Bacteriocin production and pH gradient. Some mathematical models and their problems.

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ABSTRACT

The response to stepped pH profiles obtained by successive alkalisation was examined in cultures of seven species of lactic acid bacteria. For at least four species, these conditions, combined with glucose fed-batch, promoted greater efficiency of nutrient consumption, stimulated biomass and bacteriocin production, and prolonged the productive period of the culture. In addition, they determined the appearance of heterofermentative phases absent in conventional culture, with pH either in spontaneous evolution or controlled to a stable level. The advantages and difficulties of various approaches here developed for the mathematical modeling of this system are discussed in useful terms to incorporate with control purposes to the software of a biorreactor, and the implication of the pH gradient is suggested in results from other authors, relative to the metabolic typology and production conditions of bacteriocins.

Keywords: Bacteriocins; production; pH gradient; mathematical model.

INTRODUCTION

The effect of pH on bacteriocin production is well-known, although the mechanisms involved remain controversial. It is generally accepted, sometimes implicitly, that production is inhibited by the accumulation of lactic acid. Even before bacteriocins were identified, this hypothesis was proposed in the first works on the antibiotic effects of lactic bacteria [1, 2]. The theme resurfaced after bacteriocins were described, with Hirsch [3] the most frequent reference for many subsequent workers.

Hirsch sustains that although both lactic acid and bacteriocins can inhibit the producing microorganisms, the concentrations necessary for a detectable effect are far higher than values usually present in cultures. For bacteriocins, the required concentration is 10-fold greater. For lactic acid the effects are ambiguous if added at expected culture levels, assuming stoichiometric conversion from the initial glucose and a homofermentative regime. Accordingly, with potential lactic acid levels of 4.5%, 1% totally inhibits growth if added at the start of the culture, but 10% is insufficient (produces delay, but not inhibition) if added after 6 hours incubation. Hirsch proposed, therefore, that lactic acid toxicity is negligible and that the pH should be maintained at ~6.0 for maximum nisin production. Without substantial modification, this has since been the conventional method [4-7, among others]. Accordingly, it is accepted that maximum bacteriocin production requires addition of alkali (at concentrations specific for the species considered) to compensate for the tendency of the culture towards acidification.

Nevertheless, previous studies in our laboratory with *Lactococcus lactis* sp. *lactis* [8] led to different conclusion. Essentially, the only way of interpreting unambiguously the results of cultures in buffered media at different pH was by assuming that the rate of nisin production was proportional to the pH gradient and not the initial pH. This hypothesis was confirmed for cultures subjected to successive alkalisation, a procedure that notably increased the production rate, the efficiency of nutrient consumption and the productive period.

In view of the fact that this assumption has not been addressed in the literature relating to bacteriocin production, it is not possible to ascertain the general extent of this response to pH gradient among lactic acid bacteria. Although some results (*e.g.* [9] with *Pediococcus acidilactici*, or [10] with *Lactobacillus casei*) could be interpreted accordingly, the supporting data are insufficient. The present work examines this behaviour in seven species of lactic acid bacteria, and discusses some problems posed by the mathematical modeling of cultures with stepwise-pH profiles obtained by means of repeated alkalisation.

MATERIALS AND METHODS

Microorganisms and culture media

The species used are shown in Table 1. Stock cultures were stored at -50°C in powdered skimmed milk suspension with 25% glycerol [11]. Microorganisms were grown in MRS

medium at 30°C with orbital shaking at 200 rpm. Inocula (1% v/v) consisted of cellular suspensions from 12-24 hour-aged cultures on the same medium and conditions, adjusted to an OD (700 nm) of 0.900.

100 mL of medium (160 mL for Pc 1.02) were placed in 250 mL Erlenmeyer flasks for preliminary and routine assays. The different flask loads used were the most appropriate to the assayed species, in accordance with previous works [8, 12] in which we study, using erlenmeyer flasks under orbital agitation, the relationships between flask load, oxygen saturation percentage and production of biomass and bacteriocins by several species of lactic acid bacteria. Re-alkalised cultures were performed in a 2L-bioreactor at 30°C and agitation of 200 rpm, with an aeration rate of 1 vol.vol⁻¹.min⁻¹ and with continuous pH recording. Stepwise pH profiles were obtained by repeated alkalisation of cultures up to a predetermined pH with 2N NaOH. Equal volumes of sterile distilled water were added to controls.

Analytical methods

At pre-established times, each culture was divided into two aliquots. The first was centrifuged at 5,000 rpm for 15 min, and the sediment washed twice and resuspended in distilled water to an appropriate dilution for OD measurement at 700 nm. The dry weight was then estimated from a previous calibration curve. The corresponding supernatant was used for the determination of reducing sugars [13], proteins [14], total nitrogen [15] and total phosphorus [16, 17]. Organic acids and ethanol were determined by HPLC analysis (refractive-index detector) using an ION-300 (Teknokroma) column with 6 mM sulphuric acid as a mobile phase (flow=0.4 mL/min) at 65°C. The second aliquot was used for the extraction and quantification of bacteriocin, using *Carnobacterium piscicola* CECT 4020 (Spanish Type Culture Collection) as an indicator, according to the method described in [11], and applying the equations proposed in [18]. All assays were carried out in duplicate.

Numerical methods

Fitting procedures and parametric estimations from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel 97 spreadsheet.

RESULTS AND DISCUSSION

Response to pH gradient

In view of the potential interest of the pH response in bacteriocin production, we decided to screen seven species of lactic acid bacteria, comparing the two following approaches:

- 1: As a control, a culture was performed in MRS medium at an initial pH of 6.0 and allowing the spontaneous pH evolution of the system.

2: Using the same medium and initial conditions as above, but with two re-alkalisations to pH=7.0 between 10 and 35 hours, depending on the species.

Firstly, the results (Table 2; figures 1 to 3 show three representative cases) established that the favourable effect of re-alkalisation on bacteriocin production was not atypical among the lactic acid bacteria, with four, possibly five, of the seven species tested showing increased production. Secondly, re-alkalisation increased efficiency (substrate consumed / initial substrate) for glucose and proteins. However, although the values are near to 90% in the case of the glucose, the level of proteins in the medium appears as very excedentary even in these conditions. Furthermore, for all species tested the efficiency increase was generally associated with bacteriocin production, but an acceptable linear correlation between these variables was lacking. This suggests that either the response mechanism to the pH gradient was non-linear, or that there was a species-specific response, or possibly a combination of both factors.

Finally, it was clear that despite the efficiency improvement induced by alkalisation, the carbon/nitrogen relationship of the medium was not in equilibrium. The apparent excess of proteins would thus be undesirable because it makes more expensive the process and complicates the eventual purification of the product. Now then, if the proteins were to represent a source of nitrogen only, their initial level could be simply reduced, or their consumption promoted by increasing the number of alkalisations and using glucose fed-batch. However, several authors [19, 20] suggested that an essential role of the proteins is the supply of amino acids or peptides with inducer effect on bacteriocin biosynthesis, in which case both treatments would be unsuitable. Similar appearances of heterofermentative metabolism were found by Guerra et al. [21] with *Pediococcus acidilactici*, when changing batch for fed-batch culture, as well as by Thomas [22] with *Streptococcus lactis* in anaerobiosis at low glucose levels.

Stepwise-pH profile combined with glucose fed-batch

To obtain a more detailed description of the response to pH, three additional cultures were carried out with *P. acidilactici*, *L. lactis* and *L. casei*. In each case the initial pH was 6.0, with alkalisation to pH=7.0 every 8 hours (figure 4-up). Furthermore, glucose fed-batch every 16 hours was carried out to maintain a concentration of ~15 g/L, value to which decreases the glucose level from the initial 20 g/L during the first 12 hours of incubation. For constant culture volume, glucose was added as a sterile water solution of the same volume as the extracted sample at each time interval. Figure 4-down shows the cumulative sum (extensive values) of added and consumed glucose, and figure 5 displays glucose concentrations in the medium, which were maintained between 8 and 15 g/L.

Figure 5 also shows that alkalisation associated with glucose fed-batch increased the efficiency of nutrient consumption and notably prolonged the productive period. The control cultures reached the stationary phase at ~30 hours, whereas the re-alkalised ones maintained an essentially linear increasing tendency up to ~100 hours (averaging the succession of the approximately logistical waves that show the corresponding time-courses). Analogous considerations permit comparisons between the production of lactic acid and the consumption of nitrogen (proteins) and phosphorus.

Finally, it should be noted that lactic acid production in the controls stopped (according to its nature of primary metabolite) concurrent with the biomass, whereas in the alkalisated cultures the slope changed sharply before the end of growth (~75 hours), beginning at the same time an appreciable production of ethanol, not detected in the previous period or in the controls. This shows that the homo- or heterofermentative metabolism, at times considered defining characteristics of different lactic acid bacteria, does not depend only on the species, but it is the result of enzymatic capacities modified or, maybe, induced by environmental factors.

Modeling the effects of pH

As expected, the results described until now confirmed the advantages of stepped pH profiles. Apart from this, the fact that one of the variables affected is the biomass, makes reasonable to demand that a model of this process is able to generate an acceptable description of this variable. In this respect, the main magnitudes involved in a stepped pH profile (pH jumps up to an upper limit at equal time intervals) are those shown graphically in figure 6. Besides an upper limit, pH_s , a pH step value, ΔpH_i , can be considered (difference between the upper limit and the variable minimum, pH_i , in the time interval), as well as a possible lower limit, pH_0 , representing the value below which growth or production of bacteriocin is inhibited. The results also show that the culture progressively loses recovery capacity of the minimum, pH_i .

Under the above conditions, and independent of the form adopted by the succession of the minimum pH values (pH_i , assumed linear in figure 6, although in reality non-linear: figure 4), the factor that most simply summarizes the situation is the absolute value of the pH increase, ΔpH_i . This variable includes the active pH decrease due to microbial growth and, implicitly, the maximum level, pH_s , and the decrease of the recuperation capacity of the minimum. Although it could be considered the usefulness of pH_0 , whichever term that includes it would be a function of $(pH_i - pH_0)$, according to figure 6. In this way, besides the fact that pH_0 does not contribute any useful significance other than $pH_s - pH_i$ (i.e. ΔpH_i), the model would contain a parameter which, given the kinetics of the culture, would be translated to an experimentally non-verifiable value.

Some of our previous and more limited results have been satisfactorily discussed [8] with a model worth summarizing here, since we will discuss below some of the associated problems and alternatives. This model described (see table of symbolic notations):

1: Biomass with the logistic equation:

$$X = \frac{K}{1 + \exp(c - \mu_m t)} \quad ; \quad \text{where: } c = \ln \left(\frac{K}{X_0} - 1 \right) \quad (1)$$

2: Rate of bacteriocin production with the model of Luedeking and Piret [23]:

$$r_p = \alpha r_x + \beta X \quad (2)$$

or, dividing by the biomass (where μ is the specific growth rate, or biomass formed per unit biomass present per unit time):

$$\frac{r_p}{X} = \alpha \frac{r_x}{X} + \beta ; \text{ or : } \frac{r_p}{X} = \alpha \mu + \beta \quad (3)$$

an expression that permits the metabolites to be classified as primary (formation rate depends only on the growth rate: $\beta=0$), secondary (formation rate depends only on the biomass present: $\alpha=0$) and mixed (formation rate depends simultaneously on the growth rate and present biomass: $\alpha \neq 0$ and $\beta \neq 0$).

3: Growth rate R_X as a function of an intrinsic growth rate r_X and a variable δpH :

$$R_X = r_X (1 + b \delta pH) ; \text{ where } r_X = \frac{dX}{dt} \text{ and } \delta pH = \frac{\Delta pH}{\Delta t} \quad (4)$$

Under these conditions, the logistic equation, which, in differential terms is:

$$\text{rate} = \frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) ; \text{ would adopt the form:} \quad (5)$$

$$\text{rate} = \frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) (1 + b \delta pH) \quad (6)$$

which only where μ_m and δpH are constant can an integral expression of the following type be obtained:

$$X = \frac{K}{1 + \exp[c - \mu_m (1 + b \delta pH)t]} \quad (7)$$

However, since the model contains a μ_m variable (a function of δpH , itself a function of t), the resulting dynamic situation would only be solvable numerically. The solution is obtained by applying equations (1), (2) and (4) to the description of the values of biomass and bacteriocin, and numerically integrating the corresponding rate expressions, producing:

$$X_R = \sum_{t=0}^{t=t} R_X = \sum_{t=0}^{t=t} r_X (1 + b \delta pH) \quad (8)$$

$$BT = \sum_{t=0}^{t=t} \alpha R_X + \beta X_R \quad (9)$$

These equations permit the coefficients to be derived by non-linear least squares.

In the present case, when sampling and feeding effects have been corrected, the model satisfactorily described (figure 7 and Table 3) the production of biomass and bacteriocin

(introducing a factor $(1-k_dBT_{t-1})$ in (9), which represents the spontaneous deactivation of the bacteriocin, assumed proportional to the quantity in the immediately preceding time).

To derive the solution, the biomass was firstly fit to equation (8), in such a way that the δpH effect simultaneously explained the control and alkalised cultures, *i.e.*, the coefficient b being the same in both cases. Thereafter, fitting the bacteriocin to equation (9) and using the obtained value of b , the following alternatives were tested:

- 1: All coefficients (α , β and k_d) have the same values in both types of culture.
- 2: β and k_d were allowed to vary between culture types.

In the first case the fit was poor, and improved by maintaining constant only the value of α . The estimation of the Luedeking and Piret parameters, α and β , were undoubtedly very similar (Table 3). However, whereas the best fit in the control was obtained with zero β (bacteriocin as a primary metabolite), the same was not true for the realkalised cultures. Although the value of β is of little importance, the result is suggestive of a small component of secondary metabolism. This agrees with the tendency toward metabolic secundarization noted in [8] for cultures with stepped pH profile, and which was further accentuated by increasing the upper limit of the pH step. As observed in figure 8 and Table 3, the poorer fit corresponded to bacteriocin production in the alkalized cultures. This could be simply attributed to the superior complexity of the process, which –as it was said– begins at ~ 75 hours an ethanol production phase which is absent in cultures with spontaneous pH evolution.

Simulation of the effects of pH

Simulating this observed behaviour would be useful not only to evaluate the practicality of the model, but also for deriving modifications proposed below. The simulation can be easily carried out with the following steps:

- 1: Generation of logistical growth (X) with an arbitrary group of parameters.
- 2: Numerical derivation of the growth rates at constant pH (r_X).
- 3: Acquirement of the corresponding rates with variable pH (R_X) using expression (4).
- 4: Numerical integration of R_X to obtain the biomass at stepped pH (X_R).

A series of values producing a stepped pH profile, such as that imposed in the tests described, is necessary to use (4). Although these values can be established by diverse arbitrary procedures, the results of those tests suggest an asymptotic decrease from the upper pH limit. Accordingly, an adequate approach for simulating the profile consists of a von Bertalanffy equation:

$$pH = pH_f + a \exp(-ct) \quad ; \quad \text{where: } a = (pH_0 - pH_f) \quad (10)$$

When the model represented by equations (8) and (9) is applied to a pH profile described by (10), the final biomass profile (figure 8) shows a series of progressively smooth waves, corresponding to each pH cycle and exactly of the type observed in the experimental kinetics.

Preliminary discussion of the results

An undesirable consequence of the proposed model is the lack of physical (biological) significance of the parameters, since implications of the equations (4) to (7) convert them to apparent parameters. Although we will discuss this problem in more detail below, it does not affect the phenomenological evaluation of the results. In this way, the described response again confirms and generalizes the previous conclusions of Cabo et al. [8], demonstrating that:

- 1: The culture at stepped pH obtained by successive re-alkalisation notably increases the biomass and bacteriocin production rate, the extension of the productive period and the efficiency of nutrient consumption. These effects, detected here in 4 of the 7 species tested, could constitute a fairly general response of the lactic acid bacteria.
- 2: The effects become more pronounced if the alkalisation is combined with the addition of glucose. This further raises the efficiency of nutrient consumption and induces heterofermentative metabolism in species which behave as homofermentative in control cultures with spontaneous pH evolution.
- 3: Bacteriocin production can be adequately described by means of conventional kinetic models, with the additional hypothesis that the pH gradient affects the growth rate in agreement with equation (4).
- 4: In cultures with spontaneous pH evolution, bacteriocin production follows primary metabolite kinetics in the sense of Luedeking and Piret. However, alkalisation appears to induce a small component of secondary metabolism.

These results suggest the possibility of combining and reinterpreting the conclusions of those workers who recommend constant pH, either in buffered medium or controlled by alkali addition, as well as some discrepancies about the metabolic type of the bacteriocins. In addition, both aspects appear to be interrelated. Summarising our previous discussion [8], we now point out that:

- 1: Many authors sustain that a stable pH increases bacteriocin production. However, usual buffers contain appreciable levels of nutrients such as citrate or phosphate. It is thus difficult to argue that the (relative) pH stability is the only factor contributing to the effects detected, which are frequently far lower than those described here. In addition, if pH is stabilised by alkali addition, stepped profiles are often obtained involuntarily and imprecisely, owing to the inertia of the control procedures. Finally, the relationships between bacteriocin production and pH evolution obtained here allow a satisfactory description by means of equations (8) and (9), and are incompatibles with interpretations that exclude the variable δpH .

The relevance of δpH even arises in studies focussed on other factors. Accordingly, Vignolo et al. [10] found that NaNO_2 inhibited lactocin production by *L. casei*. However, their data (represented in figure 9) indicate that an increase in nitrite determined, besides a decrease in the production of lactocin, an increase in the final culture pH (a decrease in the pH gradient). Notwithstanding the effect of nitrite, it could be concluded that δpH is a directly relevant factor.

2: With regard to bacteriocins, cases of primary and secondary metabolites have been described, sometimes for the same species and peptide. Even considering the influence of the species, the secondary character is often correctly deduced from cases in which the peptide production continues through the stationary phase of the culture. This has been associated [e.g. 9] with a decrease of the final pH, that activates enzymes involved in post-translational changes of the molecule. Without discussing this mechanism, it should be noted that these authors worked with cultures with the same initial pH, and different final pH values obtained by alkali addition. Secondary character was appreciable only in the cultures with the lowest final pH. In this way, the effect again appears compatible with the tendency toward secundarization associated with the effect of our δpH variable.

Production of lactic acid and ethanol. Induction of a heterofermentative phase

As stated previously, aside from an increase in the production of lactic acid, the stepped pH profile triggered a sharp change in process slope, before the end of growth and coincident with an appreciable ethanol production, which was absent in the precedent period and in the control cultures. It is clear, therefore, that the concepts of homo- and heterofermentative metabolism does not define different types of lactic bacteria, but rather different metabolic modalities, that can be given for the same species and medium under different culture conditions.

Although the causes for this phenomenon are unclear, it can be modelled assuming that as for other primary –e.g. citric acid: [24]– and secondary –e.g. gibberelic acid: [25]– metabolites, biosynthesis of ethanol requires the consumption of a nutrient, L , other than the carbon source, up to a limiting concentration. Accordingly, a term $0 \leq Q \leq 1$, which is zero for the initial value of L , can be introduced in the Luedeking and Piret equation for a product P :

$$r_P = (\alpha r_X + \beta X) Q ; \text{ where: } Q_t = 1 - \frac{L_t}{L_0} \quad (11)$$

being accepted that the decrease of L over the time interval Δt is proportional to the biomass produced in Δt . For simplification, we employ a unitary initial level of L and a ‘dimensionless biomass’, by dividing any biomass value by the final biomass, K :

$$L_t = L_{(t-\Delta t)} - \frac{X_t - X_{(t-\Delta t)}}{K} \quad (12)$$

Although not essential, it is more realistic to accept likewise that the biomass formation rate is also affected by the decrease in L . The Monod equation is the most appropriate expression in this respect:

$$\mu = \mu_m \frac{L}{k_L + L} ; \text{ or, making: } \frac{L}{k_L + L} = M ; \mu = \mu_m M \quad (13)$$

In this way, biomass, strictly logistical when $k_L = 0$ and approximately logistical when $L \gg k_L$, transforms to:

$$X_t = \frac{K}{1 + \exp(c - \mu_m M_t t)} \quad (14)$$

An analogous expression can be applied where the limiting level of L is a non-initiating, but interrupting, factor.

Accordingly, it could be inferred that the transition between homo- and heterofermentative phases depends on the level of a initiating or interrupting nutrient (or product) L . However, modeling of ethanol production with equations (11) to (14) did not lead to satisfactory fits for the most plausible hypotheses (excess of lactic acid or deficit of proteins), a negative result that should be underlined. On the other hand, lactic acid production (figure 10) follows the model of a primary metabolite ($\alpha=8.16$) in the control culture but not with stepped pH. From a metabolic standpoint, in this last case the product of the process could be logically considered as a linear combination of variables $La\&Et$:

$$La\&Et = \text{lactic acid} + \text{ethanol} \frac{MM \text{ lactic acid}}{MM \text{ ethanol}}$$

With this transformation (where MM are the molecular masses), the joint production over the period was satisfactorily fit to the model of Luedeking and Piret, and characterised as a primary metabolite with a small secondary component ($\alpha=12.99$; $\beta=0.04$).

It is well-known that the total linking between growth and lactic acid production ($\beta=0$) in the presence of an excess of nitrogen becomes partial ($\beta\neq0$) in nitrogen-limited cultures. This way, the secondary component observed in the glucose fed-batch cultures could be due to the establishment of an imbalance between carbon and ‘useful’ nitrogen, even in the presence of enough total nitrogen.

Problems due to the pH gradient in the kinetic models

As indicated in section 5, the model describing biomass and bacteriocin production had as unfortunate consequence the lack of biological meaning of their parameters. Specifically, when the variable δpH , that varies over the time, was introduced in the differential logistic equation, the resulting expression:

$$\frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) (1 + b \delta pH)$$

prevented the use for the calculation of its (inexistent) integral correlate. The values of K and m produced by the numerical solution do not represent the maximum biomass and specific growth rate, or the constants α and β of Luedeking and Piret, but only K the apparent correlates of these parameters (for which another notation would even be convenient, such as K' , m' , α' , β' , which we omitted earlier to postpone this discussion).

This inconvenience is serious because it prevents to control the fitting by means of restrictions over the values of the parameters between limits derived from the logic of the (biological) system under study. Given that the lack of linearity of the (mathematical) system implies a minimal quadratic function with various possible local minima, in reality there is a lack of physical feasibility criteria for choosing between approximately equivalent fits. Perhaps the best option is based on the linearity of the correlation between experimental values and model predictions and the randomness of the deviations.

In fact, some initial values drive to excellent fits between observations and predictions, but gave very high values of the b parameter, with which δpH appeared sufficient to explain the system. The treatment of equations (8) and (9) in the described sequence reduced, but not eliminated, the problem. It is thus difficult to avoid the conclusion that the proposed model, even based on structural arguments, is only quasi-empirical, suitable for interpolations, kinetic predictions and control routines, but lacking in precise biological meaning.

A possible solution: description of pH as a function of time

Without disregarding the basic model assumptions, the biological meaning may be improved by employing a formulation of the pH as a function of the time, which reasonably describes the true pH variation and can be introduced in a useful (integrable) way into the logistic equation. In this respect, it is interesting to consider an asymptotic expression of the type (10), as that applied to the simulation of pH profiles. Using the same notation as (10), a function $pH(t)$ can be written:

$$pH = pH_f + (pH_0 - pH_f)\exp(-ct) \quad (15)$$

and substituting δpH in (6) with pH (15):

$$\frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) \{ 1 + b_1 [pH_f + (pH_0 - pH_f)\exp(-ct)] \} \quad (16)$$

and integrating:

$$X = \frac{K}{1 + d \exp \left\{ - \left[\mu_m t + \mu_m b_1 pH_f t + \frac{\mu_m b_1 (pH_0 - pH_f)}{c} (1 - \exp(-ct)) \right] \right\}} \quad (17)$$

Between the limits of t , (17) takes the values:

$$\lim_{t \rightarrow 0} X = X_0 = \frac{K}{1 + d} ; \quad \lim_{t \rightarrow \infty} X = K$$

In equation (17), X_0 and K conserve the physical meaning of initial and maximum biomass (intercept and asymptotic value, respectively). Accordingly, despite that the same does not occur

with μ_m , K now constitutes an adequate physical feasibility criterion for fitting the data (equally X_0 , although is of less practical interest).

Application to the case of L. casei and alternative formulations of the pH(t) function

The fits obtained using expressions (15) and (17) for pH and biomass, respectively, are shown in figure 11-up. The difference in fit obtained when using δpH (see figure 7) is of little relevance, although this approach is preferable as it provides a true, empirically verifiable estimation of K (Table 4), useful for drawing comparisons.

Figure 11 shows that the pH model presents a definite lack of fit at low incubation times due to the “shoulder” of the real values that equation (15) cannot generate. Given the low values of biomass and bacteriocin in the initial period, the influence of this lack of fit on the production estimation is negligible. However, the “shoulder” is a relatively generalised form in this type of cultures, and (16) may be substituted by a function with a more adequate profile. The Gompertz equation:

$$Y = a \exp(-b \exp(cX))$$

modified by forcing the existence of an inferior asymptote a' :

$$Y = a' + a \exp(-b \exp(cX)) \quad (18)$$

has the required profile (figure 11, center). However, when (18) is introduced in the differential form of the logistic equation, again leads to an expression which cannot be integrated.

Another alternative is a decreasing logistic profile, formulated by subtracting to a parameter P' a conventional logistic function, whose maximum value (P) thus represents the inferior asymptote:

$$pH = P' - \frac{P}{1 + c \exp(-\gamma t)} \quad (19)$$

On substituting pH of (19) for δpH in (6) one obtains:

$$\frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) \left[1 + b_1 \left[P' - \frac{P}{1 + c \exp(-\gamma t)} \right] \right] \quad (20)$$

whose integration produces:

$$X = \frac{K}{1 + d \exp \left[-\mu_m t + \mu_m b_1 (P - P') t + \frac{\mu_m b_1 P}{\gamma} \ln \left(\frac{1 + c \exp(-\gamma t)}{1 + c} \right) \right]} \quad (21)$$

and, since: $\lim_{t \rightarrow 0} X = X_0 = \frac{K}{1 + d}$; $\lim_{t \rightarrow \infty} X = K$

again conserves the logistical significance of the parameters X_0 and K (although not that of μ_m).

The application of (19) and (21) to the profiles of pH and biomass in *L. casei* notably improves the fits with equations (15) and (17), especially in the case of pH (figure 11-down). As this approached has the additional advantage of generality, it can in principle be accepted as an adequate model for describing the cultures studied here. Table 4 summarizes the parametric values and the correlation coefficients between estimated and experimental values of the models discussed.

Other possible implications of the variable δpH

In a previous work, Cabo et al. [8] suggested the possibility that the pH gradient may specifically affect bacteriocin production without affecting growth. It was thus suggested retain the classical logistical model (5) for growth and include a new term of the type $(1+g\delta pH)$ in the Luedeking and Piret equation, where the coefficient g represents the effect of δpH on bacteriocin production:

$$r_p = (\alpha r_x + \beta X)(1 + g\delta pH) \quad (22)$$

However, the results obtained here in the basic cultures confirm that the potential effect of δpH on the rate of bacteriocin production would be in addition, and not alternative, to the effect on growth. On the other hand, even the interpretation of equation (22) is problematic. If g is positive, a specific stimulant effect on bacteriocin production could be assumed. But there are also cases in which g takes negative values. This would mean that δpH stimulates and depresses biomass and bacteriocin production, respectively, a conclusion that seems difficult to accept. In short, given the strength of terms of the type $(1+b_i X_i)$ where X_i is a variable associated with the evolution of the culture, probably the best option is to disregard additional assumptions of the stimulation of growth rate, unless there is direct experimental evidence to the contrary.

Applicability of the kinetic model to other species of lactic acid bacteria

As established (section 1) in the schematic approaches with two alkalisations, the effect of δpH on growth and bacteriocin production seems to be relatively general behaviour in lactic acid bacteria. This makes it reasonable to assume that the models discussed for *L. casei* also describe cultures of other species subjected to pH steps. The hypothesis was confirmed by the results obtained with *L. lactis* and *P. acidilactici* (figures 12 to 15), which indicate some additional peculiarities.

L. lactis responds with a late heterofermentative phase, absent in cultures with spontaneous pH profile, and characterized, as in *L. casei*, by a substantial production of ethanol after ~50 hours. In homofermentative regime the production of lactic acid obeys the model of a primary metabolite; in heterofermentative regime the equation of Luedeking and Piret produces deficient fits (figure 12) when applied separately to lactic acid and ethanol, and also for the linear combination *La&Et*, which was adequate for *L. casei*. In connection with the production of

biomass and nisin, the results (figures 12 and 13) demonstrate that the pH profile obtained by means of repeated re-alkalisation multiplies by factors of 10 and 6, respectively, the values corresponding to control cultures, with an pronounced descent of the bacteriocin levels after ~50 hours of incubation.

P. acidilactici behaves heterofermentatively with any type of pH profile. The production of the heterometabolite (in this case acetic acid) increased much less than that of lactic acid when passed from the spontaneous to forced pH profile. In both situations, the equation of Luedeking and Piret produces a satisfactory fit to the primary model when the combination of variables *La&Aa* is used, defined in analogous terms to *La&Et*. In this case (figures 14 and 15) the repeated re-alkalisation elevates more moderately the productions of biomass and bacteriocin (the factors are 2.4 and 1.4 for biomass and pediocin, respectively), without appreciable descents at long incubation times.

APPENDIX

If pH in equation (15):

$$pH = pH_f + (pH_0 - pH_f)\exp(-ct) \quad (A.1)$$

substitutes δpH in equation (6), one obtains:

$$\frac{dX}{dt} = \mu_m X \left(\frac{K - X}{K} \right) \{1 + b_1 [pH_f + (pH_0 - pH_f)\exp(-ct)]\} \quad (A.2)$$

Separating variables and integrating:

$$K \int_{x_0}^x \frac{dX}{X(K - X)} = \mu_m \int_0^t \{1 + b_1 [pH_f + (pH_0 - pH_f)\exp(-ct)]\} dt \quad (A.3)$$

The integral of the first member (I_I) can be resolved by means of separation:

$$\frac{1}{X(K - X)} = \frac{A}{X} + \frac{B}{K - X}; \text{ leading to } \begin{cases} B - A = 0 \\ AK = 1 \end{cases} \text{ and: } A = B = \frac{1}{K}$$

$$\text{Accordingly: } I_I = K \int_{x_0}^x \left(\frac{dX}{KX} + \frac{dX}{K(K - X)} \right) = \left[\ln X \right]_{x_0}^x - \left[\ln(K - X) \right]_{x_0}^x = \ln \left[\frac{X(K - X_0)}{X_0(K - X)} \right]$$

$$\text{When } \ln \left[\frac{X(K - X_0)}{X_0(K - X)} \right] = z, \text{ then } \frac{X(K - X_0)}{X_0(K - X)} = \exp(z)$$

allowing X to be cleared. Rearranging and setting $\frac{K}{X_0} - 1 = d$, one arrives at:

$$X = \frac{K}{1 + d \exp(-z)}$$

For the integral of the second component of (A.3):

$$\begin{aligned} I_2 = z &= \int_0^t \mu_m \left\{ 1 + b_1 [pH_f + (pH_0 - pH_f) \exp(-ct)] \right\} dt = \\ &= \left[\mu_m t + \mu_m b_1 pH_f t - \frac{\mu_m b_1 (pH_0 - pH_f)}{c} \exp(-ct) \right]_0^t = \\ &= \mu_m t + \mu_m b_1 pH_f t + \frac{\mu_m b_1 (pH_0 - pH_f)}{c} (1 - \exp(-ct)) \end{aligned}$$

The definitive integral expression of X is:

$$X = \frac{K}{1 + d \exp \left\{ - \left[\mu_m t + \mu_m b_1 pH_f t + \frac{\mu_m b_1 (pH_0 - pH_f)}{c} (1 - \exp(-ct)) \right] \right\}} \quad (\text{A.4})$$

Symbolic notations used

EQUATION	PARAMETER
Logistic (1)	X : Biomass (X_0 : initial biomass). Dimensions $g.l^l$.
	K : Maximum biomass. Dimensions $g.l^l$.
	μ_m : Maximum specific growth rate. Dimensions h^{-l} .
Luedeking and Piret (2)	r_P : Rate of product formation. Dimensions $BU.ml^{-l}.h^{-l}$.
	r_X : Growth rate. Dimensions $g.l^l.h^{-l}$.
	α : Luedeking and Piret parameter (to be experimentally determined). Dimensions $BU.10^{-3}.g^{-l}$.
	β : Luedeking and Piret parameter (to be experimentally determined). Dimensions $BU.10^{-3}.g^{-l}.h^{-l}$.
Effect of pH (4)	r_X : Growth rate at constant pH. Dimensions $g.l^l.h^{-l}$.
	R_X : Growth rate induced by pH variation. Dimensions $g.l^l.h^{-l}$.
	b : Parameter to be empirically determined. Dimensions h .
	δpH : Decrease in pH per unit time. Dimensions h^{-l} .
Von Bertalanffy (10)	pH_0 : Initial pH or upper level of the pH range. Dimensionless.
	pH_f : Final pH or lower (asymptotic) level of the pH range. Dimensionless.
	c : Rate of pH variation (can vary in each pH cycle). Dimensions h^{-l} .
	t : Time (zeroed at the beginning of each pH cycle). Dimensions h .
Logistic pH (19)	P' : Parameter to be empirically determined. Dimensionless.
	P : Parameter to be empirically determined. Dimensionless.
	c : Parameter to be empirically determined. Dimensionless.
	γ : Rate of pH variation (can vary in each pH cycle). Dimensions h^{-l} .
	t : Time (zeroed at the beginning of each pH cycle). Dimensions h .

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TABLES

TABLE 1: Species used

Species	Origin	Abbreviated key
<i>Lactobacillus brevis</i>	CECT 216	Lb 2.01
<i>Lactobacillus casei ssp. casei</i>	CECT 4040	Lb 3.03
<i>Lactobacillus casei ssp. casei</i>	CECT 4043	Lb 3.04
<i>Lactobacillus plantarum</i>	CECT 221	Lb 8.02
<i>Lactococcus lactis ssp. lactis</i>	CECT 539	Lc 1.04
<i>Leuconostoc mesenteroides ssp. dextranicum</i>	CECT 912	Ln 3.02
<i>Pediococcus acidilactici</i>	NRRL B-5627	Pc 1.02
<i>Carnobacterium piscicola</i>	CECT 4020	Cb 1.01

CECT: Colección Española de Cultivos Tipo.
 NRRL: Northern Regional Research Laboratory (Peoria, Illinois, USA).
 Abbreviated key: notation used in this paper

TABLE 2: Main data from seven species of lactic acid bacteria in cultures with uncontrolled pH (–) and stepped pH profile obtained by means of two re-alkalisations to pH=7.0 between 10 and 35 hours (+). Σ pH: sum of all (negative) pH increments; B: biomass; Gc: glucose consumption; Prc: protein consumption; BT: bacteriocin production; Ef: efficiency (consumed substrate/initial substrate). B, Gc and Prc in g/L; BT in bacteriocin arbitrary units (BU)/mL. All the values correspond to the point of maximum biomass.

	Lc 1.04	Lb 2.01	Lb 3.03	Pc 1.02	Lb 3.04	Ln 3.02	Lb 8.02
Σ pH–	1.32	0.72	2.01	1.43	1.69	1.21	1.98
Σ pH+	5.75	4.82	5.25	4.41	4.48	3.15	4.65
B–	0.54	0.39	1.06	1.08	2.54	4.38	1.19
B+	1.49	2.02	2.72	1.35	2.0	6.82	3.32
BT–	15.2	10.39	15.99	388.6	11.60	5.46	26.28
BT+	31.2	15.81	21.10	449.1	12.08	4.76	20.62
BT+/BT–	2.1	1.5	1.3	1.2	1.0	0.9	0.8
Gc–	6.63	1.50	10.79	7.08	18.56	13.81	9.98
Gc+	18.45	18.58	14.57	13.71	19.77	15.53	15.38
Ef G–	0.31	0.07	0.61	0.35	0.86	0.77	0.56
Ef G+	0.85	0.86	0.82	0.67	0.91	0.87	0.86
Prc–	1.47	0.75	0.63	0.38	2.10	0.06	0.28
Prc+	2.68	2.38	1.01	1.07	3.29	0.23	0.58
Ef Pr–	0.13	0.07	0.08	0.04	0.19	0.01	0.04
Ef Pr+	0.24	0.21	0.12	0.12	0.29	0.03	0.16

TABLE 3: Main parameters of the model (equations (8) and (9)) applied to the description of biomass and bacteriocin production by *Lactobacillus casei*. b : coefficient corresponding to the effect of the pH gradient; α and β : (apparent) coefficients of the Luedeking and Piret equation; k_d : coefficient corresponding to the spontaneous deactivation of the bacteriocin; r : lineal correlation coefficient between observed and expected values.

	Control (uncontrolled pH)		Stepped pH profile	
	Biomass	Bacteriocin	Biomass	Bacteriocin
b	17.045	–	17.045	–
α	–	38.285	–	38.285
β	–	0	–	0.206
k_d	–	0.017	–	7.10^{-3}
r	0.967	0.974	0.988	0.971

TABLE 4: Comparison among parametric values obtained in the description of *Lactobacillus casei* cultures (uncontrolled pH) by means of the specified models. r : lineal correlation coefficient between observed and expected values.

	($1+b \delta pH$) model: equations (8) and (9)	[$1+b_1 pH(t)$] model: equations (15) and (17)	[$1+b_1 pH(t)$] model: equations (19) and (21)
K	0.7139	1.661	1.660
X_0	0.010	0.058	0.046
μ_m	0.209	1.107	0.828
pH_0	–	6.316	–
pH_r	–	3.520	–
c	–	0.056	–
P	–	–	2.251
P'	–	–	5.976
γ	–	–	0.316
b	17.045	–	–
b_1	–	– 0.160	– 0.142
α	38.286	42.122	42.222
β	0.000	– 0.125	– 0.121
K_d	–	0.169	0.171
r_B	0.967	0.978	0.978
r_{BT}	0.974	0.964	0.961

FIGURE LEGENDS

Figure 1: Lc 1.04 cultures on MRS medium with spontaneous pH evolution (open symbols) and with stepped pH profile obtained by means of two re-alkalisations (closed symbols). B: biomass; BT: bacteriocin; RS: reducing sugars; Pr: proteins; Nt: total nitrogen.

Figure 2: Pc 1.02 cultures on MRS medium with spontaneous pH evolution (open symbols) and with two re-alkalisations (closed symbols). Keys as in figure 1.

Figure 3: Lb 3.04 cultures on MRS medium with spontaneous pH evolution (open symbols) and with two re-alkalisations (closed symbols). Keys as in figure 1.

Figure 4: Up: pH profiles of Lb 3.04 cultures with and without re-alkalisation. Down: accumulated sums of added (ΣG_{ad}) and consumed (ΣG_c) glucose.

Figure 5: Lb 3.04 cultures without (open symbols) and with (closed symbols) re-alkalisation and glucose fed-batch. B: biomass; BT: bacteriocin; La: lactic acid; Et: ethanol; RS: reducing sugars; Pr: proteins; Nt: total nitrogen; Pt: total phosphorus.

Figure 6: Main magnitudes that can be considered in a stepped pH profile.

Figure 7: Production of biomass (B) and bacteriocin (BT) in Lb 3.04 cultures without (open symbols) and with (closed symbols) repeated re-alkalisation. Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (8) and (9).

Figure 8: Simulation of the effect of pH gradient on the biomass, according to the equations (1), (4), (8) and (10). Down open symbols represent biomass at constant pH; closed symbols, biomass induced by the stepped pH profile shown up.

Figure 9: Effects of initial concentration of NaNO_2 on pH and lactocin production by *L. casei*. Data from Vignolo et al. (1995).

Figure 10: Productions of lactic acid (La: $\circ$) and ethanol (Et: $\diamond$) in control (left) and pH stepped profile cultures (right) of Lb 3.04. In this last case, the *La&Et* variable ($\bullet$, see text) is also shown. Continuous lines represent the fits of the experimental results to the Luedeking and Piret equation.

Figure 11: Treatment of the Lb 3.04 cultures with spontaneous pH evolution by means of models including a $[1+b_I pH(t)]$ term (where $pH(t)$ are the specified functions), in the description of the biomass production.

Figure 12: Lc 1.04 culture with stepped pH profile and glucose fed-batch. Keys as in figure 5.

Figure 13: Productions of biomass (B), bacteriocin (BT) and characteristic metabolites (○: lactic acid, ◇: ethanol, ●: *La&Et*), in Lc 1.04 cultures without (open symbols) and with (closed symbols) repeated re-alkalisation. Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (8) and (9).

Figure 14: Pc 1.02 culture with stepped pH profile and glucose fed-batch. Keys as in figure 5. Aa: acetic acid.

Figure 15: Productions of biomass (B), bacteriocin (BT) and characteristic metabolites (○: lactic acid, ◇: acetic acid, ●: *La&Et*), in Pc 1.02 cultures without (open symbols) and with (closed symbols) repeated re-alkalisation. Continuous lines represent the fitting functions corresponding to the experimental results (points), according to the equations (9) and (10).

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