Quality degradation of lactic acid bacteria during the freeze drying process : experimental study and mathematical modelling

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ABSTRACT

Freeze-drying (or lyophilization) is widely used to preserve thermo-sensitive active ingredient such as proteins or microorganisms. Our objective was to investigate the effect of the product thermal history during the freeze-drying process on the acidification activity of lactic acid bacteria. An empirical model describing the degradation of the acidification activity of *Lactobacillus bulgaricus* CFL1 during the process as a function of the temperature difference between the product and the glass transition temperature (Tg) was developed. This "quality" model was associated to a heat and mass transfer model of the freeze-drying process and the losses of acidification activity during the process were simulated for various operating conditions of shelf temperature and chamber pressure. Low losses of acidification activity were predicted when the process was performed at high values of product temperature, i.e. when the time the product temperature exceeds the glass transition temperature where molecular mobility increased was minimized. Two freeze-drying cycles were carried out at low and high values of product temperatures and the experimental results of the acidification activity of bacteria were in good agreement with the simulation results. This freeze-drying model including the prediction of the degradation of lactic acid bacteria appears as an interesting tool for designing optimal processes.

Keywords: lyophilisation; biological activity; lactic acid bacteria; model of heat and mass transfer; smart control

INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starters for manufacturing cheeses, fermented milks, meats, vegetables and breads products. Several species have been shown to exhibit probiotic properties i.e. positive effects on human health. The preparation of starter cultures requires production and maintenance techniques that maximise viability, activity and storage stability of bacterial cells. While frozen concentrates of lactic acid bacteria exhibit maximal survival in liquid nitrogen, the expense of these storage conditions limits the use of this method. Freeze-drying (or lyophilisation) appears as an alternative method for long time preservation of bacteria and yeasts. However, freeze drying is known to be a time consuming and expensive process. The product temperature is a key element for process optimization: the higher the product temperature, the higher the sublimation rate, and the faster the process. The product temperature results from the shelf temperature and the chamber pressure applied and is not directly controlled during the lyophilisation process [3, 10, 14]. The target product temperature during the sublimation stage (or primary drying) of an optimized lyophilisation process is usually fixed several degrees below a critical threshold value corresponding to the collapse temperature (Tcoll) [1, 11]. During sublimation, if the product temperature is higher than the collapse temperature, the amorphous material will undergo viscous flow, resulting in loss of the pore structure obtained by freezing, which is defined as the collapse phenomenon by Pikal and Shah [13]. Collapse temperatures of simple aqueous solutions of disaccharides, some protective agents widely added to bacterial suspension before lyophilisation are low and usually 1–3°C higher than their Tg′ value [13]. Fonseca et al. [7] have shown that the collapse temperature (Tcoll) *of Lactobacillus bulgaricus* suspensions, determined by freeze-drying microscopy, is different from the glass transition temperature (Tg′). Lactic acid bacterial cells gave a kind of "robustness" to the freeze-dried product and the collapse of the dried product during sublimation occurred at a temperature value 10°C higher than the Tg' value. In other words, if sublimation is carried out above Tg', then the amorphous phase will undergo viscous flow that remains undetectable (i.e., microcollapse) due to the rigid matrix provided by the bacterial cells. However, if the physical stability of the cake is preserved, bacteria degradation might exist because of the increase of molecular mobility related to the viscous flow of the amorphous phase. While there has been much speculation over the years regarding the need to lyophilize below Tg′ to maintain acceptable biological stability, few data are available, and the storage stability of the lyophilized biological products has been

largely ignored. Previous works on a strain of *Streptococcus thermophilus* have shown that performing sublimation at high product temperature close but lower than the collapse temperature resulted in higher acidification activity recovery after lyophilisation [8]. The very low Tg' value of the bacterial suspension (-37°C) made it impossible to perform a lyophilisation cycle at a product temperature lower than Tg'. The acidification activity increases to an asymptotic value of 50%, when product temperature gets closer to Tcoll. Our hypothesis was that applying higher product temperature values results in higher drying rates, thus minimizing the time the product temperature exceeds the glass transition temperature where molecular mobility is increased. Our objective was to define an empirical model describing the loss rate of acidification as a function of the temperature difference between the product temperature and the glass transition temperature of the maximally freeze-concentrated phase (Tg'). By associating this model to the heat and mass transfer model of the freeze drying process developed by Trelea et al [17], the prediction of the loss of acidification activity following the stabilisation process will be possible.

MATERIALS & METHODS

Production of concentrated and protected lactic acid bacteria suspensions

The lactic acid bacteria strain, *Lactobacillus delbrueckii sbsp. bulgaricus* CFL1, was obtained from the stock culture of the Laboratoire de Génie et Microbiologie des Procédés Alimentaires (INRA, Thiverval-Grignon, France) and used for all experiments. Inocula were stored at –80°C. Cultures were grown in supplemented whey medium (60 g.L-1 whey, 20 g.L-1 lactose, 5 g.L-1 yeast extract) in a 15 liters fermentor at 42°C. The pH was controlled at 5.5 by addition of 1.44 M NaOH. Cells were harvested by centrifugation (17000×g, 30 min, 4°C) at the end of the exponential growth phase, when the NaOH consumption rate started to decrease. After an intermediate storage period of 30 minutes at 4°C, concentrated cells were re-suspended at 4°C in a 1:2 cells/protective medium ratio. The protective medium was composed of 200 g.L-1 of sucrose and 0.15 M of NaCl. The final protected bacterial suspensions were aliquoted in plastic containers of 150 mL and frozen at -80°C in a cold air chamber.

Development and validation of a model describing the acidification activity loss rate during the freeze-drying process ("the biological model")

About 150 mL of the final protected bacterial suspension were thawed in a temperature controlled bath at 42°C, dispersed into 50 mm diameter stainless steel container (15 ml filled volume) and freeze-dried in a SMH 90 freeze-dryer (Usifroid, Maurepas, France). Conservative lyophilisation conditions were applied. Samples of freeze-dried bacteria were equilibrated at 25°C under different values of relative humidity (0, 11, 23, 33, 53, 75, 84%). After equilibration, glass transition temperature (Tg), water content and water activity (aw) were determined. The acidification activity and the viability of lactic acid bacteria were measured just after equilibrating the freeze-dried samples and after various times of storage (7, 10 and 30 days).

To validate the empirical model describing the degradation of the biological activity of bacteria during the process, various freeze-drying cycles were performed. About 450 g of the final protected bacterial suspension were thawed in a temperature controlled bath at 42°C, dispersed in a stainless steel tray and placed on the shelf of a pilot freeze-dryer (Telstar, Terrassa, Spain). The lyophilisation cycle consisted in freezing at –50°C (shelf cooling rate at 0.9°C/min and holding at –50°C for 2 h), followed by primary drying (sublimation) under various conditions of shelf temperature and chamber pressure ((-20°C; 20 Pa) and (0°C; 20 Pa)) and finally, a secondary drying phase (desorption) of 10 h at 25°C and 20 Pa. The heating rate of the shelf temperature applied during the primary and the secondary drying steps was fixed at 0.25°C/min. Five thermocouples (type K) were inserted at the bottom and at various location of the tray. Comparative pressure measurement (Pirani gauge versus capacitance manometer readings) was used to determine the sublimation end point and to allow the transition to secondary drying [12]. After secondary drying, the vacuum was broken by injection of dry air, and the freeze-dried bacteria were rapidly packed under vacuum in aluminum laminate bags. The biological activity of bacteria was determined after each step of the freeze-drying process and during the storage of the freeze-dried bacteria at 25°C.

Biological activity measurement

The samples were rehydrated in skim milk to the initial dry matter of the protected bacterial suspension before freeze-drying. Viability *of Lactobacillus bulgaricus* CFL1 was determined by plate assays on MRS (Biokar Diagnostics, France) agar plates. The Petri dishes were incubated under anaerobic conditions at 42°C for 48 h before counting.

The acidification activity of 100-µL samples was measured in milk at 42°C, in triplicate, using the CINAC System.[4]. The time necessary to reach the maximum acidification rate in milk (tm, in minutes) was used to characterise the acidification activity of the bacterial suspensions. The higher the tm, the longer the latency phase and the lower the acidification activity. The acidification activity was measured after samples equilibration at various relative humidity conditions and after various time of storage at 25°C of the equilibrated samples.

Thermal analysis and freeze-drying microscopy

Differential scanning calorimetry (DSC) measurements were performed on two different power compensation DSC equipments (Perkin Elmer LLC, Norwalk, CT, USA) depending on the moisture content of the samples: a Pyris 1 equipped with a mechanical cooling system for the low moisture content samples exhibiting thermal events at the higher temperatures (>0°C) and a Diamond equipped with liquid nitrogen cooling accessory for the high moisture content samples (lower temperatures). About 10 mg of each sample was placed in 50 µl Perkin Elmer DSC aluminium pans. An empty pan was used as a reference. Linear cooling and heating rates of 10°C.min-1 were used. The characteristic glass transition temperature (Tg) of samples was determined as the midpoint temperature of the heat flow step associated with glass transition with respect to the ASTM Standard Method E 1356-91. Results were obtained from at least four replicates.
The collapse temperature (Tcoll) was measured using a freeze drying cryo-stage (FDCS 196, Linkam Scientific Instruments, Surrey, UK) equipped with a liquid nitrogen cooling system and a programmable temperature controller according to the method described by Fonseca et al. [7]. The collapse temperature (Tcoll) determined corresponded to the lowest temperature at which overall loss of the initial frozen structure occurred during freeze-drying. The precision of the temperature measurements was 1°C, corresponding to the temperature difference between two steps.

RESULTS & DISCUSSION

Empirical model describing the degradation of acidification activity during the freeze-drying process

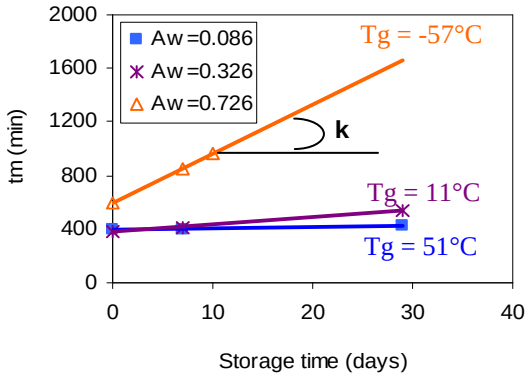

Figure 1. Acidification activity (t_m) of lyophilized *Lactobacillus bulgaricus* CFL1 in a sucrose matrix as a function of storage time at 25°C for different values of water activity (0.086; 0.326; 0.726).

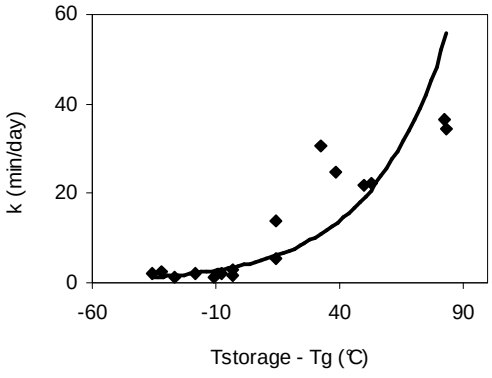

Figure 2. Rate of loss in acidification activity during storage at 25°C of lyophilized *Lactobacillus bulgaricus* CFL1 in a sucrose matrix (k in (min.day^{-1})) as a function of temperature difference between storage temperature (Tstorage = 25°C) and glass transition temperature (Tg)

Figure 1 displays the evolution of the acidification activity, characterized by the parameter t_m, with the storage time for three relative humidity conditions. The lower the t_m value, the higher the acidification activity. As expected, the t_m value increased with the storage time and this increase in t_m appeared more pronounced for the high values of water activity. Whatever the water activity of the samples, the parameter t_m increased linearly with storage time ($r^2 > 0.99$) according to the following relationship:

$$t_m = k \times \text{Storage time} + A \qquad \text{Equation 1}$$

Where k is the slope of the regression line (in min.day^{-1}) and represents the rate of loss in acidification activity during storage. A higher slope indicated a faster decrease of the acidification activity and, consequently a lower resistance to storage under various relative humidity conditions. Previous studies have

already described the acidification activity loss with storage time as a linear relationship for frozen lactic acid bacteria concentrates [6, 16].
Due to the plasticizing effect of water on the glass transition temperature (Tg), the Tg value of the freeze-dried bacteria decreased with increasing value of relative humidity. For water activity values higher than 0.24, the Tg value became lower than the storage temperature (25°C). Consequently, for the higher water activity studied (0.326 and 0.726) the freeze-dried bacteria are in a viscoelastic and unstable state thus explaining the higher degradation rates observed in the acidification activity.
The rate constants of loss of acidification activity k at storage temperature of 25°C (Tstorage) are plotted in Figure 2 as function of the temperature difference between Tstorage and the Tg of the product. Storage of the co-lyophilized matrix of *Lb bulgaricus* CFL1 and sucrose below Tg, where the molecular mobility is sharply reduced due to the very high viscosity of the amorphous state, resulted in very low rates of loss of acidification activity (low k values). The acidification activity loss rate increased with increasing temperature difference (Tstorage – Tg), corresponding to a progressively higher molecular mobility. The following exponential model was used for describing the loss of acidification activity as a function of temperature:

$$k = k_0 e^{k_1 (T_{storage} - Tg)} \qquad \text{Equation 2}$$

With k_0 = 3.88 min.day^{-1} and k_1 = 0.032 K^{-1}
To model the degradation of acidification activity of bacteria during the freeze-drying process, two assumptions were made: i) a linear relationship between t_m and freeze-drying time, in a similar way t_m varies with the storage time, and ii) the degradation rate k is an exponential function of temperature difference between the product during freeze-drying and the glass transition temperature Tg, similar to the relationship observed between k and Tstorage-Tg (eq 2). The final model describing the degradation of acidification activity of bacteria during the freeze-drying model was thus:

$$\frac{dt_m}{dt} = k_0 e^{k_1 (T_{product} - Tg)} \qquad \text{Equation 3}$$

<u>Simulation of the loss of acidification activity during the freeze-drying process</u>
Equation 3 was integrated in the heat and mass transfer model of the freeze drying process developed by Trelea et al [17], which takes into account glass transition as quality criteria of bioproducts. Briefly, the model considers one-dimensional heat and mass transfer along the shelf-product-chamber axis. At the sublimation front, the vapor pressure is assumed to be in equilibrium with the local ice temperature, and the net heat flux is assumed to contribute mainly to ice sublimation thus introducing an unavoidable coupling between heat and mass transfers. The model can accurately represent both the primary and secondary drying stages and the gradual transition between them. The evolution of the glass transition temperature with moisture content was modelled by the classical Gordon-Taylor equation, slightly modified to take into account the frozen layer. During all the sublimation stage, the glass transition temperature was kept constant and equal to the glass transition temperature of the maximally freeze-concentrated phase (Tg'). When the sublimation of ice was finished, the glass transition of the product increased with the removal of unfrozen water. Since the original freeze-drying model was developed for pharmaceutical products, all the parameters of heat and mass transfers and product properties were estimated for the bacteria product. Figure 3 displayed the simulation results of the evolution of the acidification activity (t_m) during the freeze-drying process for various combinations of operating conditions (shelf temperature and chamber pressure) applied during the sublimation step. All the four operating conditions combinations resulted in values of product temperature during the sublimation step lower than the collapse temperature (Tcoll = -27°C). However, only the operating conditions (-30°C; 20 Pa) made it possible to maintain product temperature below the glass transition temperature of the maximally freeze-concentrated phase during sublimation (Tg' = -36°C). Figure 4 illustrates the evolution of product temperature and shelf temperature during the sublimation step for both experimental conditions and the position of collapse temperature (Tcoll) and glass transition temperature of the maximally freeze-concentrated phase (Tg').
The initial value of the acidification activity t_m was arbitrary fixed at zero for clarity. The model thus represents additional degradation due to the freeze-drying step. The actual initial t_m value of the bacterial suspension before freeze-drying is highly dependent of the conditions of fermentation and concentration processes. The experimental validation of the simulation results is very difficult because many non identified physical and chemical events other than the thermal history of the product during the process are responsible of cellular damages resulting in important loss of acidification activity [2, 5, 9, 15]. During the two

experimental conditions illustrated in Figure 4, some products were sampled at the end of the sublimation step for measuring the acidification activity. The removal of unfrozen water leading to stresses on bacterial cells has not yet started at that stage of the process (or remained very limited). Consequently, we might assume that the difference of acidification activity between the two experimental cycles considered was mainly due to the thermal history of the product during the sublimation step. A higher acidification activity was observed for the combination of operating conditions (25°C; 20 Pa) with a gain of 14 min compared to the combination (-20°C; 20°C). This experimental result is in quite good agreement with the simulation results since the model predicted a difference of 19 min between both combinations of operating conditions. When considering the most conservative couple of operating conditions (-30°C; 20 Pa) resulting in product temperature never exceeding the Tg' value during the sublimation step, the highest degradation of the biological activity of bacteria was observed with a loss of acidification activity of 80 min. The high drying time required to complete the freeze-drying process, i.e. 43 hours (compared to 26 hours, 14 hours and 9 hours for the combinations (-20°C; 20 Pa), (0°C; 20 Pa) and (25°C; 20 Pa), respectively) can explain the important loss of acidification activity observed.

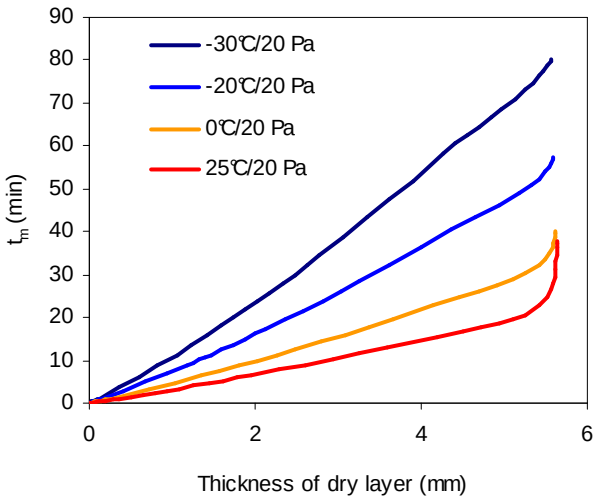

Figure 3. Simulation results of the evolution of the acidification activity (t_m) of lactic acid bacteria during the freeze-drying process for various operating conditions applied during the sublimation phase. The initial t_m value was arbitrarily fixed at zero at the beginning of the process.

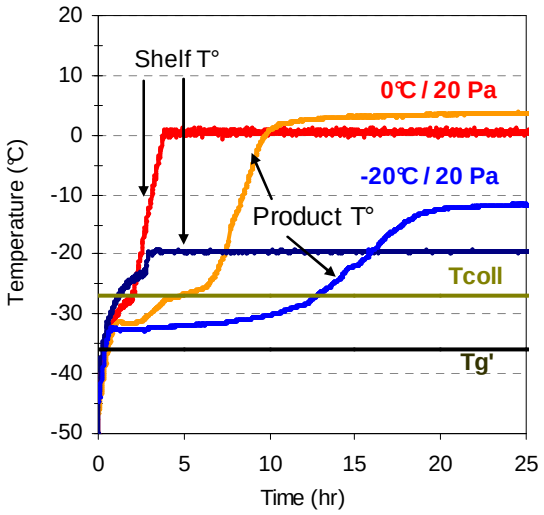

Figure 4. Shelf and product temperature profiles obtained during the sublimation step of the two freeze-drying cycles performed to validate the "biological model". The values of the collapse temperature (Tcoll) and the glass transition temperature of the maximally freeze-concentrated phase (Tg') of the protected bacteria suspension are also reported.

Experimental results

Table 1 summarizes the experimental results obtained for the two freeze-drying cycles performed at different sublimation conditions: (i) (-20°C; 20 Pa) resulting in product temperature close but slightly higher than Tg' and lower drying rate; and (ii) (0°C; 20 Pa) resulting in product temperature close but slightly lower than the collapse temperature (Tcoll) and higher drying rate. Due to fixed conditions for the desorption step, similar values of residual moisture content were obtained for both cycles.

Table 1. Degradation of the acidification activity (t_m) during the freeze-drying process for two combinations of shelf temperature and chamber pressure

	Primary Drying (Sublimation) conditions	
	-20°C / 20 Pa	**0°C / 20 Pa**
t_{m1}before freeze-drying (min)	261	240
t_{m2} after freeze-drying (min)	475	366
$dt_m=t_{m2} - t_{m1}$ (min)	214	126
k (min/days) at 25°C	2.76	1.98

t_m: time necessary to reach the maximum acidification rate in milk.
k: rate of loss of acidification activity during storage at 25°C

The lower losses of biological activity were observed when the drying steps were performed at higher values of product temperature exceeding the glass transition temperature. Applying higher values of product temperature resulted in higher drying rates, thus minimizing the time the product temperature exceeds the glass transition temperature and the product remains at increased molecular mobility. It is currently assumed that in high molecular mobility conditions the degradation reactions are accelerated. When comparing both experimental cycles, a higher t_m difference (dt_m) was obtained experimentally (214 – 126 = 88 min) than by simulation (19 min) due to additional stresses occurring during the desorption step of the process.

CONCLUSION

The process optimization requires not only minimizing the process time but also maximizing the acidification activity recovery. The product thermal history during the freeze-drying process has a significant impact of the acidification activity of bacteria. A modelling approach integrating the degradation of the activity of lactic acid bacteria was developed and could be used for optimizing the process.

ACKNOWLEDGEMENTS

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