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Multi-objective optimization of frozen and freeze-dried *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 production via the modification of fermentation conditions

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Abstract

Aim: This study investigates the individual and combined effects of fermentation parameters for improving cell biomass productivity and the resistance to freezing, freeze-drying and freeze-dried storage of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1.

Methods and Results: Cells were cultivated at different temperatures (42°C and 37°C), pHs (5.8 and 4.8) and harvested at various growth phases (mid-exponential, deceleration and stationary growth phases). Specific acidifying activity was determined after fermentation, freezing, freeze-drying and freeze-dried storage. Multiple regression analyses were performed to identify the effects of fermentation parameters on the specific acidifying activity losses and to generate the corresponding 3D response surfaces. A multi-objective decision approach was applied to optimize biomass productivity and specific acidifying activity. The temperature positively influenced biomass productivity, whereas low pH during

growth reduced the loss of specific acidifying activity after freezing and freeze-drying. Furthermore, freeze-drying resistance was favored by increased harvest time.

Conclusions: Productivity, freezing and freeze-drying resistances of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 were differentially affected by the fermentation parameters studied. There was no single fermentation condition that improved both productivity and resistance to freezing and freeze-drying. Thus, Pareto fronts were helpful to optimize productivity and resistance, when cells were grown at 42°C, pH 4.8, and harvested at the deceleration phase.

Significance and Impact of the study: Setting up predictive models for optimizing fermentation conditions is an efficient approach to guiding starter production and modulating the resistance to freezing and freeze-drying.

Keywords: *Lactobacillus*; fermentation; freezing; freeze-drying; multiple regression analysis; response surface method, multi-objective optimization, Pareto-front.

Introduction

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) is a lactic acid bacteria (LAB) that undoubtedly presents an economic interest, given its worldwide application in yogurt production (Van De Guchte *et al*. 2006). Additionally, some strains of *L. bulgaricus* have been used as probiotic cultures, exerting health benefits (Jain *et al*. 2004; Guha *et al*. 2019).

L. bulgaricus, as well as many lactic acid bacteria, are commercialized as ready-to-be-used products by food companies. The manufacturing process consists of producing bacterial concentrates via fermentation, followed by a centrifugation step. Stabilization techniques are then applied to increase the shelf life of highly concentrated bacteria.

Freezing and freeze-drying are the most currently used techniques for stabilizing lactic acid bacteria. Freeze-drying offers the advantages of low storage, transportation costs and easy handling compared to freezing. In addition, it has been recently demonstrated that for the long-term preservation of *L. bulgaricus*, the freeze-dried form is more eco-friendly than freezing (Pénicaud *et al*. 2018).

Freezing induces ice crystal formation and cryo-concentration of solutes with bacteria packed into the frozen concentrated matrix. Cryo-concentration leads to osmotic stress and cell dehydration, considered to be the primary source of cryoinjury of *L. bulgaricus* (Meneghel *et al*. 2017). Freeze-drying involves freezing the aqueous solution containing bacterial cells, followed by primary drying to sublimate ice and secondary drying to remove bound water by desorption. The removal of bound water may cause irreversible changes in the physical state of cell membrane lipids and the structure of sensitive proteins (Brennan *et al*. 1986; Castro *et al*. 1997; Romano *et al*. 2021).

Considering the harsh conditions to which these bacteria are subjected, their stabilization processes and subsequent storage provoke environmental stresses, leading to the loss of essential cell functionalities. Some strategies have been applied to limit cellular injuries and improve functional recovery, such as (i) adding protective molecules (Fonseca *et al*. 2003; Carvalho *et al*. 2003; Otero *et al*. 2007; Juárez-Tomás *et al*. 2009; Fonseca *et al*. 2016), (ii) controlling stabilization operating conditions (Fonseca *et al*. 2001; Zayed and Roos 2004; Fonseca *et al*. 2006; Kurtmann *et al*. 2009; Aragón-Rojas *et al*. 2019; Verlhac *et al*. 2020), and (iii) modulating fermentation parameters.

Modifying the fermentation conditions can induce cell-active responses to cope with the environmental stresses during the stabilization processes. Table S1 summarizes the studies that report biological adaptation following cell growth carried out in a bioreactor for bacteria of the *Lactobacillus* genus (including *L. bulgaricus*) and other LAB. The first observation is that, despite their large industrial interest, only two studies have focused on the freezing process of *L. bulgaricus* (Fonseca *et al*. 2001; Rault *et al*. 2010), and four on freeze-drying (Champagne *et al*. 1991; Li *et al*. 2009a; Li *et al*. 2012; Shao *et al*. 2014). Studies on storage stability in the freeze-dried state are also scarce (Zotta *et al*. 2013; Velly *et al*. 2014; Hansen *et al*. 2015). The second observation highlights that the fermentation conditions that induce resistant cells to stabilization processes and storage often differ from those that favor LAB growth. Thus, it is not possible to get both fair productivity and good resistance. The multi-objective optimization, like Pareto fronts, is an approach dedicated to modelling and fine-tuning the parameters in such a situation (Khorram *et al*. 2014). Our objective here was to apply this concept to optimize the fermentation parameters that produce sufficient biomass and provide fair resistance to

stabilization processes. We selected a lactic acid bacterium: *L. bulgaricus* CFL1, which belongs to a LAB species widely used in fermented dairy products, such as yoghurt. This strain represents a typical bacterium model for sensitive *L. bulgaricus* strain to freezing (Fonseca *et al*. 2000; Fonseca *et al*. 2001; Meneghel *et al*. 2017). The experimental conditions were chosen in order to (i) modulate biomass production and (ii) resistance to freezing, freeze-drying, and storage.

Data were first analyzed through a response surface methodology to identify the effect of fermentation parameters (pH, temperature and harvest time) on the functional properties of cells (acidifying activity and bacterial culturability) at different steps of the production process: fermentation, freezing, freeze-drying and freeze-dried storage. Then, for the first time, a multi-objective optimization, including bacterial cell resistance and productivity was carried out to define the best possible compromise between conflicting criteria.

Materials and methods

The experimental approach for the production and stabilization of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 (*L. bulgaricus* CFL1) cell concentrates as well as the main parameters investigated in this study are summarized in Fig. 1. All measurements were performed on at least three independent bacterial cultures. The steps corresponding to Fig. 1 are explained in the sections below.

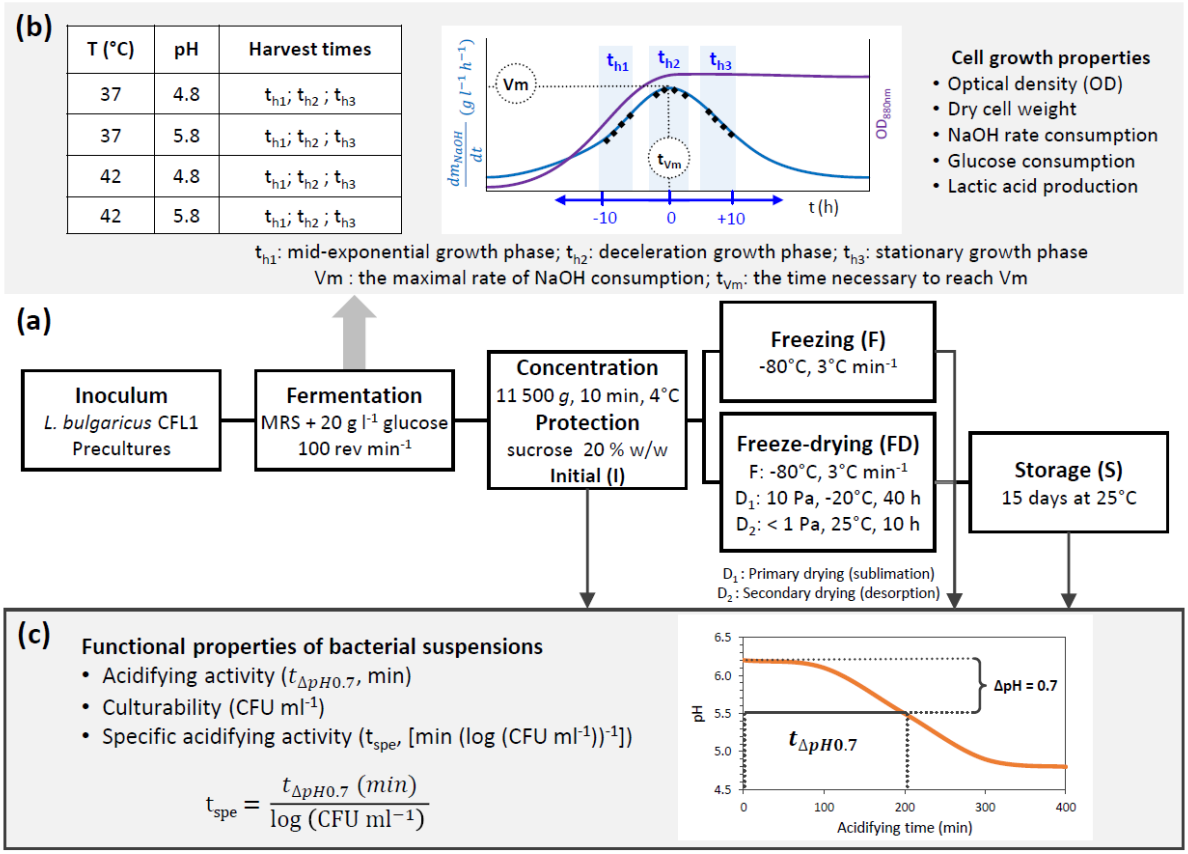


Figure 1 Diagram of the experimental approach applied to assess the effect of fermentation parameters (pH, temperature and harvest time) on the loss of specific acidifying activity of *L. bulgaricus* CFL1 after freezing, freeze-drying and freeze-dried storage: (a) Starter production process; (b) Fermentation parameters: pH and temperature values, harvest times and cell growth properties measured throughout fermentation; (c) Functional properties of *L. bulgaricus* CFL1 cells measured after concentration-protection (initial, I), freezing (F), freeze-drying (FD) and freeze-dried storage (S).

Starter production and stabilization processes

Strain and inoculum preparation

L. bulgaricus CFL1 (CIRM-BIA; Rennes, France) was used in this study. Bacterial cells were stored at -80°C in Man, Rogosa and Sharpe broth (MRS, Biokar, Diagnostics, Beauvais, France), supplemented with 15% (w/w) glycerol (VWR, Leuven, Belgium). Before inoculation of the bioreactor, inocula were first precultured twice at 42°C in 60 ml of sterilized MRS medium (121°C, 20 min) without agitation. In the first preculture, 60 ml of sterilized medium was inoculated with 300 µl of stock culture and incubated for 12 hours until reaching the stationary phase, corresponding to an optical density at 600 nm ($OD_{600\ nm}$) of approximately 4.5. Then, 1.5 ml of the resulting first preculture was used to inoculate the

second preculture, in order to begin with an optical density ($OD_{600\ nm}$) of 0.1. This second preculture contained the same amount of medium (60 ml) and was incubated for 10 hours until reaching the stationary growth phase ($OD_{600\ nm}$ ~ 5.5). The whole resulting second preculture (58-60 ml) was used to inoculate a 5.0 l bioreactor (Fig. 1a).

Fermentation

The culture medium was composed of MRS broth (Biokar, Diagnostics, Beauvais, France) supplemented with 20 g l^{-1} D-glucose (VWR, Leuven, Belgium). Culture medium was supplemented to avoid starvation stress caused by the depletion of the carbon sourced after reaching the stationary growth phase.

After filtering through a 0.22 µm polyethersulfone filter (Stericap PLUS, Millipore Express®, Merck KGaA, Darmstadt, Germany), four liters of medium were introduced into a 4.0 l working volume bioreactor (Sartorius, Biostat®A plus, Melsungen, Germany). The inoculation was performed at an initial optical density of 0.1 ($OD_{600\ nm}$), corresponding to a concentration of approximately 4×10^4 CFU ml^{-1}. Stirring was set at 100 rev min^{-1} to ensure homogenization.

The temperature and pH were set at different values according to the experimental design (Fig. 1b). These levels of the fermentation parameters were chosen to create a reasonable range of moderately stressful conditions that would induce changes in the functional properties while still permitting adequate cell growth. They have also been set according to previous studies on *L. bulgaricus* strains (Streit *et al*. 2007; Rault *et al*. 2010). For example, pH was set at either 4.8 or 5.8 while temperature was fixed at either 37°C or 42°C. One of the values of pH and temperature is respectively below the optimal conditions for growth. The optimal conditions to enhance growth was reported about pH 5.2 to 6.0 and at 40°C for different *L. bulgaricus* strains (Béal *et al.* 1989; Grobben *et al*. 1995; Burgos-Rubio *et al*. 2000; Abbasalizadeh *et al*. 2015; Aghababaie *et al*. 2015).

The pH and temperature were adjusted before inoculation and controlled throughout the fermentation. The pH was controlled by the automatic addition of 4.25 mol l^{-1} NaOH solution (VWR, Leuven, Belgium) to the bioreactor. The addition of NaOH solution was monitored throughout fermentation using SartoriusBioPAT software (SARTORIUS®, Göttingen, Germany), allowing the calculation of the

consumption rate (dm_{NaOH}/dt, in g l^{-1} of culture medium h^{-1}). The time, after inoculation, (t_{Vm}, in h) necessary to reach the maximal rate of NaOH consumption (Vm, in g l^{-1} h^{-1}), corresponding to the maximal acidification rate, was considered as the reference time (0 h) for quantitatively defining the harvest times (t_{hi}) and identifying the different bacterial growth phases (Fig. 1b).

For each couple of fermentation temperatures and pH, cell samples were taken from the bioreactor at three ranges of harvest times, corresponding to three growth phases:

(i) t_{h1}, the mid-exponential growth phase (-10 to -1.0 h from t_{Vm})

(ii) t_{h2}, the deceleration growth phase (-1.0 to +2.0 h from t_{Vm})

(iii) t_{h3}, the stationary growth phase (+2.0 to +10 h from t_{Vm})

Concentration, protection and freezing

Harvested cell suspensions were concentrated by centrifugation (Avanti® J-E centrifuge; Beckman Coulter, Fullerton, CA, USA) at 11 500 ***g*** for 10 min at 4°C. The resulting cell pellets were then re-suspended in the protective solution at a ratio of 1:2 (1 g of concentrated cells for 2 g of the protective solution) before freezing and freeze-drying (Fig. 1a). The protective solution was composed of 20% (w/w) sucrose (VWR, Leuven, Belgium) and was previously sterilized at 121°C for 20 min. One milliliter of the protected cell suspensions was distributed in cryo-tubes (Sarstedt, Nümbrecht, Germany) for freezing experiments and in five-milliliter vials (Verretubex, Nogent-Le-Roi, France) for freeze-drying trials. All samples were frozen at -80°C (freezing rate = 3°C min^{-1}).

Freeze-drying and freeze-dried storage

Five-milliliter vials containing one milliliter of frozen samples (-80°C, 3°C min^{-1}) were transferred to a pre-cooled shelf at -50°C in a REVO pilot-scale freeze-dryer (Millrock Technology, Kingston, NY, USA). After a holding step of 1.5 h at -50°C, the chamber pressure was decreased to 10 Pa, and the shelf temperature was increased from -50°C to -20°C at a heating rate of 0.25°C min^{-1} to initiate sublimation. The end of ice sublimation (ensuring the absence of remaining ice inside the product) was assessed by comparative pressure measurement (Pirani gauge vs capacitance manometer) (Passot *et al*. 2009). After 40 h of sublimation (primary drying), the shelf temperature was increased to 25°C at a heating rate of 0.25°C min^{-1}. After 10 h of desorption (secondary drying step), the vacuum was broken by injecting air

into the drying chamber. The vials were then taken out of the freeze-dryer, manually capped by inserting a rubber stopper, and packed in multi-layer aluminum bags. The bags were hermetically closed using a vacuum sealer (Bernhardt, Wimille, France). For freeze-dried samples, bags were stored at -80°C for two days until measurements of the functional properties and the residual water content. For freeze-dried storage samples bags were immediately stored at 25°C for 15 days.

Cell growth and metabolite production during fermentation

Cell growth kinetics and biomass productivity measurements

Cell growth was monitored by an infrared probe (Excell 210, CellD, Roquemaure, France) inserted into the bioreactor that continuously measured absorbance at 880 nm (data acquisition every 5 minutes) (Fig. S1a). The specific growth rate (μ, in h^{-1}) and lag growth phase duration (*lag* in h) were calculated according to the modified Gompertz equation (Zwietering *et al*. 1990), (Eqn 1):

$$y = \ln\left(\frac{OD}{OD_{\,at\ inoculation}}\right) = A\exp\left\{-\exp\left[\frac{\mu \cdot \exp(1)}{A}(lag - t) + 1\right]\right\} \qquad (1)$$

where OD is the absorbance value at 880 or 600 nm, *A* is the asymptote value of the growth curve (absorbance value), μ is the specific growth rate in h^{-1}, and *t* and *lag* are the time and the lag growth phase duration, respectively, in hours.

A correlation was established between the measurement of absorbance at 880 and at 600 nm (Fig. S1c). The absorbance values measured at 880 nm were thus converted to absorbance values at 600 nm (Fig. S1d), and the kinetic parameters at 880 and 600 nm were calculated using Eqn 1.

The dry cell weight in the bioreactor (DCW, in g l^{-1}) was determined by filtering 10 ml of culture sample through 0.20 µm hydrophilic polyethersulfone (PES). The filters (Supor®, PALL Biotech, Saint-Germain-en-Laye, France) were previously dried at 80°C for 24 h. Then, filters containing biomass samples were dried under the same conditions (80°C, 24 h). The measurements were carried out in triplicate just after inoculation of the bioreactor (t = 0) and at each harvest time.

Biomass productivity (P in g l^{-1} h^{-1}) was calculated according to the following equation:

$$P = \frac{DCW\ (at\ t = t_{hi}) - DCW\ (\ t = 0\ h_{at\ inoculation})}{t_{hi}} \qquad (2)$$

Where t_{hi} corresponds to each harvest time.

Substrate and metabolite analysis

For each harvested sample, glucose and lactic acid concentrations were quantified in duplicate using high-performance liquid chromatography (HPLC) (Waters Associates, Millipore; Molsheim, France), coupled with a Refractive Index detector (Waters, Milford, MA, USA). Before HPLC analysis, each sample was centrifuged at 16 000 *g* for 10 min at 4°C, and the supernatant was filtered through a 0.20 µm polytetrafluoroethylene (PTFE) filter (CHROMAFIL® Xtra PA, Düren, Germany). Analyses were made using a cation exchange column (Aminex Ion Exclusion HPX-87 300 X 7.8 mm; Biorad, Richmond, VA, USA) at 35°C. The mobile phase was 0.005 mol l^{-1} H_2SO_4, and the flow rate was set at 0.6 ml min^{-1} (LC-6A pump; Shimadzu, Courtaboeuf, France).

Functional properties of starters

The functional properties considered for this study were the acidifying activity and culturability of *L. bulgaricus* CFL1 cells. These properties were measured at different process steps: after cells were concentrated and protected (initial), after freezing, freeze-drying (stabilization process), and 15 days of freeze-dried storage at 25°C. (Fig. 1c).

Frozen cell samples were thawed at 42°C for 5 min in a water bath before measuring the acidifying activity and culturability. Freeze-dried samples were first rehydrated in 1 ml of skim milk solution (100 g l^{-1}, EPI-Ingredient, Ancenis, France) at 42°C and stirred for 5 min at room temperature. The skim milk solution was previously heat-treated at 110°C for 20 min,

Acidifying activity

The Cinac system (AMS Alliance, Frépillon, France) was used to evaluate the acidifying activity of the bacterial suspensions. The acidifying activity was measured in triplicate at 42°C in 100 g l^{-1} skim milk solution (EPI-Ingredient, Ancenis, France). Reconstituted skim milk solution was heat-treated at 110°C for 20 min in 150 ml flasks containing 100 ml filled volume. Each flask was inoculated with 100 µl of the bacterial suspension. The pH was continuously measured by the Cinac system and used to determine the time necessary to obtain a decrease of 0.7 pH units ($t_{\Delta pH0.7}$, in min). The descriptor, $t_{\Delta pH0.7}$, was used to characterize the acidifying activity of bacterial suspensions. The lower the value of the $t_{\Delta pH0.7}$ descriptor was, the greater the acidifying activity was.

Culturability

The cell concentration of bacterial suspensions was measured using the agar plate count method. Cell suspensions were serially diluted in saline water (NaCl, 9 g l^{-1}), then plated on MRS Agar (Biokar Diagnostics, Paris, France) and anaerobically incubated at 42°C for 48 h. The cell count was expressed in CFU ml^{-1}. Only plates containing between 30 and 300 colonies were considered for cell concentration calculation (in CFU ml^{-1}). The measurements of plate count were performed in triplicate.

Specific acidifying activity and loss of specific acidifying activity

The specific acidifying activity (t_{spe}), in [min (log (CFU ml^{-1}))$^{-1}$], was defined as the ratio of $t_{\Delta pH0.7}$ (min) to the corresponding log of cell concentration (CFU ml^{-1}) (Streit *et al*. 2007). Therefore, t_{spe} provides a meaningful measurement of the functional properties of lactic acid bacteria including acidifying activity and culturability.

The specific acidifying activity was thus determined after fermentation, concentration and protection of bacterial cells (initial, t_{spe} I), after freezing (t_{spe} F), after freeze-drying (t_{spe} FD), and after 15 days of storage at 25°C (t_{spe} S).

The determination of t_{spe} loss (dt_{spe}) after each stabilization process and freeze-dried storage was calculated using the following equations (Eqn 3-5):

$$dt_{spe}\ F_{\textit{(Freezing)}} = t_{spe\ \textit{after Freezing}} - t_{spe}\ I_{\textit{(Initial specific acidifying activity)}} \quad (3)$$

$$dt_{spe}\ FD_{\textit{(Freeze-Drying)}} = t_{spe\ \textit{after Freeze-Drying}} - t_{spe}\ I_{\textit{(Initial specific acidifying activity)}} \quad (4)$$

$$dt_{spe}\ S_{\text{(freeze-dried }\textit{Storage)}} = t_{spe\ \textit{after freeze-dried Storage}} - t_{spe\ \textit{after Freeze-Drying}} \quad (5)$$

Water content and glass transition temperature measurements

The water content of freeze-dried samples was measured by the Karl Fisher titration method using a Metrohom KF 756 apparatus (Herisau, Switzerland). At least 20 mg of powder was mixed with 2 ml of dried methanol and titrated with Riedel-de Haen reagent (Seelze, Germany) until the endpoint was reached.

Glass transition temperature (Tg) was performed as described by Velly *et al*. (2015). Briefly, Tg measurements were carried out using a power compensation Differential Scanning Calorimetry (DSC) (Pyris 1, PerkinElmer LLC; Norwalk, CT, USA) equipped with a mechanical cooling system

(Intracooler 1P, PerkinElmer). Temperature calibration was done using cyclohexane and indium (melting points at 6.5 and 156.6°C, respectively). Approximately 15 mg of each freeze-dried sample was placed in 50 µL PerkinElmer DSC sealed aluminum pans, and an empty pan was used as a reference. Linear cooling and heating rates of 10°C min^{-1} were applied. The Tg of the freeze-dried samples was determined as the midpoint temperature of the heat flow step associated with glass transition with respect to the ASTM Standard Method, E1356-91. Results were obtained from at least three replicates.

Statistical analysis

Experimental design

A full factorial design (2 × 2 × 3) was used to investigate the effect of fermentation parameters (pH, temperature and harvest time) on different responses: (i) biomass productivity (P); (ii) initial specific acidifying activity after fermentation when cells were concentrated and protected (t_{spe} I), and (iii) the loss of specific acidifying activity after freezing (dt_{spe} F), freeze-drying (dt_{spe} FD) and freeze-dried storage (dt_{spe} S).

Stepwise descending multiple regression analyses

Two independent variables were coded at a low level (-1) and a high level (+1) for pH and temperature. The harvest time was recalculated for each fermentation trial by taking the time necessary to reach the maximal rate of NaOH consumption (t_{Vm} in hours) as the reference time, corresponding to 0 h on a new time scale. The range from -10 h (low level, coded as -1) to +10 h (high level, coded as +1) was considered to code this variable (Fig. 1b).

Stepwise descending multiple regression analyses were performed to quantify the effects of three independent variables (coded pH (X_1), coded temperature (X_2), and coded harvest time (X_3)) on each response variable (P, t_{spe} I, dt_{spe} F, dt_{spe} FD, dt_{spe} S) using MATLAB® R2014b software (The MathWorks Inc, Natick, MA, USA) equipped with the Statistics Toolbox.

Measurement units are different between the three culture parameters (pH in pH units, temperature in °C, and harvest time in hours on a new time scale regarding t_{Vm}). To rank the influence of the culture variables on the response variables, coded variables were used in the stepwise descending regression analyses, thus setting the coefficients to the same scale.

The applied regression model was a second-order polynomial with interactions of the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (6)$$

where β_0, β_i, β_{ii} and β_{ij} are the intercept, linear, quadratic and interaction coefficients, respectively. X_1, X_2 and X_3 are fermentation pH, temperature and harvest time, respectively.

Stepwise descending multiple regression iteratively removed the parameters not significantly different from zero at *P*-value ≤ 0.05 from the model.

The adjusted coefficient of determination (Adjusted R^2) assessed the adequacy of the model. R^2 measures the percentage of total data variance explained by each model. The criterion for accepting a mathematical model was to exhibit an $R^2 \geq 70\%$, which explains 70% of the response (dependent) variable variability. Response surface plots were generated from the fitted polynomial equations (Eqn 6) in order to visualize the relationships between the responses and independent variables.

Multiple regression analysis allowed us to predict the specific acidifying activity (t_{spe}) after freezing and freeze-drying within the experimental domain, as well as to calculate a Pareto front by a multi-objective numerical optimization technique (NSGA II, MATLAB® R2014b software). In the Pareto front, each point corresponded to one fermentation condition and one harvest time within the experimental design (Temperature, pH, t_{hi}). These points were plotted according to biomass productivity (X-axis) and t_{spe} (Y-axis). This technique was applied to determine the set of fermentation conditions that lead to the best possible compromises between biomass productivity and the minor loss of t_{spe} after the stabilization processes.

Complementary statistical analysis

The non-parametric Kruskal-Wallis test and the post-hoc Conover Iman test were performed using XLSTAT 2020.5 (Addinsoft, Paris, France) to evaluate the effect of each independent variable (pH, temperature and harvest time) on the growth kinetic parameters (lag growth phase duration, specific growth rate, maximal rate of NaOH consumption (Vm), time to reach Vm (t_{Vm}), concentrations of lactic acid and residual glucose), as well as on the functional properties of bacterial suspensions (acidifying activity, culturability and specific acidifying activity). Harvest times were grouped according to the

previously defined three ranges (t_{h1}, t_{h2}, t_{h3}). A significance level of 95% (*P*-value ≤ 0.05) was considered. Such complementary tests were particularly useful to analyze results when multiple regression models were not adequate to describe the effect of fermentation conditions on response variables (functional properties of bacterial suspensions).

Results

Fermentation kinetics and biomass productivity

Bacterial growth (optical density) and the acidification rate (NaOH consumption rate) curves of *L. bulgaricus* CFL1 are presented in the Supplementary Information for the four fermentation conditions of pH and temperature (Figs. S1a and S1b, respectively). The curves correspond to medians and the associated interquartile ranges (IQR) of at least three biological replicates per condition. Culture reproducibility was considered satisfactory since the IQR/median ratio was lower than 30% for the fermentation conditions evaluated.

The four parameters describing the growth kinetics that were calculated using the curves in Fig. S1 (a, b, d) are summarized in Table S2 (Supplementary Information). These parameters are the lag growth phase duration (lag), the specific growth rate (μ), the maximal value of the NaOH consumption rate (Vm), and the time associated with the Vm value (t_{Vm}, time necessary in hours to reach Vm). The total lactic acid (LA) production and the residual glucose concentration (gluc) measured at the stationary growth phase harvest (t_{h3}, the latest harvest time) are also included in Table S2.

Regardless of the fermentation conditions, HPLC measurements confirmed that there was still glucose content in the fermentation medium for the latest harvest time. Therefore, there was no supplementary stress due to carbon source depletion.

Concerning the lag growth phase durations, the lag growth phase duration in the bioreactor depended on the temperature used in the precultures. The shortest ones were observed for fermentations at 42°C (pH 5.8: 0.28 h and pH 4.8: 1.31 h), while the longest were observed for fermentations at 37°C (pH 5.8: 1.33 h and pH 4.8: 1.95 h).

Specific growth rates (μ), as well as the maximal NaOH consumption rates (Vm) were about twice as high at 42°C as at 37°C. The time to reach Vm (t_{Vm}) was about 10 hours less at 42°C (t_{Vm} = 16.5 and 16.8 h) than the t_{Vm} values observed at 37°C (t_{Vm} = 24.4 and 28.8 h). The statistical analysis confirmed the temperature effect (*P*-value ≤ 0.05) for most of the calculated growth parameters (lag, μ, Vm, t_{Vm}, and LA) and highlighted an increase in bacterial growth and lactic acid production with temperature. In contrast, when two fermentation conditions at the same temperatures were compared, the pH appeared to have no significant effect on *L. bulgaricus* CFL1 for the majority of the kinetic growth parameters (μ, Vm, LA, gluc of Table S2).

The final amount of biomass is another crucial variable to be considered in LAB production. Biomass productivity (P, g l^{-1} h^{-1}) was thus calculated (Eqn 2) for all the fermentation conditions studied at each harvest time. The experimental values of productivity varied between 0.15 and 0.45 g l^{-1} h^{-1}. A stepwise multiple regression analysis was performed to quantify the linear (X_i), interactive ($X_i \times X_j$) and quadratic (X_i^2) effects of the three independent fermentation variables (pH, temperature and harvest time) on biomass productivity. The coefficients (β_i) of the multiple regression (Eqn 6) and the *P*-value of each model variable are presented in Table 1. This model also explains 84% of biomass productivity variability according to the coefficient of determination (R^2) of the multiple regression, thus satisfactorily representing this response variable. Furthermore, in Fig. S2, the predicted values calculated by the multiple regression model vs. experimental values were plotted, showing the accuracy of the model.

The most significant effect on the response variable (biomass productivity in Table 1) was found at a high absolute value of β_i coefficients. The biomass productivity (P, in g l^{-1} h^{-1}) was mainly influenced by the linear effect of the temperature and the quadratic effect of harvest time, followed by the pH and the interactive effect of pH and temperature.

The response surfaces generated with the biomass productivity (P) model (Fig. 2) show the conjugated effect of temperature and harvest time on the biomass productivity at pH 5.8 (Fig. 2a) and pH 4.8 (Fig. 2b). The values of the harvest times were normalized by considering the reference (t_h = 0 in Fig. 2) at the moment of the maximal NaOH consumption rate (t_{Vm}) (see Fig. 1b).

Table 1 Multiple regression analysis of the biomass productivity (P, in g l^{-1} h^{-1}) of *L. bulgaricus* CFL1. Cells were harvested at increasing harvest times during fermentations carried out at different pHs and temperatures. (X_1: fermentation pH; X_2: fermentation temperature (T); X_3: harvest time) (Eqn 6). Only the independent variables with a *P*-value lower than 0.05 were retained by the stepwise regression analysis.

Term	Estimated Coefficient (β_i)	95% confidence interval		*P*-value
		Min	Max	
Intercept	0.29 (β_0)	0.27	0.30	3.7×10^{-51}
X_1 (pH)	0.02 (β_1)	0.01	0.03	2.3×10^{-03}
X_2 (T)	0.07 (β_2)	0.06	0.08	6.1×10^{-21}
X_3^2 (harvest time^2)	-0.06 (β_3)	-0.10	-0.03	1.0×10^{-03}
X_1 X_2 (pH × T)	0.01 (β_{12})	0.00	0.02	1.3×10^{-02}
Adjusted R^2 = 84%; RMSE = 0.04 g l^{-1} h^{-1}				

R^2: coefficient of determination; RMSE: standard deviation of the residuals

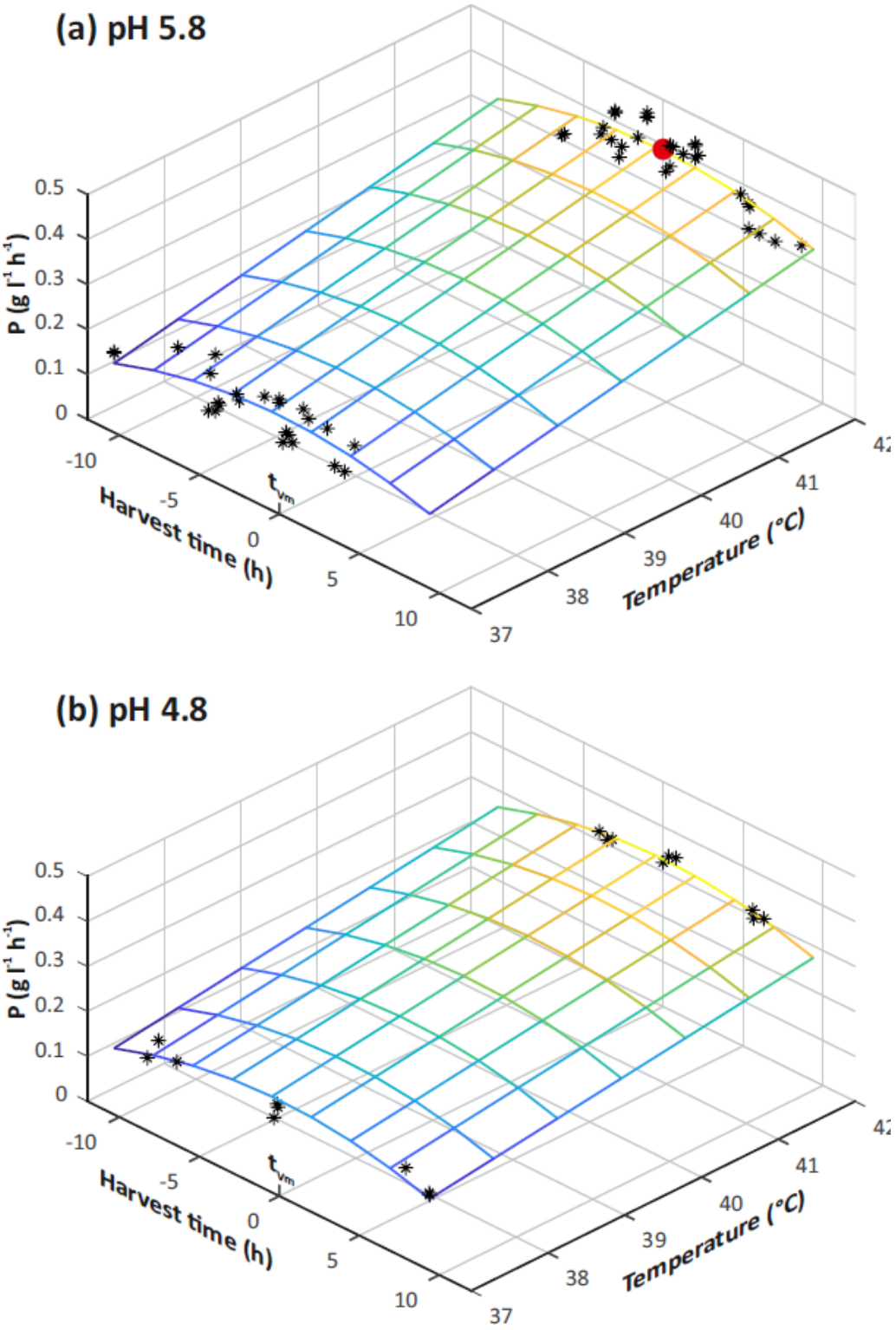


Figure 2 Response surface representations of the effect of fermentation harvest time and temperature on the biomass productivity (P, in g l^{-1} h^{-1}) of *L. bulgaricus* CFL1 produced at (a) pH 5.8, and (b) pH 4.8. Asterisks represent the experimental data points used in the model at the given pH. The red dot on the mesh of (a) pH 5.8, represents the maximal biomass productivity predicted by the biomass productivity multiple regression model. t_{Vm}: the time necessary to reach the maximal rate of the NaOH consumption.

A high fermentation temperature (42°C) had a positive effect on biomass productivity ($\beta_2 = 0.07$). Increasing the culture temperature from 37°C to 42°C resulted in an increase in productivity of approximately 70% at both pHs (from 0.24 to 0.41 g l^{-1} h^{-1} at pH 5.8, and from 0.20 to 0.34 g l^{-1} h^{-1} at pH 4.8), for cells harvested at the deceleration growth phase (t_{h2}, -1.0 to 2.0 hours). For this same harvest time and when cells were cultured at 42°C, the biomass productivity achieved at pH 5.8 was 0.07 g l^{-1} h^{-1} higher than at pH 4.8 (from 0.34 to 0.41 g l^{-1} h^{-1}). This result revealed the positive pH effect ($\beta_1 = 0.02$). At 37°C, the pH effect was less pronounced; a biomass productivity increase of 0.04 g l^{-1} h^{-1} was observed at pH 5.8 compared to pH 4.8 (from 0.20 to 0.24 g l^{-1} h^{-1}), thus illustrating the interaction effect between pH and temperature revealed by the multiple regression analysis ($\beta_{12} = 0.01$).

The negative quadratic effect of harvest time ($\beta_3 = -0.06$) explains the concave shapes of the response surfaces at both pH values studied (Fig. 2). Cells harvested at the deceleration growth phase exhibited the highest productivity values regardless of pH and temperature. The multiple regression model predicted a maximal biomass productivity of 0.39 g l^{-1} h^{-1} at 42°C, pH 5.8, and cells harvested at 0.4 h after t_{Vm}. This condition is represented by a red dot in Fig. 2a.

Initial functional properties of *L. bulgaricus* CFL1

The acidifying activity and the number of culturable cells are the main functional and technological properties of lactic acid bacteria and were measured for all fermentation conditions examined in this study. The specific acidifying activity (t_{spe}) represents the ratio of the acidifying activity ($t_{\Delta pH0.7}$, in min) and the log of the concentrated-protected bacterial suspension (culturability in CFU ml^{-1}). Thus, it was possible to combine two experimental measurements in a single descriptor to characterize the biological activity of *L. bulgaricus* before the stabilization processes (initial, t_{spe} I) (Streit *et al*. 2007).

Stepwise multiple regression analysis was performed to determine the effect of the fermentation parameters (pH, temperature and harvest time) on t_{spe} I (Table S3) and predict t_{spe} I within the fermentation conditions studied. However, the poor coefficient of determination obtained ($R^2 = 51\%$) limited the analysis of this response variable by its corresponding multiple regression model. This low coefficient of determination value suggests that the factors considered in the model explain a small part of the experimental data variations (t_{spe} I in this case).

Therefore, boxplot representations and statistical analyses were performed. For the sake of clarity, the harvest times were grouped into three categories (t_{h1}, t_{h2}, and t_{h3}) and, the effect of fermentation parameters on t_{spe} I (Fig. S3), $t_{\Delta pH0.7}$ and culturability (Fig. S4) were analyzed in these conditions.

For a given set of fermentation conditions (temperature and pH), the t_{spe} I variations (Fig. S3) were similar to those of the acidifying activity ($t_{\Delta pH0\cdot7}$) (Fig. S4a), whereas the culturability values evolved in the opposite sense (Fig. S4b). For a given fermentation condition, an increase of the t_{spe} I and $t_{\Delta pH0\cdot7}$ values (decrease of acidifying activity) is associated with a decrease in culturability.

The t_{spe} I values ranged between 20 and 45 [min (log (CFU ml^{-1})) $^{-1}$] and were affected by the three fermentation parameters studied: pH, temperature and harvest time.

The lowest t_{spe} I values (21 to 25 [min (log (CFU ml^{-1})) $^{-1}$], Fig. S3), corresponding to the highest specific acidifying activities, were observed at pH 4.8, regardless of the fermentation temperature, for cells harvested at the mid-exponential (t_{h1}) and the deceleration (t_{h2}) growth phases. Conversely, cells harvested in the stationary growth phase (t_{h3}) exhibited significant higher t_{spe} I values (*P*-value ≤ 0.05) when fermentation was performed at 42°C compared to cells cultivated at 37°C.

Effects of fermentation parameters (pH, temperature and harvest time) on the loss of specific acidifying activity after freezing, freeze-drying and freeze-dried storage

The specific acidifying activity of *L. bulgaricus* CFL1 was determined after freezing, freeze-drying and two weeks of freeze-dried storage at 25°C, to calculate the loss of specific acidifying activity (dt_{spe}) after each stabilization process and freeze-dried storage (Eqn 3, 4, and 5). Following a process, low dt_{spe} values indicate high resistance of *L. bulgaricus* CFL1.

Similarly, as biomass productivity, a stepwise multiple regression analysis allowed the quantification of the linear, quadratic and interactive effects of the three independent fermentation variables (pH, temperature and harvest time) on the loss of specific acidifying activity after freezing (dt_{spe} F), freeze-drying (dt_{spe} FD) and freeze-dried storage (dt_{spe} S). We also determined the models corresponding to the loss of acidifying activity ($dt_{\Delta pH0.7}$; Table S4) and of log CFU ml^{-1} (dlog (CFU ml^{-1}); Table S5).

The multiple regression models for dt_{spe} F and dt_{spe} FD had an acceptable value of $R^2 \geq 70\%$ (Table 2), indicating a fair representation of the loss of specific acidifying activity freezing and freeze-drying

within the experimental domain of this study. The accuracy of the model is shown in Fig. S5 in the form of a plot of predicted values calculated by the multiple regression models vs. experimental values.

A low value of the determination coefficient was observed (R^2 = 49%; Table S6) for the loss of specific acidifying activity after freeze-dried storage (dt_{spe} S). Consequently, dt_{spe} S was not adequately represented by the stepwise multiple regression analysis. Each stabilization process (freezing, freeze-drying) and freeze-dried storage is described separately in the following subsections.

Freezing

Fermentation pH (β_1 = 4.3) and the interaction between pH and temperature (β_{12} = 1.4) had the most significant effects on the loss of specific acidifying activity after freezing (Table 2, dt_{spe} F), whereas the harvest time had no influence. Response surface representations for the two pH values (Fig. 3a and Fig. 3b) illustrate the pH effect. Lower dt_{spe} F values were observed at pH 4.8 compared to pH 5.8. For instance, at 42°C, bacterial cells displayed dt_{spe} F values ten times lower at pH 4.8 than at pH 5.8, regardless of the harvest time. Consequently, bacterial cells cultivated at low pHs exhibited a significant increase in freezing resistance (low dt_{spe} F values).

The interaction between pH and temperature was remarkable. At pH 4.8, a decrease of 46% in dt_{spe} values was observed when the temperature increased from 37°C to 42°C (from 2.6 to 1.4 [min (log (CFU ml^{-1})) $^{-1}$]). Conversely, at pH 5.8, increasing the temperature resulted in a two-fold increase of dt_{spe} values (from 6.8 to 14.2 [min (log (CFU ml^{-1})) $^{-1}$]).

The minimum loss of specific acidifying activity of 0.8 [min (log (CFU ml^{-1})) $^{-1}$] after freezing was identified at 42°C, pH 4.8, regardless of harvest time, and was indicated by a succession of red dots on the surface representation (Fig. 3b).

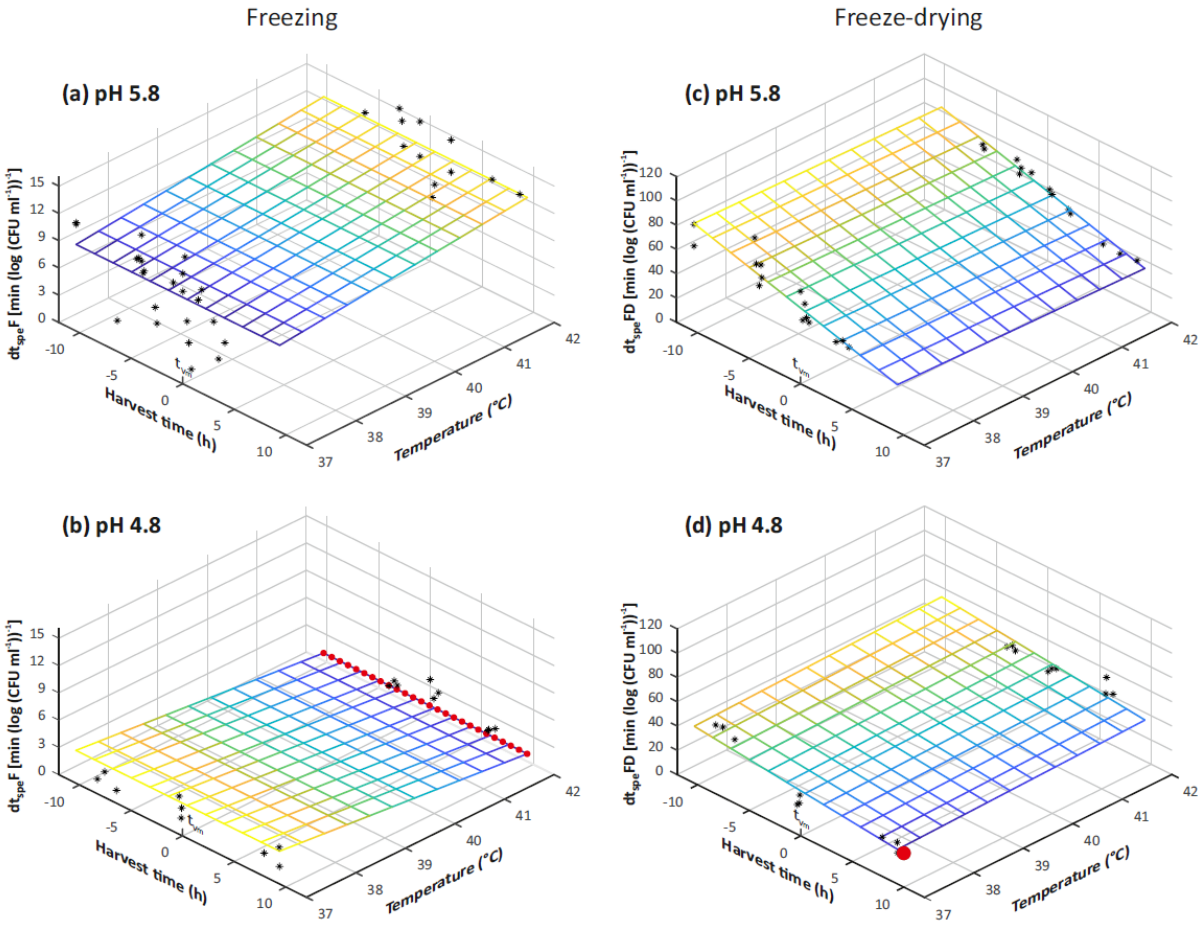


Figure 3 Response surface representations of the effect of fermentation harvest time and temperature on the loss of specific acidifying activity (dt_{spe}, in [min (log (CFU ml^{-1}))$^{-1}$]) of *L. bulgaricus* CFL1 after freezing (dt_{spe} F) at (a) pH 5.8, and (b) pH 4.8 and after freeze-drying (dt_{spe} FD) at (c) pH 5.8, and (d) pH 4.8. Asterisks represent the experimental data points used in the model at the given pH. The succession of red dots on the mesh of (b) pH 4.8 and the red dot on the mesh of (d) pH 4.8 represent the minimum loss of dt_{spe} predicted by the dt_{spe} F and the dt_{spe} FD multiple regression model, respectively. t_{Vm}: the time necessary to reach the maximal rate of the NaOH consumption.

Table 2 Multiple regression analysis of the loss of specific acidifying activity of *L. bulgaricus* CFL1 after freezing (dt_{spe} F) and freeze-drying (dt_{spe} FD). Cells were harvested at increasing harvest times during fermentations carried out at different pHs and temperatures. (X_1: fermentation pH; X_2: fermentation temperature (T); X_3: harvest time) (Eqn 6).

dt_{spe} F					dt_{spe} FD				
Term	Estimated coefficient (β_i)	95% confidence interval		*P*-value	Term	Estimated coefficient (β_i)	95% confidence interval		*P*-value
		Min	Max				Min	Max	
Intercept	6.6 (β_0)	5.8	7.4	1.2×10^{-22}	Intercept	49.9 (β_0)	47.1	52.6	5.0×10^{-33}
X_1 (pH)	4.3 (β_1)	3.6	5.1	2.7×10^{-15}	X_1 (pH)	10.0 (β_1)	7.2	12.7	5.4×10^{-09}
X_1 X_2 (pH × T)	1.4 (β_{12})	0.6	2.1	4.8×10^{-04}	X_3 (harvest time)	-17.2 (β_3)	-22.6	-11.9	8.6×10^{-08}
					X_1 X_2 (pH × T)	-2.7 (β_{12})	-5.4	-0.01	4.9×10^{-02}
					X_1 X_3 (pH × harvest time)	-8.0 (β_{13})	-13.3	-2.6	4.7×10^{-03}
Adjusted R^2 = 70% RMSE = 2.7 [min (log (CFU ml^{-1}))$^{-1}$]					Adjusted R^2 = 74% RMSE = 8.8 [min (log (CFU ml^{-1}))$^{-1}$]				

R^2: coefficient of determination; RMSE: standard deviation of the residuals.

Freeze-drying

In Table 2, harvest time had the greatest effect on dt_{spe} FD. The negative sign of the harvest time coefficient (β_3 = -17.2) indicates that low values of dt_{spe} correspond to low specific acidifying activity losses after freeze-drying, resulting from harvest time increase. Response surface representations (Fig. 3c and Fig. 3d) allow us to observed the decrease of dt_{spe} FD values when cells were harvested at increasing harvest time for both pHs (5.8 and 4.8). For example, increasing the harvest time from the mid-exponential to the stationary growth phase (from -6 h to 7 h) resulted in a decrease of dt_{spe} FD values of 39% for the fermentation conditions at 42°C, pH 5.8 (from 66 to 40 [min (log (CFU ml^{-1})) $^{-1}$]).

The loss of specific acidifying activity was also influenced by the fermentation pH (β_1 = 10.0). Lower dt_{spe} FD values were observed at pH 4.8 than at pH 5.8. For instance, when analyzing cells cultivated at 37°C and harvested at the stationary growth phase (2 to 10 h from t_{Vm}), dt_{spe} FD values were reduced when the pH was decreased from 5.8 to 4.8 (from 50 to 32 [min (log (CFU ml^{-1})) $^{-1}$]). The same tendency was observed at 42°C (from 40 to 37 [min (log (CFU ml^{-1})) $^{-1}$]).

The conjugated effect of pH and harvest time (β_{13} = -8.0, Fig. 3c and Fig. 3d) can be observed at 42°C. Cells grown at pH 5.8 and harvested at increased harvest time (from -10 to 10 h) led to a 39% decrease in dt_{spe} FD values (from 66 to 40 [min (log (CFU ml^{-1}))$^{-1}$) compared to 20% at pH 4.8 (from 46 to 37 [min (log (CFU ml^{-1}))$^{-1}$]).

The slight interaction between pH and temperature (β_{12} = -2.7) can be visualized when cells were harvested at the stationary growth phase (+2.0 to +10 h from t_{Vm}). When the temperature increased from 37°C to 42°C, at pH 4.8, dt_{spe} FD values increased by 16% (from 32 to 37 [min (log (CFU ml^{-1})) $^{-1}$]), whereas, at pH 5.8, dt_{spe} FD values decreased by 20% (from 50 to 40 [min (log (CFU ml^{-1})) $^{-1}$]).

The minimum loss of specific acidifying activity after freeze-drying (dt_{spe} FD = 29.0 [min (log (CFU ml^{-1}))$^{-1}$]) was observed at 37°C, pH 4.8, and in cells harvested at the stationary growth phase, 10 hours after reaching t_{Vm} (red dot on the surface representation in Fig. 3d).

Regardless of the fermentation conditions applied, the residual water content of the freeze-dried samples was lower than 3%. The glass transition temperature of the freeze-dried samples was measured about 50.4°C. These results indicate that the samples were kept in a glassy state.

Freeze-dried storage

Due to the low R^2 value for the multiple regression analysis of $dt_{spe}S$, the effects of fermentation parameters (pH, temperature and harvest time) on dt_{spe} S was solely visualized on boxplots in Fig. S6. Two levels of dt_{spe} S values were observed. A low level that includes the fermentation conditions carried out at 37°C, pH 5.8 and pH 4.8 (except for t_{h3}). The smallest dt_{spe} S values (the highest resistance to freeze-dried storage) were exhibited when cells were cultivated at 42°C, pH 4.8 (t_{h1} and t_{h2}: 18 [min (log (CFU ml^{-1})) $^{-1}$]). In contrast, a high level of dt_{spe} S values (49 [min (log (CFU ml^{-1})) $^{-1}$])) was mainly obtained for the fermentation condition at 42°C, pH 5.8 and no effect of the harvest time was observed.

Predictive accuracy of the multiple regression models

The relevance and the predictive capacity of the models of biomass productivity (P), loss of specific acidifying activity after freezing (dt_{spe} F), and freeze-drying (dt_{spe} FD) were validated by carrying out two independent biological replicates at fermentation conditions located at the center of the experimental range: 39°C and pH 5.3. Cells were harvested at different harvest times: the deceleration growth phase (t_{h2} = 0.6 h and 1.0 h) and the stationary growth phase (t_{h3} = 4.9 and 5.2 h). The experimental results were compared with the values predicted by the multiple regression models for P, dt_{spe} F and dt_{spe} FD (Table S7).

Most of the measured values (nine out of 12) were in good agreement with the predicted ones, within less than one residual standard deviation for the corresponding model. Concerning the biological replicate number two, the experimental values of productivity at t_{h3} and dt_{spe} F at t_{h2} and t_{h3} were higher than the predicted values. However, all measurements fell within two residual standard deviations of the predicted values, which roughly correspond to the 95% confidence interval. Model predictions were thus validated within the expected accuracy range.

Pareto front approach to produce frozen and freeze-dried *L. bulgaricus* CFL1 cells

To optimize the production of frozen and freeze-dried *L. bulgaricus* cells, a Pareto front was constructed. Pareto fronts are helpful tools for solving multi-objective optimization problem. This tool searches for the best compromise solution, minimizing or maximizing responses. In this study, we considered as responses variables the biomass productivity (P, in g l^{-1} h^{-1}) and the functional properties (acidifying activity and the number of culturable cells). The functional properties were expressed by the descriptor t_{spe}, i.e., the specific acidifying activity ($t_{spe,}$ in [min (log (CFU ml^{-1})) $^{-1}$]).

According to the coefficient of determination (R^2), the multiple regression models explained only 51% of t_{spe} I (Table S3) and 57% of t_{spe} S, whereas t_{spe} F and t_{spe} FD presented an $R^2 \geq 70\%$ (Table S8). For this reason, the further analysis only deals with freezing and freeze-drying ($R^2 \geq 70\%$).

Specific acidifying activities after freezing (t_{spe} F) and freeze-drying (t_{spe} FD) were considered as well as the t_{spe} I values to visualize the loss of specific acidifying activity after freezing (dt_{spe} F) and freeze-drying (dt_{spe} FD). Therefore, Fig. 4 illustrates the Pareto front, i.e., the set of best possible compromises between the values of t_{spe} after freezing and freeze-drying and biomass productivity (P). The displayed curves result from a numerical optimization by considering different pH, temperature and harvest time combinations within the experimental domain of this study. Note that increasing biomass productivity induces the decrease of specific acidifying activity (increasing t_{spe} values) and vice versa.

In Fig. 4, three main sections were identified and described below:

Section (i): The highest specific acidifying activity

Low t_{spe} values after freezing and freeze-drying were observed along with unfavorable biomass productivity (0.26-0.27 g l^{-1} h^{-1}). These data corresponded to cells cultivated at 42°C, pH 4.8, and harvested at the deceleration growth phase (t_{h2} = -1.7 h). Bacteria exhibited high specific acidifying activity (low t_{spe} values) following freezing (t_{spe} = 24 [min (log (CFU ml^{-1})) $^{-1}$]). Following freeze-drying, t_{spe} = 41 [min (log (CFU ml^{-1})) $^{-1}$]) was observed for cells cultured at 42°C, pH 4.8, and harvested at the mid-exponential growth phase (t_{h1} = -10 h).

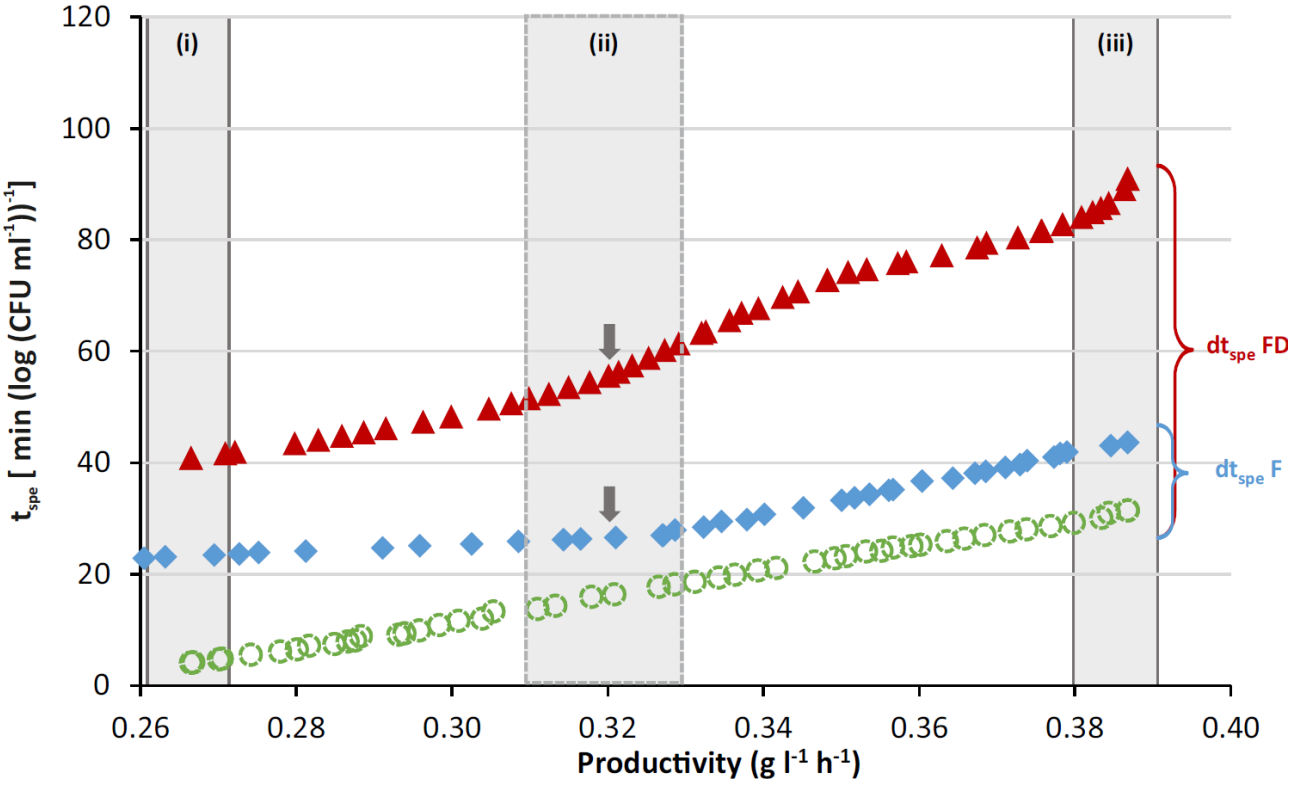


Figure 4 Pareto multi-objective optimization by maximizing the biomass productivity (high productivity values, axis x) and minimizing the t_{spe} values obtained after freezing or freeze-drying (axis y, corresponding to maximizing the t_{spe}) of *L. bulgaricus* CFL1. t_{spe} (specific acidifying activity) is defined as the ratio of $t_{\Delta pH0.7}$ (min) to the corresponding log of cell concentration (CFU ml^{-1}); $t_{\Delta pH0.7}$ corresponds to the time necessary to obtain a decrease of 0.7 pH units (Cinac system).
The values of t_{spe} were indicated after concentration and protection (initial, I) (empty green circle: ○); after freezing (F) (blue diamond: ◆); and after freeze-drying (FD) (red triangle: ▲). Section (i) represents the minimum biomass productivity and the lowest t_{spe} values (42°C, pH 4.8, t_{h1}). Section (ii) is delimited by dotted lines, representing the compromise between biomass productivity and t_{spe} (balance performance). The fermentation condition (42°C, pH 4.8, t_{h2}) leading to this compromise is indicated by full gray arrows for curves after freezing and freeze-drying. Section (iii) represents the maximum biomass productivity and the highest t_{spe} values (42°C, pH 5.8, t_{h3}).
dt_{spe} F: loss of acidifying activity after freezing and dt_{spe} FD: loss of acidifying activity after freeze-drying.

Section (ii): Balanced performance

This section represented a compromise between fair biomass productivity (0.31-0.33 g l^{-1} h^{-1}) and good specific acidification activity. In this section, a limited degradation of *L. bulgaricus* CFL1 specific acidifying activity (t_{spe}) after freezing and freeze-drying was observed. Cells cultivated at 42°C, pH 4.8, and harvested at the

deceleration growth phase (t_{h2} = -1.5 h) exhibited fair specific acidifying activity (low t_{spe} values) following freezing (t_{spe} = 27 [min (log (CFU ml^{-1}))$^{-1}$]) and freeze-drying (t_{spe} = 56 [min (log (CFU ml^{-1}))$^{-1}$]). Both results correspond to the reasonable productivity of 0.32 g l^{-1} h^{-1} (full gray arrows in Fig. 4).

Section (iii) The highest biomass productivity

Biomass productivity values between 0.38-0.39 g l^{-1} h^{-1} were reached, sacrificing the specific acidifying activity after freezing (t_{spe} = 44 [min (log (CFU ml^{-1}))$^{-1}$]) and freeze-drying (t_{spe} = 91 [min (log (CFU ml^{-1}))$^{-1}$]). For this highest biomass productivity, cells grown at 42°C, pH 5.8, and harvested at the stationary growth phase (t_{h3} = 7.0 h) represented this case.

Discussion

We performed a complete study to identify the most influential fermentation parameters affecting the functional properties at each process stage of *L. bulgaricus* CFL1. In a second step, using multiple regression analysis, we modeled the bacteria biomass productivity and the loss of their main functional properties (i.e., acidifying activity and culturability) after freezing and freeze-drying.

After freezing and freeze-drying, the functional properties of LAB are affected. Our results showed that *L. bulgaricus* CFL1 cells exhibited losses of their functional properties (dt_{spe}). Lower losses were observed after freezing than freeze-drying (1-14 [min (log (CFU ml^{-1}))$^{-1}$]) vs. (29-77 [min (log (CFU ml^{-1}))$^{-1}$]). During freezing (at a low cooling rate), the extracellular ice forms and grows, leading to the cryo-concentration of the extracellular medium without intracellular ice formation (Fonseca *et al*. 2006). The cryo-concentration of the extracellular medium induces water efflux from the intracellular compartment, which, in turn, results in cell dehydration and cell volume reduction. Additionally, the mechanical constraints applied to the bacterial membrane following cell contraction lead to membrane leakage and loss of membrane integrity (Gautier *et al*. 2013).

Following freeze-drying, the bacterial cells are exposed to the osmotic stress provoked by the previous freezing step and additional mechanical stress due to removing the water during the drying steps. Hydrogen bonds between water and cellular constituents such as membrane phospholipids and membrane proteins are broken, thus destabilizing bacterial cell membranes (Castro *et al*. 1997; Hlaing *et al*. 2017; Romano *et al*. 2021).

Multiple regression analysis helped us understand the main and the interaction effects of fermentation parameters (pH × temperature and/or pH ×harvest time) on the resistance of *L. bulgaricus* CFL1. This

combined effect is the result of studying three parameters simultaneously, which has been scarcely investigated for optimizing frozen or freeze-dried LAB (Table S1).

Our results showed that the pH was the most influential fermentation parameter on dt_{spe} for both stabilization processes. Notably, the low pH (pH 4.8) minimized the loss of specific acidifying activity during freezing and freeze-drying. Similar results were previously reported for *L. bulgaricus*. When *L. bulgaricus* cells were cultivated at low pH, bacteria improved their resistance to freezing (Rault *et al*. 2010) and freeze-drying (Shao *et al*. 2014).

For the freeze-drying process, the harvest time was another parameter that exclusively influenced the loss of the specific acidifying activity of *Lactobacillus bulgaricus* CFL1. Low dt_{spe} FD values (high resistance to freeze-drying) were observed when the harvest time was increased. To our knowledge, no study had reported the effect of harvest time on the freeze-drying resistance of *L. bulgaricus*. However, some studies confirmed our results for other LAB cells. The increase in harvest time improved freeze-drying resistance (Palmfeldt and Hahn-Hägerdal 2000; Schwab *et al*. 2007; Li *et al*. 2009b; Velly *et al*. 2015).

In this study, we showed that depending on the stabilization strategy, the fermentation parameters that affected the resistance were different: pH for freezing and pH and harvest time for freeze-drying.

Concerning the resistance to dried storage, the loss of specific acidifying activity of *L. bulgaricus* CFL1 following dried storage at 25°C (dt_{spe} S) appeared to be weakly influenced by the fermentation conditions applied in this study. Therefore, other factors during the storage step may have a more substantial effect than the fermentation conditions. For instance, several degradation reactions may occur during storage, either controlled by diffusion, such as the Maillard reaction or not controlled by diffusion, such as oxidation (Lievonen *et al*. 1998; Kurtmann *et al*. 2009).

The water content and Tg of our freeze-dried samples confirmed that they were kept in a glassy state throughout storage. Consequently, the molecular mobility and the diffusion-controlled degradation reactions were limited by embedding bacteria in a solid glassy matrix (Higl *et al*. 2007; Passot *et al*. 2012). Thus, degradation reactions controlled by diffusion were not responsible for the dt_{spe} S observed in this study. The loss of acidifying activity of *L. bulgaricus* CFL1 after storage (dt_{spe} S) at 25°C could be attributed to oxidation reactions (Kurtmann *et al*. 2009; Ying *et al*. 2011; Rodklongtan *et al*. 2022) since no antioxidants were included in the protective solution.

L. bulgaricus CFL1 strain exhibited the highest losses after freezing and freeze-drying when cells were cultivated at the fermentation condition, which enhanced high growth rate values, substrate consumption, and biomass concentration (42°C, pH 5.8). Some studies agreed with our findings: different LAB species growing under their optimal fermentation condition led to higher loss of their functional properties (or less survival) after the following stabilization processes (Palmfeldt and Hahn-Hägerdal 2000; Li *et al*. 2009a; Li *et al*. 2012; Shao *et al*. 2014; Liu *et al*. 2014). This can be understood by the fact that when bacteria are grown at their optimal fermentation condition, their enzymes function at their maximal activity, thus enhancing their metabolism and growth (Sharma *et al*. 2020).

Industrial production of lactic acid bacteria requires the maximization of the quantity of biomass and the preservation of their functional properties after the stabilization processes (freezing or freeze-drying) and the subsequent storage. Aside from determining the effect of fermentation parameters on each stage of the production of lactic acid bacteria, we proposed a universal approach consisting of a Pareto front as a complementary tool to the 3D response surfaces for optimizing frozen and freeze-dried starter culture production. This tool made it possible to select the suitable fermentation condition: 42°C, pH 4.8, t_{h2}, which allows a balanced performance between both criteria, fair biomass production and reasonable specific acidifying activity (t_{spe}) after freezing and freeze-drying. Providing a set of data is available, this methodology can be applied to any bacteria to determine the best fermentation condition tested.

In conclusion, the approaches used in this study, multi-regression analysis and Pareto font, are a breakthrough in determining the best solution to stabilize other lactic acid bacteria for two reasons. First, the multi-regression analysis allowed us to determine multivariate parameters' single and combined effects and provide models to predict different response variables. Second, the Pareto Front examined the essential criteria for LAB concentrate production, such as biomass productivity and the functional properties of bacteria that need to be preserved after the most common stabilization processes. Optimizing the fermentation conditions could help produce LAB at lower costs and time.

We speculate from the present results that *L. bulgaricus* CFL1 could have developed adaptative mechanisms to promote active biological responses under conditions other than optimal for growth (e.g., membrane lipids modification, expression of stress proteins, and changes in the morphology). These biological responses help LAB resist stressful environments induced during freezing or freeze-drying (Papadimitriou *et al*. 2016;

Fonseca *et al.* 2019; Gao *et al.* 2021). Therefore, further work is needed to understand the cellular mechanisms responsible for improving bacterial resistance to the stabilization processes. For instance, an integrative approach can be used, which combines a lipid membrane composition (lipid classes and fatty acids) and membrane fluidity.

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Conflict of interest

No conflict of interest declared.

Authors contributions

Conceptualization and investigation, MLTC, SP, FF; experiments, MLTC; formal analysis SP, FF, ICT, MLTC; data curation and statistical treatment, ICT, MLTC, FF; writing, review and editing MLTC, FF, SP, ICT, MHR, YG; supervision, FF, SP, MHR, YG; funding acquisition SP and FF. All authors provided critical feedback.

Data availability

The datasets generated and/or analyzed during the current study are available in the Data INRAE repository at

https://doi.org/10.15454/FZHIE0.

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