temperatures. *Solid line* 35°C, *dashed line* 42°C

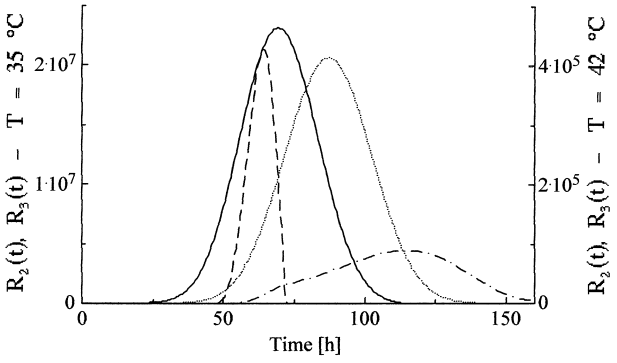

Fig. 7 $R_2(t)$ and $R_3(t)$ plotted as a function of time for the two investigated temperatures. *Solid line* $R_2(t)$ 35°C, *dashed line* $R_2(t)$ 42°C, *dotted line* $R_3(t)$ 35°C, *dash-dot line* $R_3(t)$ 42°C

value of $\left.\frac{dN(t)}{dt}\right|^{max}$ also depends on the number of cells that die during the exponential growth phase.

Figure 6 shows $\frac{\log[Q(t)]}{\log(Q_0)}$ plotted as a function of time for the two investigated temperatures. The curves obtained for the two temperatures have similar trends: microorganisms are in a non-proliferating state for a given period of time, then suddenly become adapted to the new environment. Microorganisms at 35°C adapt faster than microorganisms at 42°C to the new medium. To further highlight the difference between cell growth at 35°C and 42°C, Fig. 7 shows $R_2(t)$ and $R_3(t)$ plotted as a function of time at these two temperatures. As evident from the data shown in Fig. 7, microorganisms at 42°C have a lower growth rate than cells grown at 35°C. Moreover, $R_2(t)$ is more dependent on the limiting nutrient concentration at 42°C than at 35°C, indicating that cell growth at 42°C is much more critical than at 35°C; i.e., it can take place only in a restricted interval of limiting nutrient concentration. However, the death rate at 42°C is lower than that at 35°C, demonstrating that 42°C is a favorable temperature for the lactic acid bacteria strain tested.

Figure 8 shows $F(t)$ plotted as a function of time for the two investigated temperatures. The curves obtained for the two temperatures have similar trends. $F(t)$ remains constant for a given period of time then decreases and finally levels off to a constant value. Since the time required to adapt to the new environment at 35°C is shorter than that required at 42°C, the limiting nutrient concentration for the test conducted at 35°C starts to decrease at an earlier time compared to the test conducted at 42°C. However, the rate at which the nutrient is consumed by living cells is higher at 42°C than at 35°C, suggesting that, at the higher temperature, after adapting, the microorganisms consume the nutrient at a faster rate.

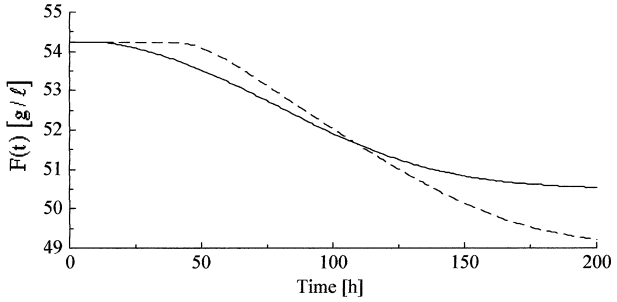

Fig. 8 $F(t)$ plotted as a function of time for the two investigated temperatures. *Solid line* 35°C; *dashed line* 42°C

Conclusions

In this paper a stochastic-deterministic model is presented to predict the growth curve of lactic acid microorganisms. To validate the model, an *L. bulgaricus* strain was tested in a medium at pH 4.6 at two different temperatures (42°C and 35°C). The curves representing the lactic acid bacteria growth curve were satisfactorily fitted by means of the proposed model. Using the values of the model's parameters obtained by fitting the data, it was possible to quantitatively predict all the phenomena involved during the cell growth cycle. In the specific case under investigation, it was evidenced that: (1) neglecting cell death during the exponential phase of growth can lead to an erroneous evaluation of the maximum growth rate; (2) the adaptation time in the conditions under investigation is shorter at 35°C than at 42°C; (3) when cells are fully adapted they consume nutrients at a faster rate.

The results obtained highlight that the mathematical complexity characterizing the proposed model is counterbalanced by the large amount of information that can be obtained.

List of symbols

$\left.\frac{dN(t)}{dt}\right\|^{max}$	the maximum of $\frac{dN(t)}{dt}$
$E\%$	the relative percent difference between experimental and predicted values
$F(t)$	the limiting nutrient concentration in the extracellular environment (expressed as g/l) at time t

F_0 the initial concentration of limiting nutrient in the extracellular environment (expressed as g/l)
$G(t)$ the concentration of generated cells (expressed as cfu ml^{-1}) at time t
k_i constant(s), to be regarded as fitting parameters
m mean value of the probability density function
$M(t)$ the concentration of death cells (expressed as cfu ml^{-1}) at time t
n_{exp} the number of experimental data points
$N(t)$ the microorganism concentration (expressed as cfu ml^{-1}) at time t
N_0 the initial concentration of microorganisms (expressed as cfu ml^{-1})
$P(t)$ the concentration of proliferating microorganisms (expressed as cfu ml^{-1}) at time t
$Q(t)$ the concentration of non-proliferating cells (expressed as cfu ml^{-1}) at time t
Q_0 initial concentration of non-proliferating cells
$R_1(t)$ probability density function of conversion of non-proliferating cells to proliferating cells (expressed as cfu ml^{-1} h^{-1})
$R_2(t)$ the proliferation rate (expressed as cfu ml^{-1} h^{-1}) at time t
R_2^{max} the maximum of $R_2(t)$ (expressed as cfu ml^{-1} h^{-1})
$R_3(t)$ the death rate (expressed as cfu ml^{-1} h^{-1}) at time t
Y^i_{exp} the experimental value
Y^i_{pred} the predicted value
σ standard deviation of the probability density function

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