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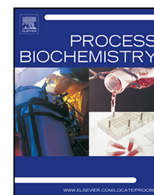

Physiological heterogeneity in *Lactobacillus casei* fermentations on residual yoghurt whey

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ABSTRACT

Lactobacillus casei is a well-known lactic acid-producer with substantial industrial interest. Currently, inexpensive lactic acid substrates such as residual yoghurt whey are being increasingly employed as revalorization strategies for such polluting food industry wastes. However, the influence of different bioprocessing conditions on the cellular functionality and physiological status of *L. casei* at single cell level has barely been evaluated to date. In the present study, monitoring the different physiological states of *L. casei* through multiparametric flow cytometry during lactic acid production from residual yoghurt whey showed that the majority of *L. casei* cells remained in healthy, metabolically active state (∼70%) under uncontrolled-pH conditions (pH <3.6), whereas a progressive increase in population heterogeneity was determined (increasing the damaged and dead subpopulations) with higher production (41.5 g/L lactate titer) and sugar consumption rates when a pH-controlled strategy at 6.5 was adopted. A segregated kinetic model was additionally developed to better describe the physiological behaviour of microbial heterogeneity, gaining deeper knowledge on the lactic acid-producing ability of each subpopulation under pH-controlled conditions in the mixed sugar co-fermentation. This study provides further understanding on the role of physiological heterogeneity in lactobacilli populations useful to enhance bioprocess performance and thus achieve efficient lactic acid production.

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1. Introduction

Production of lactic acid by fermentation holds a prime position in the biotechnological industry as cost-effective and environmentally benign approach for its large-scale manufacture [1,2]. However, the next step to consolidate the market implies the use of available, cheap non-food feedstocks for a sustainable bio-based lactic acid production. Multiple raw materials such as molasses, starchy or lignocellulosic wastes from agro-industrial residues have thus been proposed as carbohydrate-rich substrates for lactic acid fermentation [2]. Recently, residual yoghurt whey has been employed as inexpensive source for lactic acid production, reducing at the same time the end-of-cycle environmental impact of this food industry waste [3]. Unlike fermentable carbohydrate-rich materials such as agricultural-based residues, residual yoghurt whey can be converted into lactic acid with no pre-treatment in a single bioprocess carried out by *Lactobacillus casei* [3]. The availability along with the high sugar content of this food industry waste, which contains glucose, sucrose and lactose, make it particularly well-suited for being used as novel lactic acid fermentation feedstock. Nevertheless, microbial fermentations are strongly reliant on cellular functionality and viability, which may impair the metabolic ability of the microorganisms to produce the desired metabolite [4–8]. Indeed, the fermentation performance and robustness of lactobacilli's fermentations can be seriously compromised by the presence of non-producing lactic acid cells.

The characterization of healthy, damaged or nonviable microorganisms is therefore essential to determine the real metabolic *status* of the bioprocess. Changes in the underlying physiology strongly influence the biological performance which may lead eventually to reduced yields [5,9]. Multiparametric flow cytometry (FCM) has recently emerged as a powerful tool in industrial biotechnology since it enables monitoring physiological changes during industrially relevant bioprocesses in almost real time conditions [10]. Furthermore, bioprocess monitoring through FCM provides insights into the proper fermentation strategy to be adopted, enabling a better knowledge at single cell level otherwise not detected by conventional culture-dependent or biomass estimation methods in the bulk population [4]. As such, lactobacilli have shown to display significant functional challenges at population level in response to environmental stimuli such as freezing [11], pH-stress [12], and oxygen limited conditions [13].

Under batch or fed-batch conditions, lactobacilli face challenges derived from pH oscillation [14–16], which may become limiting under large-scale conditions causing either structural or intracellular changes [17,18]. Nonetheless, the impact of acidic and mild

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bioprocessing conditions on the physiological states of *L. casei* at the single cell level for lactic acid production has not been investigated yet.

In this regard, the present study aims to evaluate the physiological states of *L. casei* during lactic acid fermentation using the mixed sugar substrate composition of residual yoghurt whey as a bioprocessing revalorization strategy. The physiological status of *L. casei* was therefore monitored by FCM during uncontrolled- and controlled-pH batch cultivations, with the aim of assessing fermentation performance, cellular behaviour and efficiency of *L. casei* under different conditions. A segregated kinetic modelling was additionally undertaken in this work considering sugar uptake and lactic acid production during pH-controlled batch cultivation in order to provide deeper knowledge about bioprocess robustness. The understanding of the physiological heterogeneity may pave the way for further improvements in the lactic acid production capability of lactobacilli cultivations. The stepwise implementation of the results described here may thus result in an enhanced strain tolerance and higher viability of *L. casei* under harsh bioprocessing conditions in a high-titer lactate context.

2. Material and methods

2.1. Microorganism

Lactobacillus casei ATCC 393, obtained from the American Type Culture Collection (Virginia, USA), was maintained frozen (in 40% [v/v] glycerol at −20 °C) and subcultured on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, incubated for 48 h at 30 °C.

2.2. Yoghurt whey preparation

Whey from expired-date yoghurts was obtained by heat treatment (thermal coagulation) in order to remove fat and major proteins. The remaining fat and proteins were subsequently withdrawn from this liquid fraction by centrifugation at 12,000*g* for 10 min at 4 °C. The resulting yoghurt whey was 1-fold diluted with distilled water (1:1) and adjusted to pH 6.5 (by adding NaOH 1 M) prior to sterilization using a tangential microfiltration device equipped with a PVDF membrane-cassette of 0.22 μm pore size (Millipore, Massachusetts, USA).

2.3. Culture conditions and fermentation experiments

After reactivation on MRS under microaerophilic conditions at 30 °C for 16 h, 10% (v/v) of this culture was used to inoculate 90 mL of yoghurt whey without pH control. Precultures were incubated at 37 °C for 20 h and placed at 100 rpm in an orbital shaker (Flyer Aerotron, Infors HT, Switzerland), then used to inoculate batch fermentations. Tests for pH-control fermentations were performed in a 2-L bioreactor (Bioflo 110, New Brunswick Scientific Co. Inc., NJ, USA) with a final working volume of 1 L, controlling pH at 6.5 by computer-controlled peristaltic pumps adding 3 M NaOH. Bioreactor cultivation was conducted at an inoculation level of 10% (v/v), and agitation rate of 50 rpm, at 37 °C.

Uncontrolled-pH batch fermentation was performed with a final working volume of 1 L at 37 °C in an orbital shaker at 100 rpm. In this case, the preculture stage was supplemented with 2.5 g/L of yeast extract to achieve better fermentation performance under acidic conditions [3]. Samples from fermentation broths were withdrawn periodically to determine bacterial growth and physiological status by FCM, while cell-free supernatants were stored (at −20 °C) until further analysis. Fermentations were carried out in duplicate as independent experiments.

2.4. Staining procedures

Cells were stained with a mixture of two fluorescent dyes, ChemChrome V6 (CV6, Chemunex, France) and Propidium Iodide (PI, Molecular Probes), as previously reported by Quirós et al. [19] with slight modifications. Whereas CV6 stains viable cells which are featured by enzymatic activity, PI is a fluorescent nucleic acid dye which stains damaged and dead cells. Samples from cultures were harvested by centrifugation at 16,000*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 μm), and adjusted to cellular densities corresponding approximately to 4×10^5 cells/mL in the same buffer. For viability assessment, 200 μL of cellular suspension were added to the different staining solutions previously prepared. CV6 stock solutions were prepared by dilution (1:10) in sterile distilled water (0.22 μm filtered), adding 8 μL to the cell suspension which was incubated for 15 min in the dark at room temperature. PI stock solution (1 mg/mL solution in water) was diluted in sterile distilled water and then added to the cell suspension at a final concentration of 5.25 μg/mL. This mixture was incubated for 30 min under the same conditions as CV6 staining. Gates in the FCM dot plots were established according to control samples: heat-killed cells treated at 90 °C for 30 min and immediately cooled on ice; early exponentially growing cells; and mixtures containing exponential and heat-killed cells (1:1). Unstained samples were additionally used as controls.

2.5. Multiparametric flow cytometry

FCM measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with 488- and 633-nm excitation light source from an argon ion laser. Green fluorescence from CV6-stained cells was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4000 events/s). For ratiometric count, fluorescent microspheres (Perfect Count, Cytognos, Spain) were used as internal standards in each sample. For each analysis 2,000 microspheres were acquired at low flow rate setting. Both data acquisition and analysis were carried out using Cytomics RXP software (Beckman Coulter).

2.6. Analytical determinations

Bacterial growth was measured spectrophotometrically as optical density at 660 nm (Shimazdu, UV 1203 model) after centrifugation of culture samples at 16,000*g* for 5 min. Optical density data was converted to cell dry weight (expressed in g/L) using the corresponding calibration curve previously obtained.

Lactic acid, lactose, glucose and sucrose content of cell-free culture samples were measured by high performance liquid chromatography (HPLC) as previously described [20]. The liquid chromatographic system (Agilent 1200, Agilent Technologies Inc., California, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., California, USA) coupled to a refractive index detector. Sulphuric acid (0.450 mM, pH 3.1) was employed as the mobile phase at a flow rate of 0.3 mL/min with the column temperature set at 75 °C. Data acquisition and analysis were performed with ChemStation software (Agilent).

2.7. Segregated kinetic model based on flow cytometric data for pH-controlled conditions

A segregated kinetic model involving metabolically active, damaged and dead cells was developed according to the proposed scheme (Fig. 1) in which compromised cells (damaged and dead

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