

Growth and Freeze-Drying Optimization of *Bifidobacterium crudilactis*

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Abstract

Bifidobacterium crudilactis FR62/b/3 belongs to a new population of bifidobacteria isolated from raw milk and raw milk cheese. The objective of this work was to study the large scale culture of the strain and its stability in a dry formulation. Growth rate of *Bifidobacterium crudilactis* FR62/b/3 was optimal at a pH of 5.0 and a temperature of 37°C. At a temperature growth of 33°C and a pH of 5.0, the stationary phase was reached after 22 h, the viable cell number and the mean dry biomass concentration were respectively of 8.3×10^9 CFU/mL and of 2.1 g/L. Resistance of *Bifidobacterium crudilactis* FR62/b/3 to freeze-drying and effect of a variety of cryoprotectants to maintain the viability were also evaluated. Sorbitol was the most suitable cryoprotectant for freeze-drying as well as storage whereas sucrose and monosodium glutamate were only efficient during storage.

Keywords

Probiotic, Bifidobacteria, Crudilactis, Cryoprotectant, Freeze-Drying

1. Introduction

Probiotics are living microorganisms, which beneficially affect the health of the host, when consumed in adequate quantities [1]. Bifidobacteria as well as lactic acid bacteria are currently the most widely used probiotic bacteria. In the genus *Bifidobacterium*, the most described probiotic species are *B. animalis* ssp *lactis*, *B. bifidum*, *B. longum*, *B. infantis*, *B. breve* and *B. adolescentis* [2] [3].

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A new population of bifidobacteria was isolated from a production chain of raw milk cheese (L'Etoile du Vercors, St Marcellin, Vercors area, France). This group was separated and classified into a new species named *crudilactis*. The strain *B. crudilactis* FR62/b/3 was phenotypically the closest to all the other *Bifidobacterium crudilactis* strains and was then retained as the reference strain of this species [4]. According to previous studies [4] [5], *B. crudilactis* FR62/b/3 was able to grow in Tryptone Yeast medium at 4°C and to form colonies under aerobic conditions. A weak growth was observed in Tryptone Yeast medium at pH 4.4 and no growth at pH 3.8 within 15 days. Therefore, contrary to the other bifidobacteria which are mostly unable to grow at low temperatures and tolerate oxygen, *B. crudilactis* FR62/b/3 may present a great technological potential as probiotic [4] [5]. However, the development of a probiotic formulation of *B. crudilactis* depends on its large scale culture potential and its resistance to drying technologies used to maintain viability during manufacturing and storage [6] [7]. Growth of bifidobacteria is known to depend on growth medium and specific nutrient like carbon sources, nitrogen sources and presence of low redox potential components. Furthermore fermentation conditions such as pH, temperature and residual oxygen concentration are also of fundamental importance to obtain high cell density. According to the literature, bifidobacteria are able to grow between 20°C and 46°C. They are acid-tolerant microorganisms, and their optimal pH of growth is observed between 6.0 and 7.0. Although they are described as anaerobic, their sensitivity to oxygen varies according to species [2] [8] [9]. Freeze-drying has been reported by different authors as gentle and suitable technology for the production of dried probiotic formulations. Nevertheless, damages due to ice crystals formation during freezing, osmotic stress, important dehydration by biological molecules and membrane lipids oxidation phenomenon are described to affect probiotics survival during this drying process and storage of the final product [10]-[12]. Resistance to freeze-drying and subsequent storage depend on many factors like intrinsic characteristics of each strain such as cell size and membrane composition but also on external conditions such as presence of nutrients and assimilation of compatible solutes. Sub-lethal treatments which lead to a physiological state of enhanced resistance and freeze-drying process parameters impact also the viability of freeze-dried bacteria [6] [13]. In addition to the aforementioned factors, protective additives have important role in reducing damages on cells. Cryoprotectants such as skim milk are commonly used [14], but the demand for non milk-based probiotic is required to avoid lactose intolerance and presence of allergen in the final product [15]. Further alternative cryoprotectants have already been identified by many authors, including penetrating compounds (e.g. monovalent alcohols, amino-acids), slowly penetrating compounds such as glycerol and non-penetrating compounds (e.g. polysaccharide, polyalcohols, proteins). The mechanism of protection depends on many factors like the type and the concentration of cryoprotectant, cell density and the ratio cryoprotectant/cell [6] [16] [7]. The effect of each protectant appears to be strain specific and has to be investigated on a case-by-case basis [6] [17]. Moreover, cryoprotectants reveal synergistic effects. It's often advisable to use a mixture of rapidly penetrating and non- penetrating (or slowly penetrating) agents [18].

The main purpose of this study was to evaluate the resistance of *Bifidobacterium crudilactis* FR62/b/3 to an industrial process combining cultivation in bioreactor and freeze-drying. Indeed, as a first step, the effects of pH and temperature on the growth kinetics of the strain were studied. Then efficiency of different cryoprotectants was separately investigated on survival of the strains after freeze-drying, and under two different storage conditions.

2. Methods

2.1. Microorganism and Growth Medium

Bifidobacterium crudilactis FR62/b/3 was provided by the Food Sciences Department, Faculty of Veterinary Medicine, University of Liege. The strain was kept frozen at -80°C in growth medium and 40% (v/v) glycerol. The growth medium (adapted from MRS medium) composition is: glucose 17.5 g/L, yeast extract 15 g/L, casein pepton 15 g/L, KH₂PO₄ 1 g/L, K₂HPO₄ 1 g/L, MgSO₄·7H₂O 0.2 g/L, MnSO₄ 0.007 g/L, FeSO₄ 0.01 g/L, Tween 80 1 g/L, NaCl 0.01 g/L, cystein 0.5 g/L.

2.2. Preparation of Inocula

To prepare the subculture, 330 mL of growth medium (adjusted to pH 6.5 with NaOH 10 M) in flask were inoculated with 0.1 mL of the cell suspension obtained from the suspension stored in glycerol at -80°C. The subcultures were carried out in anaerobic conditions generated by nitrogen sparging. Flasks were maintained at 33°C under stirring (150 RPM) for 42 h.

2.3. Effect of Temperature and pH on Growth Kinetics

2.3.1. Fermentation Experiments

Batch cultures were carried out in 2 liters bioreactors (Biostat B, Sartorius B. Braun) with working total volume of 1.75 L of growth medium. pH was adjusted to the set value using a 10 M NaOH and H₃PO₄ 30% (v/v) solution. The inoculum rate was 6% (v/v) and all fermentations were performed under stirring (350 RPM), anaerobically by previously sparging the medium with nitrogen and maintaining the culture in closed system.

In a first set of experiments the effect of pH was studied at 33°C from a range of 4.5 to 6.5 with a step of 0.5. Afterwards, effect of temperature (30°C, 33°C, 35°C, 37°C, 39°C, 41°C), on growth was investigated at a pH of 5.0. Each experiment was performed in triplicate.

2.3.2. Sampling and Analysis

Samples were withdrawn from the bioreactors every 2 h for dry cell weight measurement until stationary phase. Concentrations of glucose, lactic acid and acetic acid were measured 2h after the beginning of stationary phase by HPLC (Waters 515 Pump, Waters 717 plus Autosampler) using a Shodex™ SH-1011 column cation H⁺ exchanging resin, mobile phase H₂SO₄ 0.01 N, flow rate 0.8 mL/min at 90°C, refractive index detector (Waters 2414 refractometer).

2.4. Effect of Protective Agents on Viability after Freeze-Drying and Storage

2.4.1. Cells Culture

Subculture was used to inoculate two bioreactors (Biostat ED, Sartorius B. Braun) containing each 10 L of growth medium (inoculation rate was 6% v/v). The bioreactor was stirred at 350 RPM, the temperature was maintained at 33°C and the pH controlled at 5.0 using a 10 M NaOH and H₃PO₄ 30% (v/v) solution. Fermentation was performed under anaerobic conditions and was stopped after 26 h (2 h after the beginning of the stationary phase).

2.4.2. Formulation of Protective Medium

Twenty millilitres of five different protective media at a concentration of 15% (w/v) were prepared in 0.1 M phosphate buffer at pH 6.2 using the following protective agents: betaine, monosodium glutamate, sorbitol, sucrose and trehalose. A solution of phosphate buffer without any cryoprotective agent was used as control.

For each formulation, 3 L of growth medium at the early stage of the stationary phase were centrifuged (Sorvall RC 12B) at 2270 g for 45 min at 4°C. The resulting pellet was resuspended in 20 mL of the previously prepared protective solution and adjusted to 50 mL with phosphate buffer in order to obtain 12% (w/v) of dry cell and 6% (w/v) of cryoprotectants in each preparation. A contact time of 30 minutes with cryoprotectants has been carried out.

2.4.3. Freeze-Drying Conditions

Three milliliters of bacterial suspension was dispensed into vials and freeze dried in a tray freeze dryer (EPSILON 2-8 D Martin Christ®). The applied freeze-drying program was: freezing step at -45°C for 5 h, first step of sublimation at -42°C for 6 h and a second step of sublimation for 58 h during which temperature was progressively raised to 10°C (0.95°C/h) and finally a secondary drying at 10°C for 6 h. At the end of drying, the vials were closed under vacuum and sealed. They were kept in sealed plastic bags under vacuum at 4°C or at room temperature (20°C ± 3°C) in the dark.

2.4.4. Determination of Cell Viability

Before freeze drying, two samples were taken from each suspension and the viable cell concentration was calculated as CFU/mL by plate dilution on MRS agar medium (Merck Millipore) containing cysteine hydrochloride 0.5 g/L. Viable cell concentration CFU/mL was determined in triplicate samples, after freeze-drying and after 1 and 3 months of storage for each condition, by reconstituting the initial volume (3 mL) with physiological water.

2.4.5. Statistical Analysis

Statistical difference was determined by analysis of variance (ANOVA) using the statistical program R. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Temperature and pH Effect on Growth Kinetics

The growth of *Bifidobacterium crudilactis* during batch culture in 2 liters bioreactor at different regulated pH was studied. As presented in **Figure 1**, growth curves revealed that the cultures reached the stationary phase after about 22 h with an inoculation level of 2×10^8 CFU/mL. After a short adaptation phase, the maximum specific growth rate (μ_{\max}) was obtained between 4 h and 10 h of culture for each tested pH excepted at pH 6.5, for which μ_{\max} ($0.22 \pm 0.01 \text{ h}^{-1}$) was determined between 6 h and 10 h of culture. A pH regulation of the medium at pH 5.0 allowed the highest maximum specific growth rate of $0.30 \pm 0.01 \text{ h}^{-1}$ (**Table 1**). Based on these results, the influence of culture temperature on the growth rate at this optimal pH was studied. As shown in **Figure 2**,

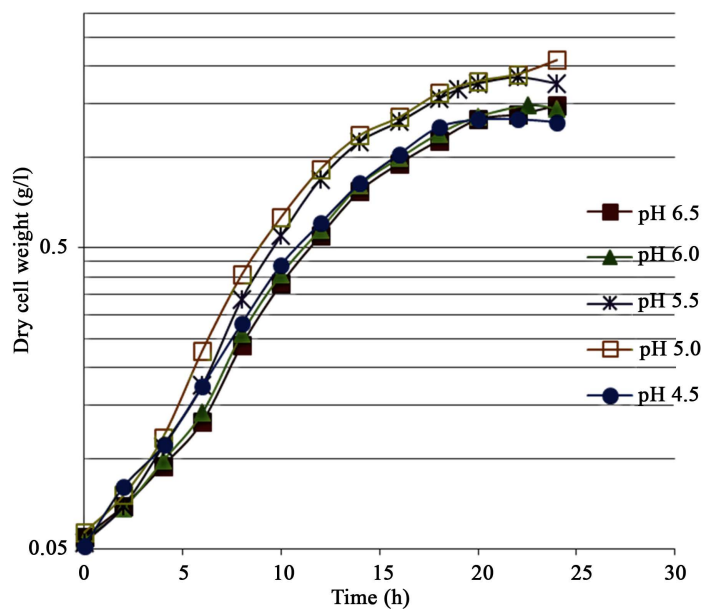


Figure 1. Effect of pH on growth kinetics.

Table 1. Optimal specific growth rate related to pH and incubation temperature.

Growth conditions	$\mu_{\max} (\text{h}^{-1})$
33°C	
pH 4.5	0.25 ± 0.00
pH 5.0	0.30 ± 0.01
pH 5.5	0.27 ± 0.00
pH 6.0	0.24 ± 0.01
pH 6.5	0.22 ± 0.01
pH 5.0	
30°C	0.25 ± 0.01
33°C	0.30 ± 0.01
35°C	0.32 ± 0.01
37°C	0.33 ± 0.00
39°C	0.31 ± 0.01
41°C	0.25 ± 0.01

Each value is expressed as the mean value of three experiments \pm standard deviation.

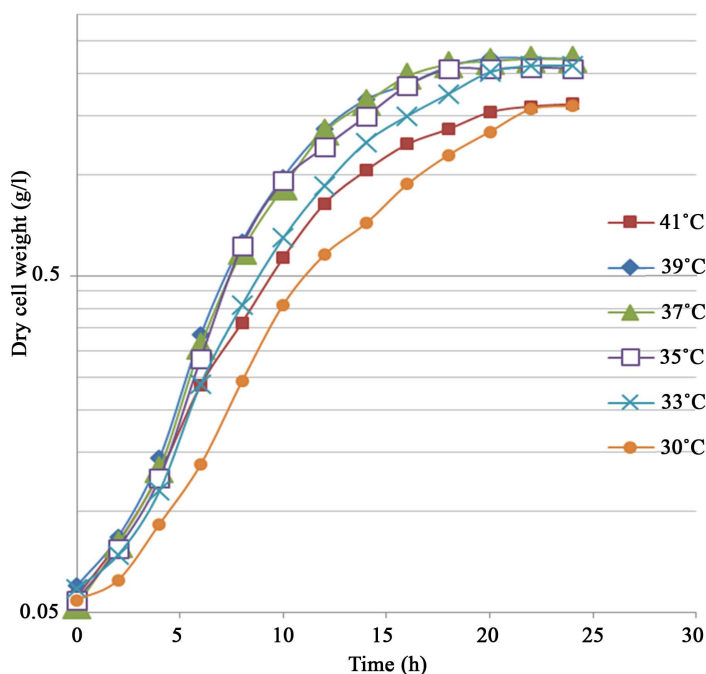


Figure 2. Effect of temperature on growth kinetics.

the specific growth rate was maximum between 4 h and 10 h of culture for all the temperature conditions. The optimal specific growth rate was measured at 37°C ($0.33 \pm 0.00 \text{ h}^{-1}$), but no significant difference was noticed between 35°C ($0.32 \pm 0.01 \text{ h}^{-1}$) and 39°C ($0.31 \pm 0.01 \text{ h}^{-1}$). The final biomass concentration ($2.1 \pm 0.1 \text{ g/L}$) at the stationary phase was similar at the temperatures of 33°C, 35°C, 37°C and 39°C. For the culture performed at extreme temperatures (30°C and 41°C), a slower growth rate was observed and a lower biomass concentration was measured at the stationary phase (1.6 g/L).

Overall, growth rate of *Bifidobacterium crudilactis* FR62/b/3 was optimal at a pH of 5.0 and a temperature of 37°C. However, additional studies have demonstrated that the viability preservation of the strain prior freeze-drying was better when the culture was performed at 33°C compared to 37°C (data not shown). With regard to this result, growth characteristics of the strain at 33°C and a pH of 5.0 after 22 h fermentation have been assessed (Table 2).

Under these culture conditions, the viable cell number reached an average value of 8.3×10^9 CFU/mL at the end of growth. Glucose was not completely consumed and was mainly converted into acetate and lactate. The molar ratio acetate/lactate was of 1.5, as classically described for bifidobacteria pathway. The average biomass concentration was of 2.1 g/L dry cell weight.

3.2. Effect of Protective Agent on Viability after Freeze-Drying and Storage

The freeze-drying of *B. crudilactis* was carried on in the presence of different cryoprotective agents to study their effect on viability. As shown in Table 3, viable cell concentration before freeze-drying was similar for all the tested formulations, revealing that the addition of cryoprotective agents had little impact on cell viability prior freeze-drying.

The survival rate after freeze-drying for each formulation was expressed as the ratio between the number of viable cell after freeze-drying and the number of viable cell before freeze-drying (Figure 3). The highest viability loss (89.5%) was measured for the control. All five preparations containing cryoprotective agents increased significantly the survival rate comparing to the control. Sorbitol offered the best protection to the strain during freeze-drying (80.5%) and its effect was significantly different than the other cryoprotective agents. Besides sorbitol, trehalose and sucrose allowed an appreciable viability (respectively 44.5% and 39.5%), followed by hydrochloride betaine and monosodium glutamate (respectively 37.1% and 33.1%). Even though there were no significant differences on survival rate between trehalose, sucrose, hydrochloride betaine and monosodium glu-

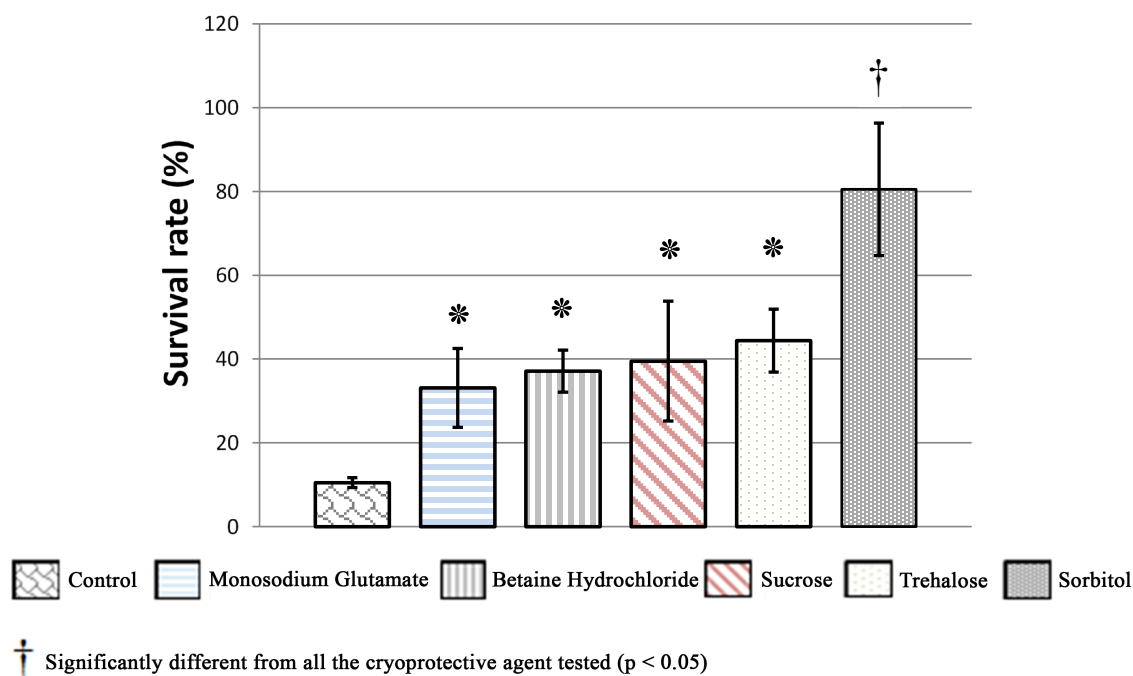


Figure 3. Effect of cryoprotective agents on survival rate of *B. crudilactis* after freeze-drying.

Table 2. Growth characteristics of *Bifidobacterium crudilactis* FR62/b/3 at 33°C and pH 5.0.

Growth characteristics	Values
μ_{\max} (h^{-1})	0.30 ± 0.01
Viable cell concentration (CFU/mL)	$8.3 \times 10^9 \pm 0.9$
Dry cell weight (g/L)	2.1 ± 0.1
Glucose consumed (g/L)	14.5 ± 1.2
Residual glucose (g/L)	2.7 ± 0.5
Lactic Acid (g/L)	6.9 ± 0.3
Acetic Acid (g/L)	6.8 ± 0.4
Ratio acetic/lactic (mol/mol)	1.5 ± 0.1

Each value is expressed as the mean value of three experiments \pm standard deviation.

Table 3. Effect of cryoprotective agents on the viable cell concentration of *Bifidobacterium crudilactis* after freeze-drying process.

Protective formulation	Viable cell concentration before freeze-drying (CFU/mL)	Viable cell concentration after freeze-drying (CFU/mL)	Survival rate (%)
Control	$3.4 \times 10^{11} \pm 0.3$	$3.6 \times 10^{10} \pm 0.3$	10.5 ± 1.2
Betaine	$3.5 \times 10^{11} \pm 0.4$	$1.3 \times 10^{11} \pm 0.2$	37.1 ± 5.0
Glutamate	$3.2 \times 10^{11} \pm 0.2$	$1.1 \times 10^{11} \pm 0.3$	33.1 ± 9.4
Sucrose	$3.5 \times 10^{11} \pm 1.4$	$1.4 \times 10^{11} \pm 0.5$	39.5 ± 14.3
Sorbitol	$3.4 \times 10^{11} \pm 0.6$	$2.7 \times 10^{11} \pm 0.5$	80.5 ± 15.8
Trehalose	$4.0 \times 10^{11} \pm 0.2$	$1.8 \times 10^{11} \pm 0.3$	44.4 ± 7.5

Each value is expressed as the mean value of three measurements \pm standard deviation. Phosphate buffer was used as a control.

tamate, these results suggest that non penetrating compounds (sorbitol, trehalose and sucrose) might tend to a more effective protection than penetrating compounds (hydrochloride betaine and monosodium glutamate) during freeze-drying.

After 6 months of storage at 4°C in sealed flasks under vacuum (Figure 4), the viable cell concentration was stabilized whatever the type of cryoprotectant. Mean survival rate values exceeding 100% are explained by variability between measurements. As might be expected, an important loss of viability (60.1%) was observed for freeze-dried cells *Bifidobacterium crudilactis* without protective agent. Nevertheless, when storage was performed at 23°C (Figure 5), a loss of viability between 99.0% and 45.1% was observed after 6 months storage. As a general trend, survival decreased most importantly within the first month meaning that some cells had been weakened by freeze-drying. Sorbitol and sucrose were confirmed to provide the most significant protection (respectively 54.9% and 50.1% survival rate after 6 months at 23°C) followed by monosodium glutamate (34.1% of survival after 6 months at 23°C). No significant difference was measured on the survival rate after 6 months between, hydrochloride betaine and trehalose but all the tested protective agent offered a significant effect comparing to the control.

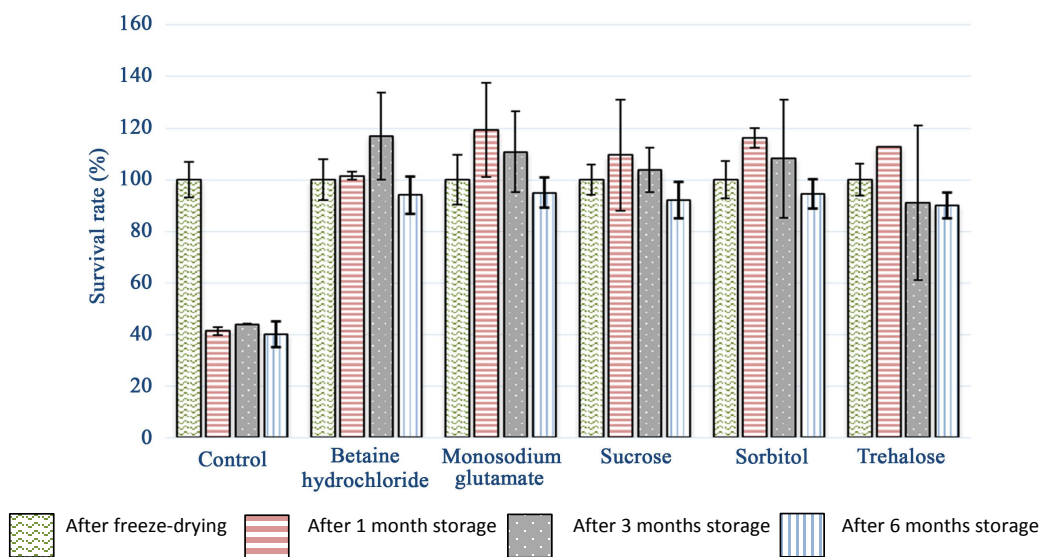
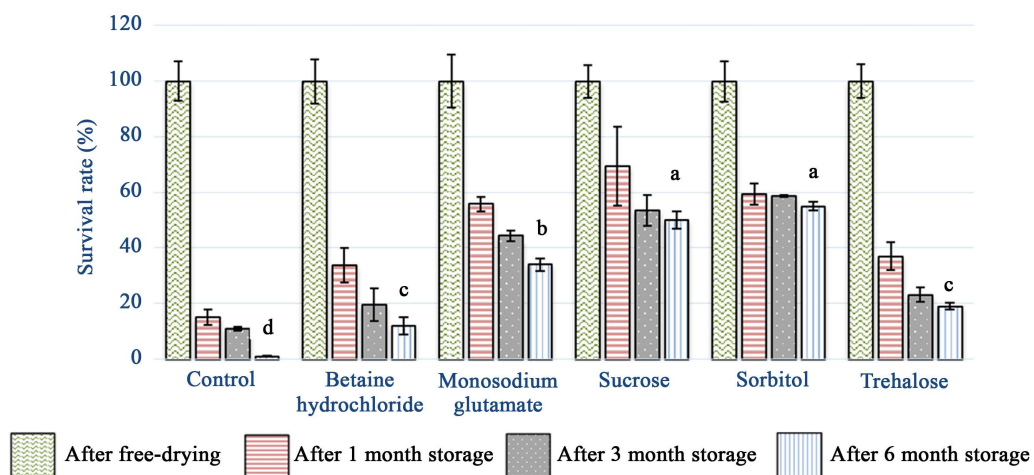


Figure 4. Effect of cryoprotective agent on survival rate of *B. crudilactis* during storage at 4°C.



The columns with different letters indicate a significant difference ($p < 0.05$) between the survival rate obtained after 6 months storage.

Figure 5. Effect of cryoprotective agent on survival rate of *B. crudilactis* during storage at 23°C.

Even though sucrose and monosodium glutamate exhibited a significant preservation level during storage, these protectants were less efficient during freeze-drying. These observations indicate that only sorbitol could be used as global protectant for freeze-drying and storage of *Bifidobacterium crudilactis* FR62/b/3 resulting in a global viability of 80.5, 94.5% and 54.9% respectively after freeze-drying, after six months of storage at 4°C and 23°C.

4. Discussion

In order to develop a probiotic formulation containing *Bifidobacterium crudilactis* FR62/b/3, growth and resistance of this strain to freeze-drying has been assessed. *Bifidobacterium crudilactis* FR62/B/3 exhibited an optimal pH of growth of 5.0. This pH is lower than the optimum pH range (5.5 - 7.0) described in the literature for the majority of bifidobacteria [2] [19] [20]. However, a similar optimum pH has been reported for *B. animalis* subsp. *lactis* [21]. This characteristic might confer a better resistance to acidic conditions as encountered in food product and in the digestive system to *Bifidobacterium crudilactis* FR62/b/3. The optimal temperature was determined at 37°C and is in accordance with the optimum temperature range (37°C - 41°C) reported in literature for bifidobacteria [1] [2] [9].

Additional studies (data not shown) showed a better resistance of *Bifidobacterium crudilactis* FR62/b/3 to freeze-drying, when the culture was performed at 33°C compared to 37°C. An influence of growth temperature on cryotolerance has already been reported with *Lactobacillus acidophilus* RD758. This strain showed a better tolerance to freezing when its culture was conducted at a temperature lower than its optimal growth temperature due to the modification of the membrane fatty acid composition [22].

Growth characteristics of *Bifidobacterium crudilactis* FR62/b/3 were determined under optimal conditions (pH 5.0°C and 33°C) for freeze-drying, it appeared that, glucose at initial concentration of 17.5 g/L was partially consumed and was mostly converted into lactate and acetate (94.4% of consumed glucose was converted into those two organic acids). Supplementation of medium with yeast extract and casein peptone did not allow extending the growth, supposing an inhibitory effect of these organic acids as main explanation for the early termination of the growth (data not shown). At the stationary phase, the measured viable cell concentration was of 8.3×10^9 CFU/mL. Freeze-drying has long been considered as a suitable dehydration process for long-term preservation of probiotics, achieving a stable powder formulation [12] [23]. Unfortunately, not all microorganisms are able to resist to such drying process [7] [24]. For each strain, it is thus necessary to optimize the process and the formulation prior drying. A large number of studies have demonstrated the essential importance of cryoprotectants to preserve cell from irreversible damages. For this reason, freeze-drying tolerance of *Bifidobacterium crudilactis* and effect of a variety of cryoprotectants to maintain the viability have been assessed. It appeared from this study that the tested cryoprotective agents (sorbitol, monosodium glutamate, sucrose, trehalose and hydrochloride betaine) showed different level of efficiency. Sorbitol was the most efficient during freeze-drying for viability preservation of *Bifidobacterium crudilactis* FR62/b/3 as well as during storage.

Although there are few studies reporting the efficiency of sorbitol for bifidobacteria protection during freeze-drying and storage, some previous studies revealed that this cryoprotectant offered excellent stability during storage to *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Enterococcus durans* and *Enterococcus faecalis* [6] [11] [17]. The protective effect of sorbitol could mainly be attributed to its ability to stabilize the protein structure and functionality by the formation of complexes [25]. Its anti-oxidant properties reducing membrane lipid oxidation phenomenon might also be involved for cell preservation. The ability of sorbitol to maintain turgor, due to its high hygroscopic behavior might prevent irreversible damages to occur to the cells [6] [21]. Other studies have shown that addition of sorbitol prior to freeze-drying, was able to increase the ratio of unsaturated/saturated fatty acids in the membranes of *Lb. plantarum*. Double bounds present in unsaturated acids enhance membrane permeability at low temperature and thereby improving resistance to freezing and long-term storage. However the mechanism of protection by polyols is not fully understood [11].

As observed in this study, sucrose has been identified by different authors, to preserve viability during storage. Indeed, sucrose provided a good stability to dried cells of *B. animalis* and *Lb. bulgaricus* [14] [17] [26] [27]. The protective effect of this cryoprotectant has been associated to its ability to trap salts in a highly viscous or glass like-phase, preventing damages caused by freezing [8]. This cryoprotectant is also able to decrease membrane phase transition [19]. It prevents the protein denaturation by the formation of hydrogen bonds which stabilize the tridimensional structure after removal of water [28].

Regarding monosodium glutamate, mechanisms for preservation of distinct microorganisms are also well documented [6] [29]-[31]. Different authors suggested that glutamate is able on one hand, to stabilize the proteins structure through reaction between its amino group and the carboxyl group of the protein. On the other hand, glutamate is able to retain important amount of residual moisture [6] [14]. Moreover, it has been demonstrated that, the resistance of freeze-dried *Lb. plantarum* storage in the presence of monosodium glutamate was related to the increase of unsaturated/saturated fatty acids ratio [11].

The effect of two storage conditions demonstrated that, the storage at 4°C was better than the one at room temperature for the viability and long-term preservation of freeze-dried *Bifidobacterium crudilactis* FR62/b/3. Indeed, storage at high temperature is referenced to increase the chance of glass transition, molecular mobility and the detrimental reactions like oxidation and Maillard reactions [32]. This result is in agreement with Bruno and Shah (2009), but these authors recorded a better preservation of viability at a storage temperature of -18°C [33]. In order to optimize conservation after freeze-drying, many other parameters like optimal water activity and atmosphere of storage (nitrogen versus vacuum) could be considered.

The stability of the viability of the preparations with cryoprotectants, observed after six months of storage revealed that this strain could be stabilized in a freeze-dried formulation.

5. Conclusion

The technological potential of *Bifidobacterium crudilactis* FR62/b/3 for its exploitation as a probiotic strain in an industrial process was assessed in this study. The optimization of the culture conditions revealed that the strain could be cultured in bioreactors and high viable cell density could be obtained at the stationary phase. Its low optimum pH of growth might confer a better resistance to acidic conditions to *Bifidobacterium crudilactis* FR62/b/3 comparing to other probiotic strains. Further investigation on the effect of a variety of cryoprotective agents on the survival to freeze-drying and storage revealed that sorbitol could be used as an efficient protective agent for a dry formulation.

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