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ORIGINAL PAPER

The Joint Effect of pH Gradient and Glucose Feeding on the Growth Kinetics of *Lactococcus lactis* CECT 539 in Glucose-Limited Fed-Batch Cultures

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Abstract

Two glucose-limited realkalized fed-batch cultures of *Lactococcus lactis* CECT 539 were carried out in a diluted whey medium (DW) using two different feeding media. The cultures were fed a mixture of a 400 g/l concentrated lactose and a concentrated mussel processing waste (CMPW, 101.72 g glucose/l) medium (fermentation I) or a CMPW medium supplemented with glucose and KH_2PO_4 up to concentrations of 400 g glucose/l and 3.21 g total phosphorus/l, respectively (fermentation II). For an accurate description and a better understanding of the kinetics of both cultures, the growth and product formation by *L. lactis* CECT 539 were both modelled, for the first time, as a function of the amounts of glucose (*G*) added and the pH gradient (*VpH*) generated in every realkalization and feeding cycle, by using an empirical polynomial model. With this modeling procedure, the kinetics of biomass, viable cell counts, nisin, lactic acid, acetic acid and butane-2,3-diol production in both cultures were successfully described (R^2 values >0.970) and interpreted for the first time. In addition, the optimum *VpH* and *G* values for each product were accurately calculated in the two realkalized fed-batch cultures. This approach appears to be useful for designing feeding strategies to enhance the productions of biomass, bacteriocin, and metabolites by the nisin-producing strain in wastes from the food industry.

Key words: fed-batch fermentation, empirical modeling, probiotic biomass, nisin, glucose-limited cultures

Introduction

In recent years, there has been an increasing interest in using probiotic cultures from lactic acid bacteria (LAB) as additives in animal feed to prevent or treat diseases (Alvarez-Olmos and Oberhelman 2001) and enhance the production results, e.g. weight gain and feed conversion efficiency (Guerra et al. 2007a).

For a successful application in animal feed, the probiotic cultures should contain a high concentration of viable cells, bacteriocins and fermentation metabolites to control the growth of pathogenic bacteria in both the animal feed and gut of the animals (Guerra et al. 2007a; Costas et al. 2018). Three alternatives for this purpose are: i) the use of cheaper fermentation and feeding media (like wastes from the food industry), ii) the selection of an appropriate strain, and iii) the design of an adequate fermentation procedure.

Whey (a waste from the cheese-making process) and mussel processing wastes (MPW) contain lactose in the relatively high concentration (~ 50 g/l in case of whey), glycogen (~ 10 g/l in case of MPW), proteins (~ 5.0 g/l in case of whey and 3.5 g/l in case of MPW), as well as micronutrients, including amino acids, vitamins and minerals (Murado et al. 1994; Costas et al. 2018). For these reasons, both substrates have been used for productions of probiotic biomass (Costas et al. 2018) and bacteriocins (Garsa et al. 2014; Costas et al. 2018) by different LAB.

The comparison of the antibacterial activity of 38 bacteriocin-producing LAB (including *Lactococcus*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc* strains)

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against a set of 19 indicator bacteria (including *Listeria*, *Enterococcus*, *Carnobacterium*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* strains) showed that the nisin produced by *L. lactis* CECT 539 was the most potent bacteriocin with the broadest antibacterial spectrum (Guerra and Pastrana 2002). In addition, previous studies showed that the strain CECT 539 has potential as a probiotic additive for animal feed (Guerra et al. 2007a), or as a food products' preservative to extend their shelf life (Guerra et al. 2005).

Considering that fed-batch fermentation allows obtaining cultures with a high concentration of viable cells (Cho et al. 2010; Costas et al. 2018), the use of this fermentation technique for production of highly concentrated probiotic cultures on whey and MPW could be very advantageous. With this approach, the high mean chemical oxygen demand of these wastes, about 70 g/l in case of whey (Slavov 2017) and 25 g/l in case of MPW (Murado et al. 1994), and consequently their contamination effects could be considerably reduced.

Previous studies showed that the growth and nisin synthesis by LAB in realkalized fed-batch cultures depend on the stepwise pH profiles generated in the cultures, due to i) the effect of pH on nutrient uptake, ii) the inhibitory effect of low pH values on biomass and product formation, and iii) the specific effect of pH on bacteriocin synthesis (Cabo et al. 2001; Costas et al. 2018). Then, the use of an adequate mathematical model describing the joint effect of the culture pH and nutrient (glucose) addition on the productions of biomass, nisin and fermentation products could provide a better understanding for controlling and optimizing the fermentation process.

However, to our knowledge, no information is available on the quantification of the joint effect of glucose addition with the feeding media and the pH gradients (*VpH*) generated in the cultures on the synthesis of biomass, nisin, lactic acid, acetic acid and butane-2,3-diol by *L. lactis* strains.

For these reasons, in the present study, a first glucose-limited realkalized fed-batch culture in DW medium was designed by using a medium prepared with mussel processing wastes (CMPW) and a 400 g/l concentrated lactose (CL) as feeding media. From the results obtained in this culture, the effects of glucose addition and *VpH* on biomass and product synthesis by *L. lactis* CECT 539 were quantified, for the first time, by using empirical modeling. To validate the effectiveness of this modeling procedure, a second glucose-limited realkalized fed-batch culture in DW medium was carried out by using the CMPW medium supplemented with glucose and KH_2PO_4 up to concentrations of 400 g glucose/l and 3.21 g total phosphorus/l as feeding medium.

Experimental

Materials and Methods

Bacterial strains and culture media. The LAB strains used in this study were selected as the best nisin producer (*Lactococcus lactis* CECT 539) and the most nisin-sensitive bacterium (*Carnobacterium piscicola* CECT 4020) in a previous study (Guerra and Pastrana 2002). Both strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Working cultures of both strains were grown on MRS as agar slants.

The culture media used in this study (Table I) were prepared with diluted whey (DW) and a concentrated mussel processing waste (CMPW) obtained from local dairy and mussel processing plants, respectively. The pretreatment of both substrates was described by Costas et al. (2018).

Inoculum preparation and fermentation conditions. A loop of cells of *L. lactis* CECT 539 from a 1-day old MRS slant was used to inoculate 10 ml of MRS broth, which was incubated at 30°C and 200 rpm for 12 h. Subsequently, 1 ml of this pre-inoculum was transferred aseptically to a 250 ml Erlenmeyer flask containing 50 ml of DW medium and incubated at 30°C/12 h (200 rpm). An aliquot of this culture (containing 7.4×10^9 CFU/ml) was used as the inoculum for the realkalized fed-batch fermentations to obtain an initial viable cell count of 1.5×10^9 CFU/ml (0.03 g of cell dry weight/ml) in the fermentation medium (Costas et al. 2018).

The two fixed-volume realkalized fed-batch fermentations (cultures I and II) were carried out in duplicate in a 6-l bench-top fermenter (New Brunswick Scientific, Edison, NJ, USA) containing 4 l of sterilized DW medium (pH 7.0), at 30°C, 200 rpm and with an aera-

Table I
Mean composition (g/l) of the substrates used as culture media.

Nutrient	DW medium	CMPW medium	CMPWGP medium
Lactose	22.62±0.05	–	–
Glucose	–	101.72±0.17	400.00±0.01
Total nitrogen	0.433±0.02	0.540±0.01	0.431±0.02
Total phosphorus	0.227±0.02	0.060±0.00	3.210±0.06
Proteins	2.07±0.01	3.47±0.03	2.75±0.02

tion level of 0.5 l/h. The first culture was fed a mixture of CMPW medium and 400 g/l concentrated lactose (CL), and the second fermentation was fed a CMPW medium supplemented with glucose and KH_2PO_4 up to concentrations of 400 g glucose/l and 3.21 g total phosphorus/l (CMPWGP medium) and sterile distilled water (if needed). In the two realkalized fed-batch cultures the feeding media were used to bring the cultures up to the initial total sugars (TS) concentration (22.62 g/l) in the fermentation medium in every realkalization and feeding cycle.

However, the CMPW medium has a mean total sugars concentration (101.72 g/l) considerably lower than that (400 g/l) of the CL substrate and a total phosphorus (TP) concentration (0.060 g/l) lower than that (0.227 g/l) of the fermentation medium (Table I). Therefore, the use of the unsupplemented CMPW medium as the unique substrate to feed the growing culture has two drawbacks. First, high volumes of the feeding substrate could be necessary to replenish the initial TS levels in the culture medium in every realkalization and feeding cycle, increasing considerably the volume of fermentation medium and provoking the dilution of the culture. Second, the use of CMPW medium jointly with the sample extraction every 12 h, could lead to the exhaustion of the phosphorus source in the fermentation medium, thus limiting the growth of *L. lactis* CECT 539 (Costas et al. 2018).

For these reasons, the CMPW medium was firstly supplemented with KH_2PO_4 up to 3.21 g TP/l to obtain the same C/TP relationship (31.7) as in the MRS medium, because this salt was found to be the best TP source for nisin synthesis (De Vuyst and Vandamme 1993). Then, the medium was supplemented with glucose up to a concentration of 400 g/l.

Samples were taken from the culture medium in the corresponding fermenter every 12 h and divided into three aliquots to measure the viable cell counts (first aliquot), the culture pH and concentrations of biomass, nutrients and fermentation products (second aliquot), and also the nisin activity (third aliquot).

Realkalization and feeding procedure. After determining the TS concentration in the samples withdrawn at every sampling time (12 h), the fermentation medium was realkalized up to the initial pH of 7.0 with a volume of 5 N NaOH (V_{NaOH}) to generate different pH gradients. Then, the volumes of feeding substrates (*VFS*) needed to bring the culture up to the initial TS concentration (22.62 g/l) in each culture were calculated by developing the corresponding mass balance equations, as indicated below.

The volume of fermentation medium (*VFM*) in the two fed-batch fermentations was kept constant ($(dVFM/dt) = 0$) by matching the volumes added to the fermenter ($VFS_{tn} + VNaOH_{tn}$) with the sampling volume (VS_{tn}):

$$VS_{tn} = VFS_{tn} + VNaOH_{tn} = (VCMPW_{tn} + VCL_{tn}) + \\ + VNaOH_{tn} \quad \text{(for fermentation I)} \qquad [1.1]$$

$$VS_{tn} = VFS_{tn} + VNaOH_{tn} = (VCMPWGPt_{n} + VdW_{tn}) + \\ + VNaOH_{tn} \quad \text{(for fermentation II)} \qquad [1.2]$$

Where $VNaOH_{tn}$, $VCMPW_{tn}$, VCL_{tn}, $VCMPWGP_{tn}$ and VdW_{tn} are respectively, the volumes (in l) of 5 N NaOH, CMPW medium, concentrated lactose (CL), CMPWGP medium and distilled water added to the fermenter at the beginning of every realkalization and feeding cycle.

From equations [1.1] and [1.2] it follows that:

$$VCMPW_{tn} = VS_{tn} - VNaOH_{tn} - VCL_{tn} \\ \text{(for fermentation I)} \qquad [2.1]$$

$$VCMPWGP_{tn} = VS_{tn} - VNaOH_{tn} - VdW_{tn} \\ \text{for fermentation II)} \qquad [2.2]$$

The reduction in the mass (in grams) of TS in the medium due to the joint effect of the extraction of samples and TS consumption by the growing strain (TS_{E+C}) was calculated by applying a mass balance equation for the total sugars in the fermenter:

$$TS_{E+C} = V \cdot [TS]_{tn} - (V - VS_{tn}) \cdot [TS]_{tn+1} \qquad [3]$$

Where $[TS]_{tn}$ and $[TS]_{tn+1}$ are the total sugars concentration (in g/l) at the beginning and at the end of every feeding cycle. The difference ($V–VS_{tn}$) represents the remaining volume (l) in the fermenter after the extraction of samples.

Therefore, the mass of TS (in grams) that must be added to the fermenter to restore the initial TS concentration in the DW medium was calculated by the following expressions:

$$VCMPW \cdot [TS_{CMPW}] + VCL \cdot [TS_{CL}] = TS_{E+C} \\ \text{(for fermentation I)} \qquad [4.1]$$

$$VCMPWGP \cdot [TS_{CMPWGP}] = TS_{E+C} \\ \text{(for fermentation II)} \qquad [4.2]$$

Where $[TS_{CMPW}]$, $[TS_{CL}]$ and $[TS_{CMPWGP}]$ are the total sugars concentration in the CMPW, CL and CMPWGP media, respectively.

Substituting Eq. [2.1] into Eq. [4.1] gives:

$$(VS_{tn} + VNaOH_{tn} - VCL_{tn}) \cdot [TS_{CMPW}] + \\ + VCL_{tn} \cdot [TS_{CL}] = TS_{E+C} \\ \text{(for fermentation I)} \qquad [5.1]$$

Thus, the V_{CL} was calculated as:

$$VCL_{tn} = \frac{(TS_{E+C} - VS_{tn} \cdot [TS_{CMPW}] + VNaOH_{tn} \cdot [TS_{CMPW}])}{([TS_{CL}] - [TS_{CMPW}])} \qquad [6.1]$$

The *VCMPW* was obtained by introducing the values of *VCL* and *VNaOH* into Eq. [2.1].

The $VCMPWGP_{tn}$ in fermentation II was obtained by rearranging Eq. [4.2]:

$$VCMPWGP_{tn} = \frac{TS_{E+C}}{[TS_{CMPWGP}]} \quad [6.2]$$

The *VdW* was obtained by introducing the values of *VCMPWGP* and *VNaOH* into Eq. [2.2].

These sampling, feeding, and realkalization procedures were repeated every 12 h in all the cultures.

Viable cell counts. The first aliquot of culture medium was used to count the total viable cells [VCC, as colony forming units (CFU)/ml] by plating serial dilutions of this aliquot (in triplicate) onto MRS agar and incubation at 30°C for 48 h (Costas et al. 2018).

Biomass, nutrients and fermentation products. Triplicate equivolume subaliquots from the second aliquot of the culture medium were centrifuged (12 000 × g for 15 min at 4°C) and after separation of the supernatants, the sedimented cells were washed with saline (0.8% (w/v) NaCl) and centrifuged (12 000 × g for 15 min at 4°C) two times. The rinsed cells were again resuspended in saline to measure the optical density at 700 nm of the three subaliquots using the sterile saline as a blank. Then, the biomass concentration (X, as g dry cell weight/l) was determined from a standard curve (Costas et al. 2018). The supernatants were used to measure the culture pH and the concentrations of nutrients (total sugars, nitrogen, phosphorus, protein, glucose and lactose) and fermentation products (lactic acid, acetic acid and butane-2,3-diol).

The concentration of total sugars was measured using the phenol/sulfuric acid method (Dubois et al. 1956) according to Strickland and Parsons (1968a), with glucose (at concentrations between 12.5–125.0 µg/ml) as standard. Total nitrogen was quantified by the micro-Kjeldahl method, replacing distillation by the spectrophotometric method of Havilah et al. (1977), with ammonium sulfate (at concentrations between 12.5–500.0 mg/l) as standard. Total phosphorus was determined by the molybdate reaction (Murphy and Riley 1962) according to Strickland and Parsons (1968b), with KH_2PO_4 (at concentrations between 0.2–2.0 mg/l) as standard. Protein was measured by the method of Lowry et al. (1951), with bovine serum albumin (at concentrations between 0.05–0.50 g/l) as standard.

Concentrations of glucose (G), lactose (L), lactic acid (LA), acetic acid (AA) and butane-2,3-diol (B) were quantified by a high-performance liquid chromatography (HPLC) system equipped with an ION-300 Organic Acids column (length 300 mm, internal diameter 7.8 mm) with a precolumn IONGUARD™ (polymeric guard column), both obtained from Tecknokroma S. Coop. C. Ltda, Barcelona, Spain. Sugars and fermentation products were separated at 60–65°C using a 0.012 N sulfuric acid aqueous mobile phase flowing at 0.4 ml/min and detected using a refractometer with a refractive index detector. Solutions of glucose, lactose, lactic acid, acetic acid and butane-2,3-diol at a concentration between 0.5 and 10.0 g/l were used as standards. Prior to HPLC analysis, all samples and standards were filtered using syringe filters (0.22-µm pore size, 25-mm diameter disk filters, Membrane Solutions, Dallas, TX, USA) (Costas et al. 2018). All the analytical determinations were performed in triplicate.

Nisin. Triplicate samples of the third aliquot of the culture samples were adjusted to pH 3.5 with 5 N HCl to prevent the adsorption of molecules of bacteriocin onto the producer cell surfaces, heat-treated in a boiler water bath for 3 min to kill the cells and centrifuged (27 200 × g for 15 min at 4°C). The antibacterial activity of the cell-free supernatants (CFS) adjusted at pH 6.0 was determined by a photometric bioassay method (Cabo et al. 2001) using *C. piscicola* CECT 4020 as indicator strain (Costas et al. 2018). Nisin activity (Nis) was expressed as bacteriocin units (BU) per ml CFS, being one BU/ml defined as the inverse of the dilution of CFS causing 50% growth inhibition of the target strain compared with control tubes (Murado et al. 2002).

Statistical analyses. The effects of the pH gradients (*VpH*) generated in the culture and the addition of glucose (*G*) with the feeding with CMPW or CMPWGP media on the synthesis of biomass, viable cells and products (Nis, LA, AA and B) in every realkalization and feeding cycle in the two realkalized fed-batch cultures were described with a second-order polynomial model [7]:

$$Ps = A_1 + A_2 \cdot VpH + A_3 \cdot VpH^2 + A_4 \cdot G + A_5 \cdot G^2 + A_6 \cdot VpH \cdot G \quad [7]$$

Where *Ps* represents the levels of X, VCC, Nis, LA, AA or B synthesized in every realkalization and feeding cycle. A_1, A_2, A_3, A_4, A_5, and A_6 are constants.

The corresponding values for the constants and their standard errors were obtained by using the nonlinear curve-fitting software of the SigmaPlot program, version 12.0 (Systat Software Inc., 2012). The coefficients of the models were considered statistically significant if their *p* values were lower than 0.05.

The goodness-of-fit of model [7] for each product was evaluated by analyzing the determination coefficient (R^2) and the mean relative percentage deviation modulus (*RPDM*) (Guerra et al. 2007b):

$$RPDM = \frac{100}{n} \sum_{i=1}^{n} \frac{|P_t - cP_t|}{P_t} \quad [8]$$

Where P_t and cP_t are respectively, the experimental and calculated X, VCC, Nis, LA, AA or B concentrations (with model [7]) and *n* is the number of experimental data points. *RPDM* values < 10% and R^2 values

> 0.95 indicate a good fit for practical purposes (Guerra et al. 2007b).

The experimental concentrations of X, VCC, Nis, LA, AA or B synthesized (Ps_t) in every realkalization and feeding cycle were calculated as follows:

$$Ps_t = P_t - P_{t-12\text{h}} \cdot \frac{(VFM - VS_{t-12\text{h}})}{VFM} \quad [9]$$

Where $P_{t\text{-}12\text{h}}$ and P_t are the experimental concentrations of biomass or products at the beginning and at the end of every realkalization and feeding cycle, respectively.

Subsequently, the calculated X, VCC, Nis, LA, AA or B concentrations at the end of every realkalization and feeding cycle (cP_t) were obtained as:

$$cP_t = cPs_t + cPs_{t-12\text{h}} \cdot \frac{(VFM - VS_{t-12\text{h}})}{VFM} \quad [10]$$

Where $cPs_{t\text{-}12\text{h}}$ and cPs_t are respectively, the X, VCC, Nis, LA, AA or B concentrations at the beginning and at the end of every realkalization and feeding cycle calculated with model [7].

The differences between the predictions of model [7] and experimental data were minimized according to the sum of squares of errors (*SSE*) of the model fit:

$$SSE = \sum_{i=1}^{n} \sum_{j=1}^{m} \left(P_t - cP_t\right)^2 \quad [11]$$

Where *m* represents the number of variables. Other terms are as previously described.

Results and Discussion

Growth kinetics of *L. lactis* CECT 539 in the realkalized fed-batch cultures. The evolution of the culture variables (pH and concentrations of nutrients: glucose, lactose, proteins, total nitrogen, and phosphorus) in the cultures I and II are shown in Figures 1 and 2, respectively. Although the pH gradients decreased progressively in both cultures, *L. lactis* CECT 539 did not lose completely its capacity to recover the acidic pH at the end of fermentation. In both cultures, glucose was completely consumed in every realkalization and feeding cycle, but relatively high concentrations of lactose remained in the media (Figs. 1 and 2). The proteins and total nitrogen and phosphorus were consumed in parallel with the production of biomass, but the complete exhaustion of these nutrients was not observed in the cultures (Figs. 1, 2 and 3).

The nisin-producing strain was grown in two growth cycles (Fig. 3) composed of two exponential growth phases, separated by an intermediate lag phase, as observed before in previously realkalized fed-batch cultures in DW medium (Guerra et al. 2007b; Costas et al. 2016, 2018).

Since *L. lactis* CECT 539 exhibited a diauxic growth in a batch culture in DW medium supplemented with glucose (Costas et al. 2016), it could be logical to suppose that the nisin-producing strain presented two growth phases within every realkalization and feeding cycle of 12 h in the realkalized fed-batch cultures I and II. However, the diauxic growth pattern observed in both cultures would not be related with the use of glucose- and lactose-containing substrates (culture I) or with a glucose-containing substrate (culture II), as feeding media. This is mainly because the feeding media were added at the beginning of every realkalization and feeding cycle to bring the culture up to the initial total sugars concentration (22.62 g/l) of the fermentation (DW) medium (Guerra et al. 2007b; Costas et al. 2016, 2018), and the experimental X concentrations were obtained at the end of every cycle (Figs. 1, 2 and 3).

The TN and TP concentrations decreased progressively in the first culture as a consequence of their consumption by the nisin-producing strain and the sample collection every 12 h. However, in the second fermentation, the growth slowed down at 144 h of incubation, even though the culture was fed with a substrate (CMPWGP) containing a relatively high TP concentration (3.21 g/l) that produced an increase in the concentration of this nutrient in the fermentation medium (Fig. 2).

Thus, culture II was stopped at 168 h because the cells entered in the second stationary phase of growth after 144 h of incubation and, taking into account the profiles described by the variables X and Nis in fermentation I (left side of Fig. 3), it is logical to consider that the extension of the fermentation would not produce significant increases in the concentration of both variables.

In addition, the TN source could be considered as a limiting substrate for the growth of *L. lactis* CECT 539 in fermentations I and II, rather than the two carbon (glucose and lactose) or TP sources. Thus, the nisin-producing strain probably grown first consuming the free amino acids and utilizable oligopeptides (Letort et al. 2002) present in the DW medium and/or the inorganic nitrogen (Guerra et al. 2007b) added with the feeding media (CMPW or CMPWGP). In the second exponential growth phase, the strain CECT 539 probably utilized the less preferred nitrogen sources, for example, the proteins present in both the DW and CMPW media (Murado et al. 1994; Letort et al. 2002).

Nisin production paralleled both the biomass production and pH gradients generated in the two cultures (Figs. 1, 2 and 3), because this metabolite was produced as a pH-dependent primary product in this kind of realkalized fed-batch fermentations (Cabo et al. 2001; Guerra et al. 2007b; Costas et al. 2016; 2018). Lactic acid

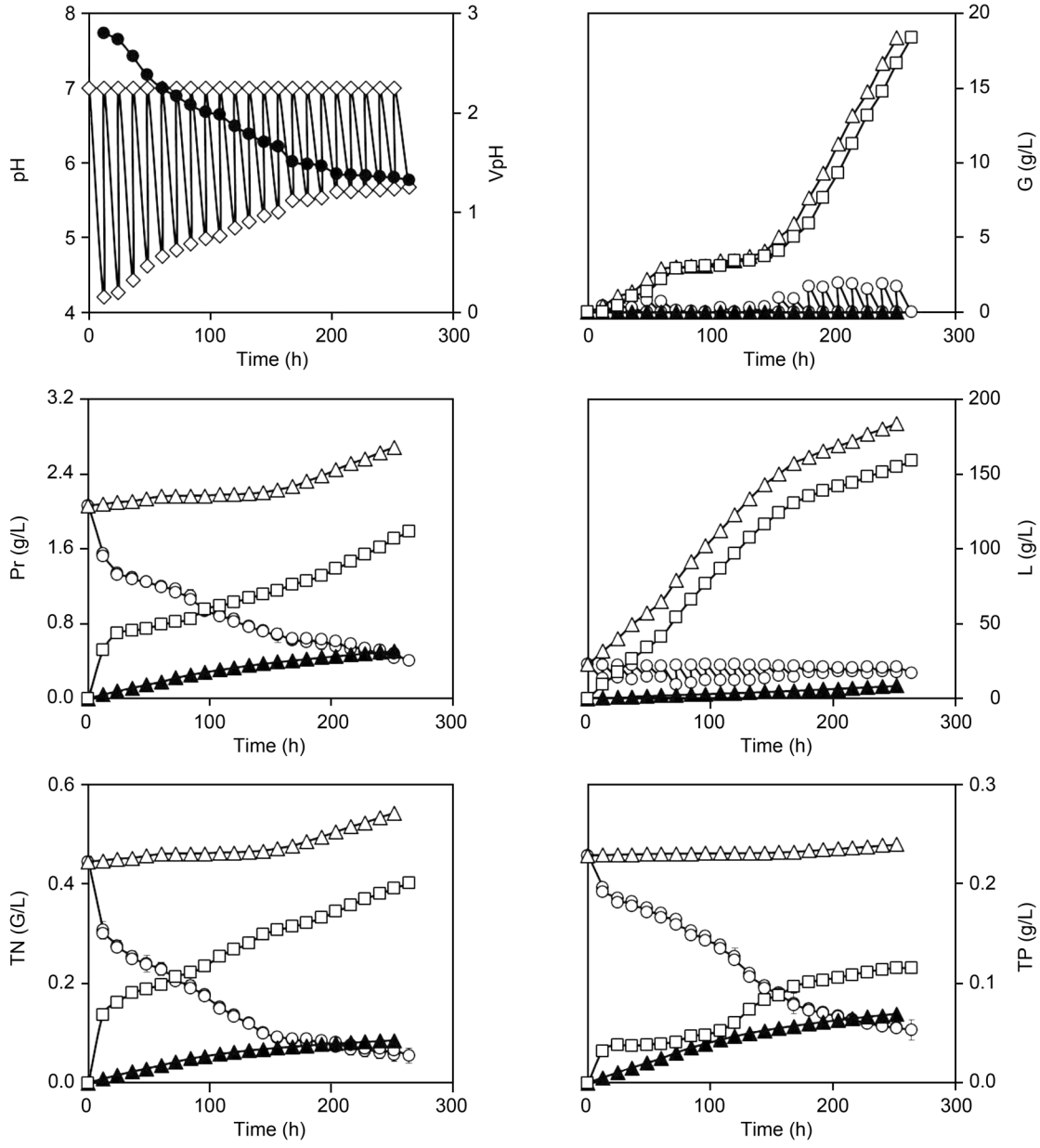

Fig. 1. Time course of the culture pH (◇), pH gradient (VpH, ●), and remaining (○), consumed (⊠), added (Δ) and extracted (▲) concentrations of glucose (G), lactose (L), proteins (Pr), total nitrogen (TN), and phosphorus (TP) in the realkalized fed-batch culture I. The data reported are means ± standard deviations of two repeated experiments and three replicate measurements.

was also synthesized in parallel with biomass synthesis in both cultures, but the productions of acetic acid and butane-2,3-diol triggered after 84 h of fermentation (left and right sides of Fig. 3).

Although the incubation times in fermentation I (264 h) and fermentation II (168 h) were different, the final levels of X, VCC, Nis and AA synthesized in the first culture (3.07 g/l, 1.75×10^{10} CFU/ml, 105.61 BU/ml, and 1.78 g/l, respectively) were almost similar ($p > 0.05$) to those (3.08 g/l, 1.75×10^{10} CFU/ml, 103.68 BU/ml, and 1.66 g/l, respectively) obtained in fermentation II. Only the LA and B concentrations in the first culture (16.98 and 4.96 g/l) were higher ($p < 0.05$) than those (13.62 and 2.24 g/l) obtained in fermentation II (Fig. 3).

This observation indicates that the CMPWGP medium can be used as an appropriate feeding substrate for the production of probiotic biomass and nisin. However, further studies based on optimizing its TP concentration are required to avoid the accumulation of this nutrient in the fermentation medium.

Empirical models obtained for biomass and product synthesis in the first fed-batch culture. Table II shows the statistically significant values obtained for the constants of model [7] for the dependent variables (X, VCC, Nis, LA, AA and B) in fermentation I. In all cases, *RPDM* values < 6 % and R^2 values > 0.994 were obtained, indicating that the fitted models described appropriately the trend observed for all the dependent variables in

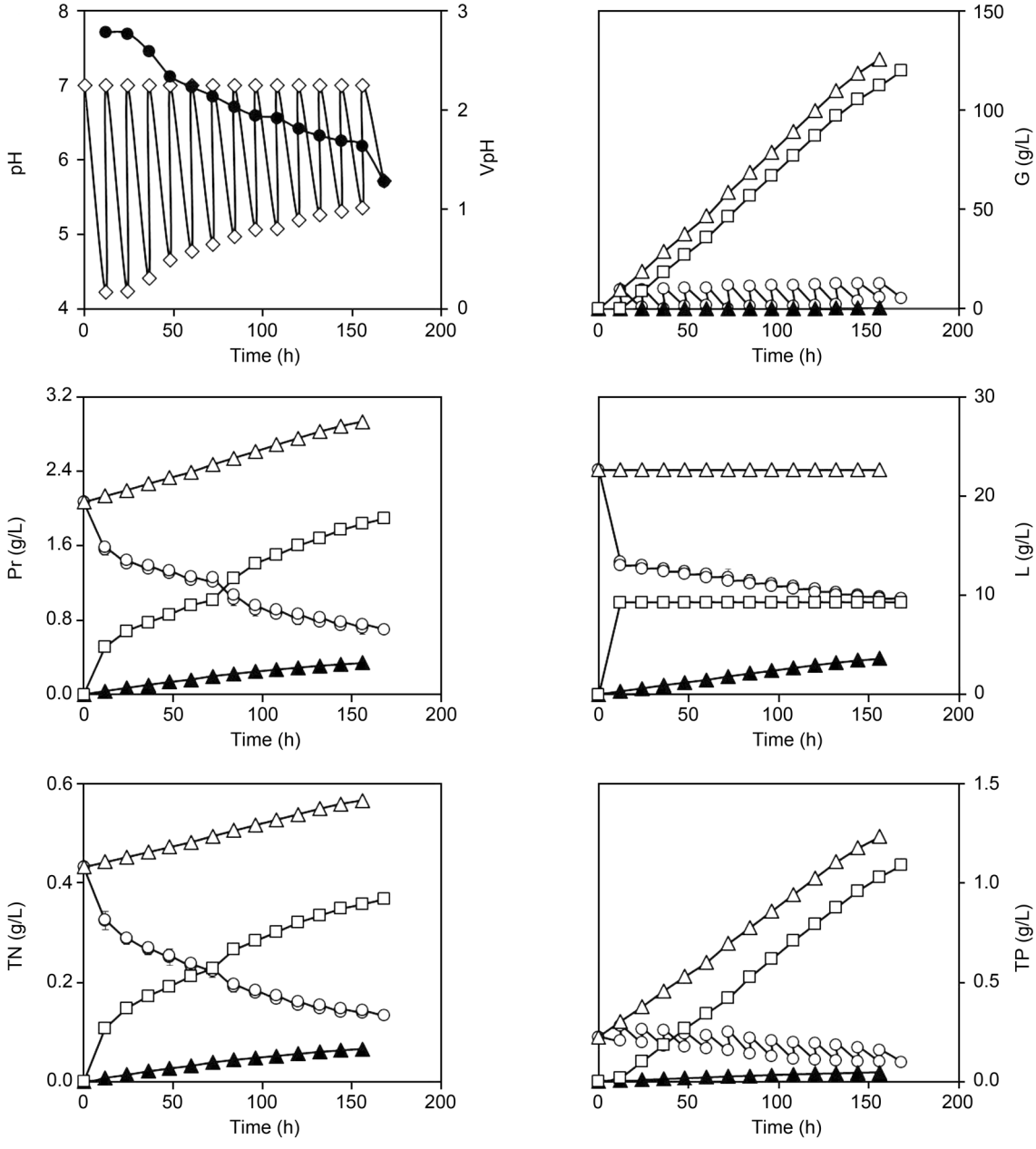

Fig. 2. Time course of the culture pH (◇), pH gradient (VpH, ●), and remaining (○), consumed (⊠), added (Δ) and extracted (▲) concentrations of glucose (G), lactose (L), proteins (Pr), total nitrogen (TN), and phosphorus (TP) in the realkalized fed-batch culture II. The data reported are means ± standard deviations of two repeated experiments and three replicate measurements.

response to the addition of glucose and the pH gradients generated in the culture. The fitted models obtained for each dependent variable are discussed below:

Biomass and viable cell counts. For both dependent variables (X and VCC), the values of the coefficients for the lineal (*VpH* and *G*) and quadratic (VpH^2 and G^2) terms had a negative and positive sign, respectively (Table II). However, while the coefficient of the binary interaction between *VpH* and *G* had a positive sign for X, it was not statistically significant for VCC.

From a mathematical point of view, these results indicate that X and VCC increased for *VpH* and *G* values lower and higher than the corresponding optimum values (*VpHopt* and *Gopt*) for both independent variables. However, from the results obtained in previous works (Cabo et al. 2001; Guerra et al. 2007b), it could be reasonable to consider the obtained optimum *VpH* values (1.81 for X and 1.92 for VCC) as the minimum pH gradient that allows the growth of *L. lactis* CECT 539 in the first culture. In fact, the corresponding maximum final pH values, 5.19 for X and 5.08 for VCC (*pHopt* = 7.0 – *VpHopt*), were slightly higher than that (pH = 4.86) observed in the previous batch cultures of the nisin-producing strain in DW medium (Guerra et al. 2007b). These maximum final pH values for the growth of *L. lactis* CECT 539 are lower than that (5.50)

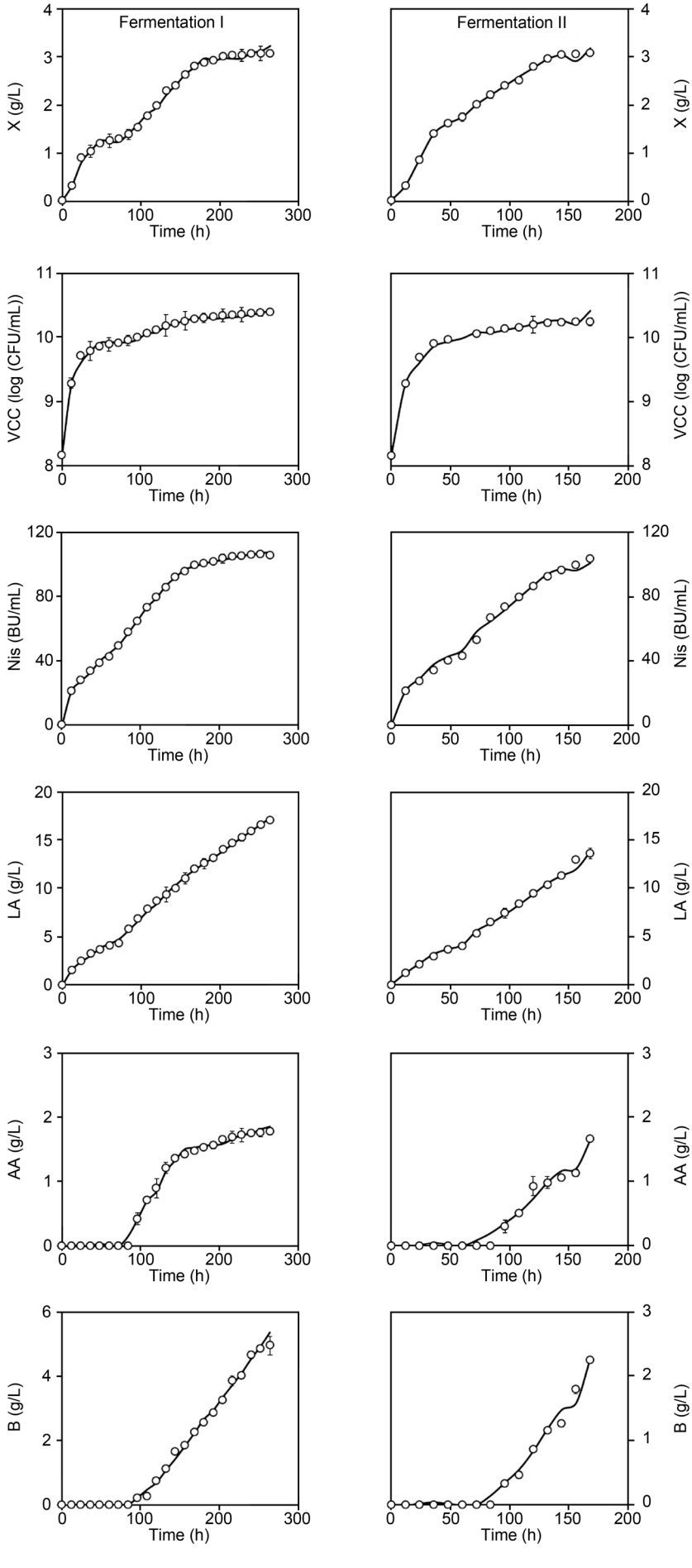

Fig. 3. Time course of the biomass (X), viable cell counts (VCC), nisin (Nis), lactic acid (LA), acetic acid (AA), and butane-2,3-diol (B) in the realkalized fed-batch cultures I (left side), and II (right side). The data reported are means ± standard deviations of two repeated experiments and three replicate measurements. The solid lines drawn through the experimental data for each variable were obtained according to the empirical model [7].

Table II
Significant values ($p < 0.05$) for the constants in model [7] for the different dependent variables in the first fed-batch culture.

Parameter	Biomass	Viable cell counts	Nisin	Lactic acid	Acetic acid	Butane-2,3-diol
A_1	4.12 ± 0.631	1.86 ± 0.084	1.13 ± 0.113	5.71 ± 0.235	1.16 ± 0.003	2.75 ± 0.277
A_2	−3.60 ± 0.545	−1.56 ± 0.072	8.67 ± 0.4244	−4.04 ± 0.225	−0.70 ± 0.012	−2.00 ± 0.265
A_3	0.83 ± 0.116	0.41 ± 0.015	−2.07 ± 0.206	0.88 ± 0.054	0.12 ± 0.005	0.37 ± 0.064
A_4	−1.09 ± 0.383	−0.15 ± 0.051	−9.25 ± 1.547	−0.92 ± 0.040	−0.46 ± 0.021	−0.19 ± 0.021
A_5	0.14 ± 0.062	0.03 ± 0.008	2.93 ± 0.314	0.26 ± 0.019	0.15 ± 0.011	0.03 ± 0.003
A_6	0.35 ± 0.168	NS	0.74 ± 0.203	NS	NS	NS
R^2	0.9950	0.9948	0.9993	0.9986	0.9949	0.9954
RPDM (%)	3.335	0.228	1.121	3.760	1.701	5.809
VpHopt	1.81	1.92	2.32	2.30	2.83	2.40
pHopt = 7.0 − *VpHopt*	5.19	5.08	4.68	4.70	4.17	4.60
Gopt (g/l)	1.64	2.72	1.29	1.76	1.41	2.80

observed for *L. lactis* ATCC 11454 in MRS broth (Penna and Moraes 2002).

The decrease in the bacterial growth at pH values lower than 5.19 or 5.08 could be related to a reduction in the metabolic activity of *L. lactis* CECT 539 probably caused by a limitation in micronutrients or nutrient transport (Poolman and Konings 1988).

Using the same argument as that used for the *VpHopt*, it can be also reasonable to consider the values of *Gopt* (1.59 g/l for X and 1.63 g/l for VCC) as the minimum concentrations of glucose that should be in the culture medium to stimulate the growth of *L. lactis* CECT 539.

Nisin. The statistically significant coefficient for the quadratic term VpH^2 with a negative sign indicates that the optimum *VpH* value (2.32) for maximum nisin production (Table II) is within the pH gradient range (from 2.80 to 1.33) generated in the first culture (Fig. 1). Thus, the calculated minimum final pH value (4.68) is in perfect agreement with the optimum final pH values (4.90 and 4.27) obtained for nisin synthesis in DW medium in batch and realkalized fed-batch cultures, respectively (Guerra et al. 2007b).

With regard to the addition of glucose, it can be noted that the *Gopt* value obtained (1.29 g/l) is the minimum value that favored bacteriocin production (Table II). Then, more addition of glucose in every realkalization and feeding cycle is needed to enhance nisin synthesis in fermentation I.

Lactic acid, acetic acid, and butane-2,3-diol. The signs of the lineal and quadratic terms in the empirical models obtained for these three metabolites were similar to those obtained for the dependent variables X and VCC. Thus, according to the predictions of model [7], the maximum final pH values for LA, AA and B productions were 4.70, 4.17 and 4.60, respectively.

Lactic acid production in the realkalized fed-batch culture I was minimal at final pH values between 4.62 and 4.83 (Fig. 2), which includes the maximum pH value (4.70) calculated from model [7]. Similarly, when the culture reached final pH values between 4.22 and 4.97 (12–84 h of incubation), the lowest AA and B productions were obtained, because, at these incubation times, the two fermentation metabolites had not been detected in the culture medium (Fig. 2). In fact, AA and B productions started when the culture reached a final pH value of 4.99 after 84 h of incubation (left side of Fig. 3).

In the same way, the minimum concentrations of glucose in the processes for high LA, AA and B production were 1.76, 1.41 and 2.80 g/l, respectively (Table II).

Validation of model [7]. The effectiveness of model [7] to describe the productions of biomass, bacteriocin and fermentation metabolites by *L. lactis* CECT 539 was validated by using the experimental data obtained from the second fed-batch culture. As can be observed, the empirical model [7] satisfactorily described the evolution of the dependent variables X, VCC, Nis, LA, AA and B (right side of Fig. 3), providing *RPDM* values < 4% and R^2 values > 0.979 (Table III). In addition, the maximum final pH values were similar (for X, VCC, LA and B) or almost similar (for AA) to the corresponding calculated values in the fed-batch culture I (see Tables I and II).

However, in the case of nisin synthesis, the optimum pH value calculated (4.88) could be considered as the maximum final pH value for high bacteriocin production in every realkalization and feeding cycle, since the value of the coefficient for VpH^2 had a positive sign. This indicates that nisin synthesis is favored in this type of fed-batch cultures in the pH range of 4.68 and 4.88, which is within the optimum pH range observed in previous studies for nisin production by *L. lactis* CECT 539 (Guerra et al. 2007b; Costas et al. 2016, 2018).

As shown in Table IV, the optimum final pH values for nisin production by other *Lactococcus* strains (e.g. *L. lactis* ATCC 11454 and ATCC 7962) were not always within the optimum final pH range calculated

Table III
Significant values ($p < 0.05$) for the constants in model [7] for the different dependent variables in the second fed-batch culture.

Parameter	Biomass	Viable cell counts	Nisin	Lactic acid	Acetic acid	Butane-2,3-diol
A_1	0.46 ± 0.043	0.58 ± 0.053	0.42 ± 0.040	10.19 ± 0.405	0.41 ± 0.032	0.23 ± 0.062
A_2	–3.32 ± 0.351	–1.75 ± 0.062	–27.69 ± 1.011	–8.10 ± 0.621	–2.19 ± 0.027	–3.15 ± 0.333
A_3	0.58 ± 0.059	0.45 ± 0.011	6.53 ± 0.462	1.79 ± 0.109	0.44 ± 0.024	0.63 ± 0.097
A_4	0.53 ± 0.038	0.26 ± 0.065	4.73 ± 0.516	–0.35 ± 0.017	0.42 ± 0.023	0.72 ± 0.065
A_5	–0.04 ± 0.005	–0.01 ± 0.003	–0.10 ± 0.013	0.03 ± 0.006	–0.02 ± 0.001	–0.04 ± 0.004
A_6	0.11 ± 0.006	NS	NS	NS	NS	NS
R^2	0.9973	0.9906	0.9950	0.9959	0.9794	0.9856
RPDM (%)	0.987	0.326	3.775	2.609	3.988	3.602
VpHopt	1.82	1.96	2.12	2.26	2.47	2.49
pHopt = 7.0 – *VpHopt*	5.18	5.04	4.88	4.74	4.53	4.51
Gopt (g/l)	10.55	11.10	22.83	5.30	11.17	10.27

Table IV
The optimal final culture pH values for different bacteriocins produced by lactic acid bacteria.

Bacteriocin	Producing strain	Optimum final pH	Culture medium	Reference
Mesenterocin 5	*Leuconostoc mesenteroides* UL5	4.24 to 4.34	Whey	Daba et al. 1993
Pediocin AcH	*Pediococcus acidilactici* LB42-923	3.70	TGE broth	Yang and Ray 1994
Nisin	*Lactococcus lactis* ATCC 11454	5.80	TGE broth	Yang and Ray 1994
Leuconocin Lcm1	*Leuconostoc carnosum* Lm1	5.00	TGE broth	Yang and Ray 1994
Sakacin A	*Lactobacillus sake* LB 706	4.50	TGE broth	Yang and Ray 1994
Nisin	*Lactococcus lactis* ATCC 7962	4.90	Whey permeate	Flôres and Alegre 2001
Carnocin KZ213	*Carnobacterium piscicola* 213	4.80 to 5.08	MRS broth	Khouiti and Simon 2004
Nisin	*Lactococcus lactis* ATCC 11454	4.60	MRS + milk	Penna et al. 2005
		4.80	M17 + milk	
Nisin	*Lactococcus lactis* ATCC 11454	4.65 to 4.96	Whey + YE (5 g/l)	Jozala et al. 2011

YE: yeast extract

for *L. lactis* CECT 539. In addition, other lactic acid bacteria, including the genera *Leuconostoc*, *Pediococcus*, *Lactobacillus*, and *Carnobacterium* produced the highest bacteriocin concentrations at final pH levels different to those observed for *L. lactis* strains. These results indicate that the effect of the final pH on the bacteriocin production depended on both the strain and culture medium used.

This different effect of the final pH values on the bacteriocin synthesis has been related with the need of an appropriate final pH range for the post-translational conversion of prebacteriocin to active bacteriocin (Yang and Ray 1994).

With regard to the glucose addition, it could be noted that with the exception of LA, the signs for the coefficients of G and G^2 terms in the models for the other dependent variables, had a positive and negative sign, respectively (Table III). Therefore, the calculated *Gopt* values are the maximum added glucose levels to obtain the maximum concentrations of X, VCC, Nis, AA and B. Therefore, with the use of a feeding medium (CMPWGP) with a concentration of glucose (400 g/l) higher than the CMPW medium (101.72 g/l), the amounts of glucose added to the growing culture in fermentation II were considerably higher than those added in fermentation I (Figs. 1 and 2). Thus, glucose levels higher than the calculated *Gopt* value in the second culture could produce substrate inhibition on the growth and product (Nis, AA and B) formation (Pongtharangkul and Demirci 2006; Costas et al. 2016, 2018) by *L. lactis* CECT 539. In addition, the inhibitory effect produced by higher levels of glucose on nisin production could be also related with the regulation that the carbon source produced on the synthesis or activity of prenisin-modifying enzymes (De Vuyst 1995).

These observations are consistent with the results obtained for the fed-batch production of nisin by *L. lactis* ATCC 11454 in a complex medium, with sucrose feeding at rates of 6, 7, 8, and 10 g/l/h (Lv et al. 2005). In this study, the growth was slightly affected by the different feeding rates, but the bacteriocin synthesis was different in the four cultures. Thus, the optimal

feeding rate for nisin production was 7 g/l/h, but the addition of sucrose at feeding rates of 8 and 10 g/l/h led to the accumulation of the carbon source in the culture medium during feeding that inhibited the bacteriocin synthesis. For a feeding rate of 6 g/l/h, the sucrose was almost completely consumed by the nisin-producing strain and its remaining level in the culture medium was close to zero in the period of feeding. This low sucrose availability limited the growth and bacteriocin production (Lv et al. 2005).

The results obtained with the use of model [7] are in perfect agreement with the affirmation that nisin is produced as a pH-dependent primary metabolite since its production depends on both the biomass synthesis and the final culture pH in the medium (Yang and Ray 1994; Guerra et al. 2007b). Thus, biomass production by *L. lactis* CECT 539 was favored at culture pH values above 5.0, but a final pH range of 4.68–4.88 was needed for high nisin synthesis.

Conclusions

From a practical point of view, the modeling procedure used in this work could allow: i) determining the optimum pH and glucose ranges to obtain high levels of biomass, nisin and fermentation metabolites in realkalized fed-batch cultures, ii) providing an accurate interpretation of the fermentation kinetics taking into account the effects of the amount of glucose added and the pH gradient generated in every realkalization and feeding cycle on the growth and product synthesis by *L. lactis* CECT 539 and, iii) design feeding strategies to produce highly concentrated probiotic products with high concentrations of viable cells and nisin.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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