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## Study and Optimization of Freeze--Drying Cycles of Model Probiotic: Morphology Characterization of Lyophilisates by SEM

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### **Abstract**

*These experimental data are concerning the optimization of freeze-drying cycles of a model type Casei probiotic bacteria with water based formulations using lactose and polymer polyvinylpyrrolidone (PVP) as cry/lyo protectants.*

*After a previous study devoted to the influence of the main freeze-drying parameters (freezing rates; shelf temperature; total gas pressure) on the bacteria survival ratios, this paper reports some morphological data obtained by scanning electron microscopy (SEM) with the final lyophilisates.*

*The numerous and original SEM images realized with different formulations of water binary or ternary mixtures with fixed operating conditions proved to be very useful to explain and to understand the different bacteria survival ratios observed.,*

*Thus, with bacteria or micro-organisms systems, this powerful investigation method of characterization of lyophilisates morphology should be more largely used in the future for setting up the optimal formulations parameters (composition) and the connected operating physical parameters of freeze-drying steps (freezing rate, shelf temperature, total gas pressure).*

**Keywords:** *freeze-drying, probiotics, SEM, lyophilisate morphology characterization..*

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## 1. Introduction

These last decades, the number of researches on the probiotics has been increased in the food and pharmaceutical industries.

Unfortunately, probiotics are thermosensitive bacteria, which loss a part of their viability after their manufacturing, storage and transport processes. Many factors such as temperature, oxygen level, osmotic, pressure and pH stresses, etc. have important impacts on the viability ratios and, by the way, on the health promoting effects [1]. Probiotics are usually stabilized by two main techniques, namely by freezing (cryo-preservation) and by drying techniques such as spray-drying, fluidized bed drying and freeze drying [2].

Freeze-drying is known as the method that usually leads to the best results in term of many quality attributes of the freeze dried cake (viability ratios, rehydration rate, etc.) despite the various complex and damaging effects generated by the freezing and sublimation/desorption phenomena (crystal growth; cryo-concentration, etc.) [2]. Nevertheless, this long and complex process, has to be carried out at very low levels of temperature and total gas pressure during which living bacteria have to endure numerous stresses more or less interdependent and very deeply understood (thermal, osmotic, pH, sub-cooling, intra and extra-cellular crystallization, etc.) [3].

That's why it is necessary to define protection strategies in order to carry out this process by trying to minimize these losses as far as possible [4].

For our study, we have selected the strain *Lactobacillus casei* ATCC 393 as a model probiotic bacteria [5]. Simultaneously, the viability of the bacteria in the drying medium was assessed with the addition of two types of cryo-protectants. First, the lactose, a well-known disaccharide used as pharmaceutical excipient as well as an ingredient for dairy or nutrition? products for its protective effect [6]. Next, the polyvinylpyrrolidone (PVP), a pharmaceutical grade excipient widely used in different pharmaceutical formulations [7]. The combination of these two protectants offer an interesting perspective for long time conservation, because the PVP is known as a glass forming agent with a high Tg value, and, moreover, it can inhibit the lactose crystallization [8]. The phase diagrams of water mixtures of these three components have been previously deeply investigated in our laboratory by determining the vitreous transition curves of these formulation leading to the Tg' values which are known to be key parameters for setting up the optimal sublimation and desorption conditions [2],[9]. First, we investigated some freezing protocols (cooling's rate, nucleation temperature, annealing treatment) leading to the highest survival ratios [10]. Next, with the preselected formulation and the optimal freezing rate we studied the sublimation step by investigating the influence of the sublimation operating parameters, namely the total gas pressure inside the sublimation chamber and the shelf temperature on the bacteria survival ratios and on the sublimation times

Finally, in the last part of our project, the morphology of the freeze-dried cakes was investigated by using Scanning Electron Microscopy (SEM) images in order to observe the location of the bacteria inside the glassy matrix and to verify their integrity, their shape and



their characteristic dimensions. These data that contribute to explain some defects in the main quality attributes (survival ratios) of the final lyophilisates. are presented in this paper.

## 2. Materials and Methods

### 2.1 Material

MRS broth (Man Rogosa Sharpe) and bacteriological agar type E was purchased from Biokar Diagnostics (Beauvais, France). Kollidon®30 was generously donated by BASF (Ludwigshafen, Germany). The monohydrate lactose was provided by Cooper (Melun, France) and the ultra-pure sodium chloride (NaCl) was obtained from Across Organics (Geel, Belgium).

### 2.2 Bacteria and growth conditions

The strain *Lactobacillus casei* ATCC® 393™ (Alliance Bio Expertise, Guipry, France) was rehydrated from the Kwik-Stik®. All other details of the bacteria culture and preparation are given in our previous paper [10].

### 2.3 Formulation composition

A concentrated bacteria was suspended into different protectant solutions before processing. These solutions were made with a Kollidon®30 and monohydrate lactose water solutions and with mixtures of these two cryoprotectants (from 1% to 10% (w/w) in the final formulations) or only with the saline buffer in order to target the best quality parameters after process. These compositions of water binary or ternary mixtures of lactose and PVP and their referred labels are presented on Table 1 hereafter.

Nomenclature	Weight PVP composition (m/V)	Weight Lactose composition (m/V)
K5	5%	0%
L5	0%	5%
KL10-5	10%	5%
KL1-1	1%	1%
KL5-5	5%	5%
KL10-10	10%	10%

Table 1. Index of the different lactose and PVP formulations investigated

### 2.4 Freezing and freeze-drying parameters

All the experiments were carried out by using 1mL of bacterial suspension filled into 6mL standard glass vials. The formulations were cooled from ambient temperature until - 45°C at the less harmless cooling rate equal to 0.5°C/min [10]

The lyophilization of the bacteria suspension was realized using a pilot freeze-dryer (Cryotec, Saint Gely du Fesc, France) with a control shelf temperature and a total pressure regulation devices. All the other details of our freeze-dryer are given in one of our previous papers on these topics [2,10].

In order to investigate the impact of the type and amount of solute on the viability ratios and on the quality of the freeze-dried cake we chose very soft sublimation and desorption

protocols. Sublimation was performed slowly under vacuum at selected values from 10 to 15 Pa until the pressures indicated by the Pirani and by the capacitive gauges reached the same values and when the products temperature inside the instrumented vial started to rise. The secondary drying step was achieved for 24 h at 20°C and 5 Pa. After the end of the process, vials were closed inside the freeze-dryer and hermetically sealed and stored at 4°C.

### **2.5 Enumeration of viable bacteria**

The freeze-dried bacteria were rehydrated at 20°C by addition of the same amount of saline solution as the weight loss during freeze-drying and immediately diluted and plated on MRS agar plate. The survival bacterial rate was obtained by counting 30 to 300 colony forming unit before and after the process. Every enumeration was conducted in triplicate.

### **2.6 Scanning Electron Microscopy (SEM)**

Scanning Electron Microscopy was performed with a FEI Quanta 250 FEG microscope at the “Centre Technologique des Microstructures” (CTμ) at the University of Lyon (Villeurbanne, France). The freeze-dried cakes were cut with a scalpel to explore the state of the section and deposited on a flat steel holder. Freeze-dried cakes were deposited on a flat steel holder. The sample was coated under vacuum by cathodic sputtering with copper. The samples were observed by SEM technique under an accelerating voltage of 5 kV. Images were then analyzed with the software ImageJ.

### **2.7 Statistical analysis**

The statistical significances of the differences in viability of the freeze-dried probiotics in their protective media were analyzed using an ANOVA-test. This statistical analysis was performed using Microsoft Excel 2016). The statistical tests were achieved with a significance level  $\alpha = 0.05$ .

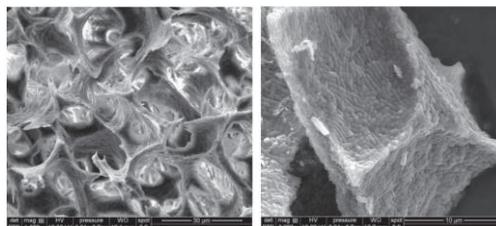
## **3. Results and discussion**

### **3.1 Effect of formulation composition on lyophilisate morphology**

In order to try to explain the observed viability results presented in a previous paper [10] we have determined by Scanning Electron Microscopy (SEM) the morphology of the lyophilisates obtained after the sublimation realized in the same operation conditions of freezing and of sublimation. This characterization was realized with two types of samples, firstly with a core cut of the lyophilisates and secondly with grinding samples of lyophilisates. These experimental data have been successively obtained with saline solution with the two binary water formulations K5 et L5 and with ternary water formulation KL10-5, KL1-1 KL5-5 and KL10-10

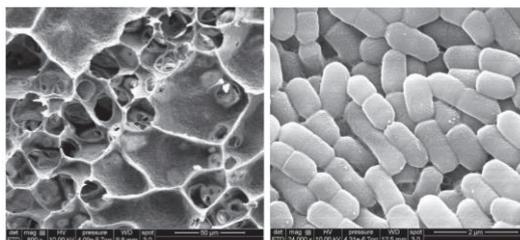
**Saline solutions:** The images obtained with the saline solution are presented on Figure 1. The vitreous phase is clearly visible and seems constituted essentially with bacteria clustered together during the ice crystallization. The right image shows that the bacterial structure was maintained after grinding so that it seems quite robust despite of the low solute content. This means that freeze-dried bacteria are forming a solid network without the presence of any protective solute.





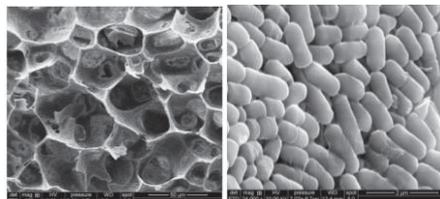
*Fig. 1 SEM picture of saline bacteria formulation. Solid lyophilizate magnification X 1 600 (left) and grinded lyophilizate X 6 000 (right).*

**PVP formulation – K5:** The images of figure 2 show the same porous morphology as with the saline solution with a dendritic structure of the pores directed from the bottom to the top of the freeze-dried cake along the direction of the heat flux during the freezing step. Nevertheless, the pore size is not uniform and the polymer seems not visible on this image [2]. The same image with a larger zoom, presented on the right picture, shows clearly a bacteria cluster - with probably their original shapes and sizes - and some cavities corresponding probably to the bacteria that took down from the pores surface during the grinding operation.



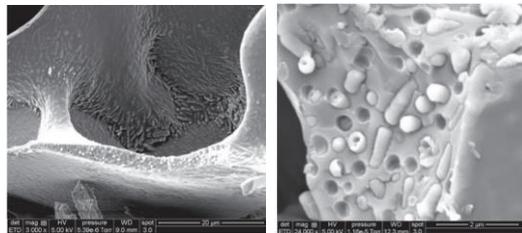
*Fig.2 SEM picture of K5 formulation containing bacteria, solid lyophilizate magnification X 800 (left) and grinded lyophilizate X 24 000 (right).*

**Lactose formulation – L5:** The images obtained with this formulation, presented on Figure 3, are very similar to the images describing the formulation K5. The vitreous porous structure covered with the bacteria can be clearly observed, but nevertheless this structure appears thinner and more fragile than with formulation K5. The right image, presented with a larger zoom shows again a bacteria cluster with some little aggregates of solid matter around a few bacteria, aggregates that could correspond to lactose cryo-concentrated vitreous phase.



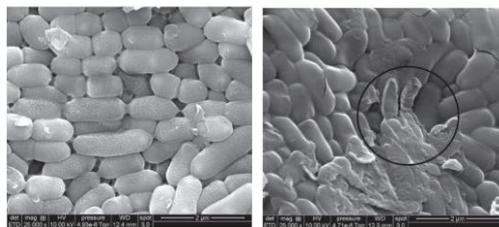
*Fig.3 SEM picture of L5 formulation containing bacteria, solid lyophilizate magnification X 800 (left) and grinded lyophilizate X 24 000 (right).*

**Lactose+PVP formulation. KL10-5:** The images of Figure 4 show the porous morphology of KL10-5 lyophilisate. This ternary water formulation with the two cryo-protectants is thicker than the previous ones. The bacteria are more or less inserted inside the vitreous phase of the solid matrix constituted by the two cryoprotectants. Thus, this structure could explain the long-term high values of bacteria stability (viability ratios) previously observed with this formulation [10], the bacteria being still preserved from oxidation degradation reactions. The morphology observed with grinded samples is also presented on the right of this Figure 4. This configuration of dense bacteria network could explain why the bacteria are more impacted by the freeze-drying process than in the case of lactose formulation L5 because the higher proximity between bacteria could lead to lower survival ratio. However, this statement needs to be verified.



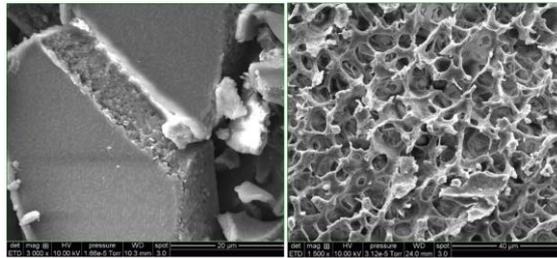
*Fig.4 SEM picture of KL10-5 formulation containing bacteria, solid lyophilizate magnification X 3000 (left) and grinded lyophilizate X 24 000 (right).*

**Mixed Lactose +PVP formulation – KL1-1 :** The morphology of this formulation is presented on the images of Figure 5 at high zoom. On the right image one can observe a fine layer of matter that could proceed from the bacteria surface (CF formulation L5). Nevertheless, these characteristics cannot explain the low values of the viability ratios observed with this composition. However, the freeze-dried cake macroscopic structure was not very mechanically strong, probably due to the low amount of solute in the solution.



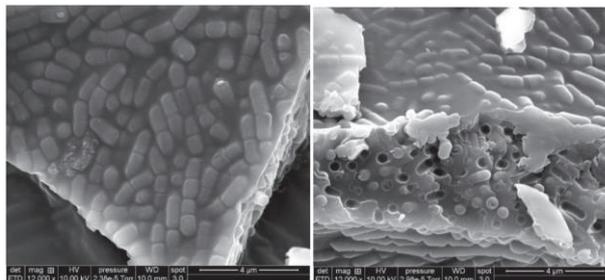
*Fig. 5 SEM picture of KL1-1 formulation containing bacteria, magnification X 25 000 (left) and X 25 000 (right). Grinded lyophilizate.*

**Mixed Lactose+ PVP formulation – KL5-5 :** The images of Figure 6 show that the structure of KL 5-5 formulation is quite similar with the structure observed with the formulation KL 10-5. These two cryo-protectants formulation, at these concentrations, led to a thick and robust porous matrix inside which the bacteria are deeply inserted.



*Fig.6 SEM picture of KL5-5 formulation containing bacteria, magnification X 3 000 (left) and X 1500 (right). Solid lyophilizate.*

**Mixed Lactose + PVP formulation – KL10-10:** The images of Figure 7 show that this formulation presents the same morphological characteristics as the formulation KL 10-5 and the previous KL 5-5 with inserted bacteria inside the vitreous solid phase of the porous matrix. But these morphologies cannot explain the significant differences on bacteria survival ratios previously observed on the final lyophilisates with these formulations [10].



*Fig.7 SEM picture of KL10-10 formulation containing bacteria, magnification X 12 000 (left) and X 12 000 (right). Grinded lyophilizate.*

### 3-2 Global comparison

Finally, all these data shows that with mixed formulations containing both PVP and lactose, the bacteria appear to be included and completely covered by the solid matrix. While for the formulation containing only lactose or PVP, the bacteria appeared to form themselves the solid matrix. . This could explain the survival which is lower with the mixture PVP+lactose than with the lactose alone. When the matrix is formed during freezing, the bacteria may experience a high local pressure due to the growth of ice crystals. [2,11]. On a long time scale, the conformation of the solid matrix of the mixture KL10-5 could allow a better survival ratios due to the thick layer of solid which can prevent water and oxygen diffusion to reach the cells [4]. These results were unexpected since some previous research data seemed to consider that the freezing step was the most lethal step [12]. Nevertheless, the freezing is an important key step of the whole freeze-drying process that has to be controlled precisely because it govern directly the upcoming drying steps [2,11, 12].

### 3.3 Influence of the amount of solute protectants on the cells viability

In the case of low amounts of excipients, the bacteria could directly be exposed to water during the rehydration process. Indeed, the rehydration rate had probably a great influence on the final viability ratio of the cells and the amount of solute exerted probably an important osmotic pressure buffer effect on the mechanisms of water transfer to the cells. In another hand, a too important content of excipients could unbalance the osmotic equilibrium of bacteria with their surrounding solution during the formulation and freezing steps and, by the way, could generate too important water flows crossing the cells walls which resulted to important cell death ratios or viability losses. That's why a balance should be established between too low content of cryo- or lyo-protectant resulting in a loss of efficiency and, a too high content, providing high stresses to the cells. Anyway, more experiments are still necessary to conclude on these hypotheses. Nevertheless, the KL5-5 formulation presented the highest survival ratios with this soft freeze-drying protocol [13].

#### **4. Conclusion**

This study presents the effect of composition of PVP (Kollidon®30) and lactose based formulations on the morphology of a *L. Casei* 393 strain after freezing and after freeze-drying. SEM images showed that the type and the concentration of solute in the cells formulation have an important impact on the bacteria location inside the freeze-dried matrix and, consequently, on the survival ratio. *L. Casei* bacteria entrapped in a glassy matrix showed better stability results than those located on the surface. The KL5-5 formulation led to the best bacteria survival ratio with less than 0.5 log reduction after a standard freeze-drying cycle and less than 0.9 log reduction after 6 months of storage at 4°C. Nevertheless, it is still difficult to conclude on the mechanisms explaining the best observed viability at this composition. The KL10-10 formulation appeared to be more stable in the time but did not present the highest values of bacteria survival ratios just at the end of freeze-drying process. We believe that optimal conditions should be looked for between the amount of these protectants in the formulation and physical operating parameters of the process (shelf temperature and total gas pressure).

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