Supplementary materials for the article:

Costas Malvido M, et al. The Joint Effect of pH Gradient and Glucose Feeding on the Growth Kinetics of *Lactococcus lactis* CECT 539 in Glucose-Limited Fed-Batch Cultures. Pol J Microbiol. 2019, Vol. 68, No 2, 269–280.

Materials and Methods

Realkalized fed-batch cultures. 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 to a 250 ml-Erlenmeyer flask containing 50 ml of DW medium and incubated at 30°C/12 h (200 rpm). Then, an aliquot of this culture (containing 7.4×10^9 CFU/ml) was used as the inoculum for the realkalized fed-batch fermentation 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. 2016).

The fixed-volume realkalized fed-batch fermentations I and II were carried out in duplicate in a 6-l bench-top fermentor (New Brunswick Scientific, Edison, NJ, USA) containing 4 l of sterilized DW medium (pH 7.0), at 30°C with agitation at 200 rpm and with an aeration level of 0.5 l/h. The first culture was fed a mixture of CMPW medium (Table S-I) 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 (CMPW+G+P medium) (Table S-I) or sterile distilled water (if needed).

Table S-I

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

Nutrient	DW	CMPW	CMPW+G+P
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

The cyclic fed-batch fermentations were initiated as batch cultures during 12 h when the cultures reached the optimum final pH values for nisin production in DW medium (Guerra et al. 2001). At this time, a sample (~100 ml) was aseptically taken from the fermentation medium and the concentration of total sugars was measured. After realkalizing the fermentation medium up to initial pH (7.0) with 5 N NaOH, the volume of feeding substrate needed to restore the initial total sugar concentration (22.62 g/l) in the fermentation medium was calculated by applying the mass balance equations around the fermenter (see below).

The calculated volumes of the feeding substrate (CL and CMPW medium in fermentation I and CMPW+G+P medium in fermentation II) were then added to the fermenter using a peristaltic pump (LKB Pharmacia). The volume of culture medium in the fermentor was kept

constant by matching the volumes added to the fermentor (sum of the volumes of 5 N NaOH and feeding substrates) with the corresponding sampling volume. The cultures were realkalized and fed every 12 h until the nisin-producing strain was able to reduce the initial culture pH (Guerra and Pastrana 2003).
The samples taken from the fermentation medium every 12 h were divided into two aliquots. The first was used to measure the culture pH and the concentrations of biomass (as cell dry weight and viable cell counts per ml), total sugars, nitrogen, phosphorus, proteins, glucose, lactose, lactic acid, acetic acid, and butane-2,3-diol. The second aliquot was used to quantify the nisin activity. All the analyses were carried out in triplicate (Costas et al. 2016).

Mass balance equations. In the first fed-batch culture, the growing culture was fed a mixture of CL and CMPW medium:

1. The CMPW medium was used to supplement the growing cultures in every realkalization and feeding cycle with the additional sources of carbon (glucose), nitrogen, and phosphorus, which were different to those of the DW medium. This could allow preventing the nutrient (total nitrogen, total phosphorus, and proteins) exhaustion observed in the control cultures fed concentrated solutions (400 g/l) of lactose or glucose (Costas et al. 2016).
2. The CL substrate was used to i) feed the realkalized fed-batch culture with the same carbon source (lactose) as the fermentation medium, ii) reduce considerably the volumes of feeding substrate to kept constant the fermentation volume, and iii) avoid masking the effect of glucose addition with the CMPW medium.

The volumes of feeding media were calculated by developing the corresponding mass balance equations (Fajardo et al. 2008):

Realkalized fed-batch fermentations I and II:

As indicated above, the volume of the fermentation medium (V) in the two fed-batch fermentations was maintained constant ((dV/dt) = 0) by matching the volumes added to the fermenter (feeding volume (VF) plus the volume of 5 N NaOH) with the sampling volume (VS_{tn}).

$$VS_{t_n} = VF_{t_n} + VNaOH_{t_n} = \left(VCMPW_{t_n} + VCL_{t_n}\right) + VNaOH_{t_n} \text{ for fermentation I} \quad (1.1)$$

$$VS_{t_n} = VF_{t_n} + VNaOH_{t_n} = \left(VCMPW + G + P_{t_n} + VdW_{t_n}\right) + VNaOH_{t_n} \text{ for fermentation II} \quad (1.2)$$

Where $VCMPW_{tn}$, VCL_{tn}, $VCMPW+G+P_{tn}$, and VdW_{tn} are respectively, the volumes (in l) of CMPW medium, concentrated lactose (CL), CMPW+G+P medium and distilled water added to the fermenter at the beginning of every feeding cycle. $VNaOH_{tn}$ is the volume (l) of 5 N NaOH added to the fermenter for re-alkalizing the fermentation medium up to the initial pH value of 7.0.
The sum of the volumes of feeding substrates that must be added for restoring the initial total sugars (TS) in the fermentation medium can be calculated from Eqs (2.1) and (2.2):

$$VCMPW_{t_n} + VCL_{t_n} = VS_{t_n} - VNaOH_{t_n} \text{ for fermentation I} \quad (2.1)$$

$$VCMPW + G + P_{t_n} + VdW_{t_n} = VS_{t_n} - VNaOH_{t_n} \text{ for fermentation II} \quad (2.2)$$

from which it follows that:

$$VCMPW_{t_n} = VS_{t_n} - VNaOH_{t_n} - VCL_{t_n} \text{ for fermentation I} \quad (3.1)$$

$$VCMPW + G + P_{t_n} = VS_{t_n} - VNaOH_{t_n} - VdW_{t_n} \text{ for fermentation II} \quad (3.2)$$

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

$$TS_{C+E} = V \cdot [TS]_{t_{n-1}} - (V - VS_{t_n}) \cdot [TS]_{t_n} \quad (4)$$

Where $[TS]_{tn-1}$ and $[TS]_{tn}$ 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 (in 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 fermentation medium can be calculated by the following expressions:

$$VCMPW \cdot [TS_{CMPW}] + VCL \cdot [TS_{CL}] = TS_{C+E} \text{ for fermentation I} \quad (5.1)$$

$$VCMPW + G + P \cdot [TS_{CMPW+G+P}] = TS_{C+E} \text{ for fermentation II} \quad (5.2)$$

Where $[TS_{CMPW}]$, $[TS_{CL}]$ and $[TS_{CMPW+G+P}]$ are the total sugars concentration in the CMPW, CL and CMPW+G+P media, respectively.
Substituting Eq. (3.1) into Eq. (5.1) gives:

$$(VS_{t_n} - VNaOH_{t_n} - VCL_{t_n}) \cdot [TS_{CMPW}] + VCL_{t_n} \cdot [TS_{CL}] = TS_{C+E} \text{ for fermentation I} \quad (6.1)$$

Thus, the V_{CL} can be calculated as:

$$VCL_{t_n} = \frac{(TS_{C+E} - VS_{t_n} \cdot [TS_{CMPW}] + VNaOH_{t_n} \cdot [TS_{CMPW}])}{([TS_{CL}] - [TS_{CMPW}])} \quad (7.1)$$

Now, the *VCMPW* can be obtained by introducing the values of *VCL* and *VNaOH* into Eq. (3.1).

The $VCMPW+G+P_{tn}$ in fermentation II can be obtained by rearranging Eq. (5.2):

$$VCMPW + G + P_{t_n} = \frac{TS_{C+E}}{[TS_{CMPW+G+P}]} \quad (7.2)$$

Then, the *VdW* can be obtained by introducing the values of *VCMPW+G+P* and *VNaOH* into Eq. (3.2).

On the other hand, the accumulated concentrations of substrates (total sugars (*TS*), nitrogen (*TN*), phosphorus (*TP*), and protein (*Pr*)) consumed ($\sum[S_{cons}]$) in every re-alkalization cycle were calculated by using the following mass balance equations for fermentations I and II:

$$\sum[S_{cons}]_{tn} = \frac{(V - VS_{tn-1})\cdot[S]_{tn-1} + (VCMPW_{tn-1}\cdot[S_{CMPW}]) + (VCL_{tn-1}\cdot[S_{CL}]) - (V\cdot[S]_{tn})}{V} + [Scons]_{tn-1} \quad (8.1)$$

$$\sum[S_{cons}]_{tn} = \frac{(V - VS_{tn-1})\cdot[S]_{tn-1} + (VCMPW + G + P_{tn-1}\cdot[S_{CMPW+G+P}]) - (V\cdot[S]_{tn})}{V} + [Scons]_{tn-1} \quad (8.2)$$

Where $[S]_{tn-1}$ and $[S]_{tn}$ are the nutrient (*TS*, *TN*, *TP* and *Pr*) concentrations (in g/l) in the fermenter at the beginning and at the end of every feeding cycle, respectively. $[S_{CMPW}]$, $[S_{CL}]$ and $[S_{CMPW+G+P}]$ are the nutrient (*TS*, *TN*, *TP* and *Pr*) concentrations (in g/l) in the feeding substrates (CMPW, CL and CMPW+G+P).

Since the values for *VS*, *VCMPW*, *VCL*, *VCMPW+G+P* and $[S_{cons}]$ are zero in the first 12 h of incubation, the concentrations of nutrients consumed in this period in the two cultures were calculated as follows:

$$[S_{cons}]_{12h} = \frac{V\cdot([S]_{0h} - [S]_{12h})}{V} \quad (9)$$

The accumulated concentrations of nutrient extracted ($\sum[S_{ext}]$) from the fermentation medium as well as the accumulated concentrations of nutrient added ($\sum[S_{added}]$) to the fermentation medium with the feeding substrates were calculated as follows:

$$\sum[S_{ext}]_{tn} = \frac{VS_{tn}\cdot[S]_{tn}}{V} + [S_{ext}]_{tn-1} \text{ for both cultures} \quad (10)$$

$$\sum[S_{added}]_{tn} = \frac{VCMPW\cdot[S_{CMPW}] + VCL\cdot[S_{CL}]}{V} + [S_{added}]_{tn-1} \text{ for fermentation I} \quad (11.1)$$

$$\sum[S_{added}]_{tn} = \frac{VCMPW + G + P\cdot[S_{CMPW+G+P}]}{V} + [S_{added}]_{tn-1} \text{ for fermentation II} \quad (11.2)$$

The accumulated concentrations of products (biomass, lactic acid, acetic acid, butane-2,3-diol and nisin) extracted ($\sum[P_{ext}]$) at the end of every feeding cycle were calculated by the following mass balance equations:

$$\sum[P_{ext}]_{tn} = \frac{VS_{tn}\cdot[P]_{tn}}{V} + [P_{ext}]_{tn-1} \text{ for both cultures} \quad (12)$$

Where $[P]_{tn}$ is the concentration of product (in g/L) at the end of every feeding cycle.

Then, the accumulated concentrations of products formed ($\sum[P]$) at the end of every feeding cycle were calculated as the sum of the concentrations of product synthesized at the end of every feeding cycle and the total amounts of products extracted in the previous feeding cycle:

$$\sum [P]_{tn} = \frac{VS_{tn-1} \cdot [P_{ext}]_{tn-1}}{V} + [P]_{tn} \text{ for both cultures} \quad (13)$$

Nutrients. Concentrations of total sugars were measured using the phenol/sulfuric acid method (Dubois et al. 1956) as described by Strickland and Parsons (1968a), with glucose (Panreac, Barcelona, Spain) as standard. Protein was measured by the method of Lowry et al. (1951), with bovine serum albumin (Sigma, St. Louis, MO, USA) as standard. Total nitrogen was estimated by the micro-Kjeldahl method, replacing distillation by the spectrophotometric method of Havilah et al. (1977), with ammonium sulfate (Panreac, Barcelona, Spain) as standard. Total phosphorus was determined by the molybdate reaction (Murphy and Riley, 1962) as described by Strickland and Parsons (1968b), with KH_2PO_4 (Panreac, Barcelona, Spain) as standard. Patterns were used as solutions of glucose, bovine serum albumin, ammonium sulfate and KH_2PO_4 at concentrations between 12.5–125.0 µg/ml, 0.05–0.50 g/l, 12.5–500.0 mg/l and 0.2–2.0 mg/l, respectively. All the analytical determinations were performed in triplicate.

Glucose, lactose, and fermentation products. The supernatants were filtered through cellulose acetate membrane syringe filters (0.22-µm pore size, 25-mm diameter disk filters, Membrane Solutions, Dallas, TX, USA). Concentrations of glucose, lactose, lactic acid, acetic acid, and butane-2,3-diol were quantified using a high-performance liquid chromatography (HPLC) 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 (Costas et al. 2016).

The mobile phase consisted of 0.012 N sulfuric acid at a flow rate of 0.4 ml/min at 60–65°C. The refractive index of the peaks was measured by a refractometer with a refractive-index detector (Guerra and Pastrana 2003). Patterns were used as solutions of glucose, lactose, lactic acid, acetic acid and butane-2,3-diol at a concentration between 0.5 and 10.0 g/l.

Nisin. The nisin activity assay was carried out as follows (Cabo et al. 1999): Aliquots from cultures of *L. lactis* CECT 539 were adjusted to pH 3.5 with 5 N HCl to avoid the adsorption of molecules of bacteriocin onto the producer cell surfaces. Thereafter, the acidified samples were heated in a boiler water bath for 3 min to kill the cells and centrifuged (27 200 × g for 15 min at 4°C) to remove dead cells from the supernatants.

The cell-free supernatants (CFS) containing nisin activity are adjusted to pH 6.0 and frozen until further use. Nisin activity was determined by a photometric bioassay method (Cabo et al. 1999) using *C. piscicola* CECT 4020 as indicator organism (Guerra and Pastrana 2002). The CFS were serially diluted as needed in sterile distilled water and 2.5 ml of these samples were added into sterile culture tubes. Each tube was inoculated with 2.5 ml of a culture of the indicator strain diluted to an absorbance of 0.2 at 700 nm with sterile MRS broth (buffered at pH 6.3 with 0.1 M potassium hydrogen phthalate-NaOH), and incubated for 6 h at 30°C and 200 rpm. Controls consisted of three culture tubes in which the diluted cell-free supernatant was replaced by sterile distilled water. The absorbance of each sample was measured

spectrophotometrically at 700 nm (Cabo et al. 1999) and the growth inhibition percentage (GIP) was calculated (Murado et al. 2002).

Dose/response curves were built by plotting the GIP values against the inverse of the corresponding dilution (1/D) of each sample. Then, a dose-response model was set up to obtain the nisin activity titres, which were expressed as bacteriocin units (BU) per ml cell-free supernatant (Murado et al. 2002). One bacteriocin unit (BU) per milliliter was expressed as the inverse of the dilution that produced a 50% growth inhibition (inhibitory dose 50 obtained from triplicate samples) of the indicator bacterium compared with control tubes (Cabo et al. 1999; Murado et al. 2002).

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