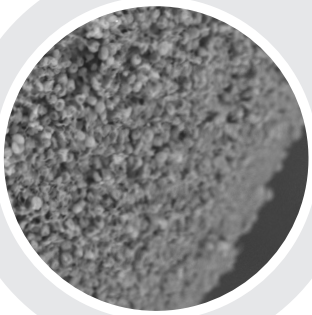
Application Note
No. 294/2017

Freeze drying of beads containing yeasts

Lyovapor™ L-200 Pro



1. Introduction

Freeze drying or lyophylisation is a very well-known dehydration method commonly used to preserve microorganisms, food or pharmaceuticals such as protein-based drugs. It can create high quality final dry products by combining freezing and drying in a unique operation[1].
Freeze drying is regularly used to preserve microbial culture collections[2,3] since it offers non-negligible advantages such as the convenience of storage and the possibility to transport the microorganisms by mail[4]. Moreover, the product only requires low maintenance, the cultures are protected from contamination during storage and the microorganisms remain viable for long periods of time[2].
It is however common knowledge that freeze drying is critical for microorganisms as it affects both their viability and physiological state negatively[1]. A large variety of survival rates can be found depending on methods and organisms; viability levels are however significantly lower than for liquid nitrogen storage[2]. The observed decrease in viability is mainly due to some undesirable side effects such as the formation of ice crystals within the cells[1], the denaturation of sensitive proteins or some irreversible changes in the physical state of the membrane lipids during the process[3,5]. In order to prevent such effects, protective substances such as skim milk, sucrose, glycerol, DMSO or trehalose are commonly used before freezing or freeze drying[1,3].
Trehalose is reported to exert a protective effect on yeasts and bacteria under extreme environments such as desiccation, freezing, osmotic stress and heat shock. Those protective effects are linked to the stabilization of membranes and the preservation of enzyme activity. Several hypotheses concerning trehalose protective effects have been reported. Some reports assume it acts by replacing water molecules involved in the maintenance of the tertiary structure of proteins through multiple external hydrogen bonds, others that it forms glassy structures which assure physical stability; specical interactions at molecular level therefore probably have to operate to assure physical stability[3,6].
Next to fermentation processes or transformation of foods, microorganisms such as *Saccharomyces cerevisiae* or lactic acid bacteria are of economic importance in the field of probiotic dietary food and feed supplements. These applications however require the preservation of cell viability during storage[7]. By combining granulation and freeze drying, dust free particles homogeneous in size and composition can be obtained. This will enable a good particle flowability, an easier dosage and a faster reconstitution of the product due to a higher surface area. Despite the above challenges, freeze drying remains a convenient method of preserving yeasts, sporulating fungi and bacteria since the long term viability remains usually rather good and the requirements for storage and distribution of the strains are quite simple[8].
This Application Note therefore aims to produce *Saccharomyces cerevisiae* particles as a model microorganism using the Encapsulator B-390 as a granulator to prill the yeasts suspension into liquid nitrogen and form monodispersed beads that will then be freeze dried using the Lyovapor™ L-200.

2. Equipment, Chemicals and Material

- Equipment
  - ESCO NordicSafe, Biosafety Cabinet Class II
  - BUCHI Encapsulator B-390
  - BUCHI Lyovapor™ L-200 Pro
  - BUCHI Lyovapor™ Software
  - BUCHI Lyovapor™ Drying chamber with heatable shelves

- Chemicals
  - YPD Medium, Sigma Aldrich
  - Trehalose, Sigma Aldrich
  - Skimmed milk powder
  - Agar
  - Deionized water
  - Liquid nitrogen

- Material
  - Glass petri dishes
  - Benchtop liquid nitrogen container

3. Experimental

The work described in this Application Note was performed under aseptic conditions. 84 g of commercially available baker's yeast were suspended in 50 mL of sterile YPD Medium (Sigma Aldrich). 50 mL of sterile lyoprotectant medium containing 5 g of trehalose (Sigma Aldrich) and 5 g of skimmed milk in deionized water was then added to the yeast suspension before extrusion with the Encapsulator B-390 (Table 1). The extruded droplets were collected and frozen in a liquid nitrogen bath before being transferred in stainless steel trays and stored in a -25°C freezer until freeze drying.

Table 1: Encapsulation parameters

	300 µm nozzle	1 mm nozzle
Frequency [Hz]	680	60
Electrode [V]	750	2500
Pressure [mbar]	500	500

The freeze drying steps (primary and secondary drying) were programmed using the Lyovapor™ Software as listed in Table 2. The Lyovapor™ L-200 Pro was used with the drying chamber with heatable shelves and ambient air.

Table 2: Parameters of the primary and secondary drying

Step		1	2	3	4
Phase	Loading	Primary drying	Primary drying	Secondary drying	Secondary drying
Duration hh:mm		02:00	20:00	02:00	04:00
Shelf temperature °C	-35.0	-15.0	-15.0	30.0	30.0
Shelf temperature gradient °C/min		0.17	0.00	0.38	0.00
Pressure zone		Regulated	Regulated	Regulated	Regulated
Pressure mbar		0.200	0.200	0.200	0.200
Safety pressure mbar		0.500	0.500	0.500	0.500
Safety pressure duration sec		10	10	10	10

The beads without yeasts were prepared using the same media composition and the same parameters as the beads containing yeasts.

After freeze drying, 1 mL of sterile water was added to 1 mL of beads in order to reconstitute the sample. For the bead containing yeasts, serial dilution of 10x, 100x and 1000x were performed for each reconstituted solution. The reconstituted solution and the dilutions were then plated on YPD agar plate as shown in Figure 1. The agar plate were then incubated at 28°C for 24h to evaluate cell viability.

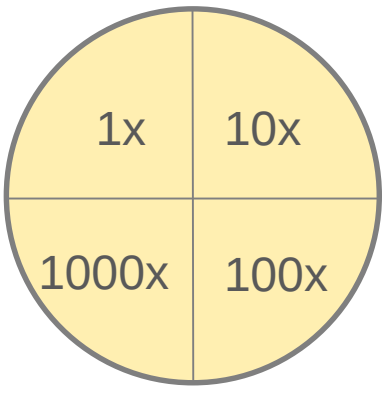


Figure 1: Yeast viability test on agar plate

4. Result and discussion

Microbeads containing yeasts can be produced by extruding a mixture made of yeasts and lyoprotectant medium in liquid nitrogen, using the Encapsulator B-390. Results showed that yeasts could be granulated by dripping it in liquid nitrogen with the Encapsulator B-390; beads of around 700 µm and 1500 µm were produced with the 300 µm nozzle and the 1 mm nozzle respectively. Similar results were obtained using solution containing lyoprotectant medium only. As shown in Figure 2, after lyophylisation the beads remained similar in shape and size than the wet frozen beads.

Figure 2: Yeast microbeads produced with the Encapsulator B-390 and the 300 µm nozzle before (left) and after (right) freeze drying

The analysis of the bead structure was performed through SEM microscopy. In Figure 3, a difference in beads morphology can be observed between beads containing yeasts (bottom) and beads made of lyoprotectant medium only (top). The beads containing yeasts exhibit a rough structure made of 5 µm agglomerated particles that can be assumed to be the microorganisms, while the beads containing only lyoprotectant have a smoother structure.

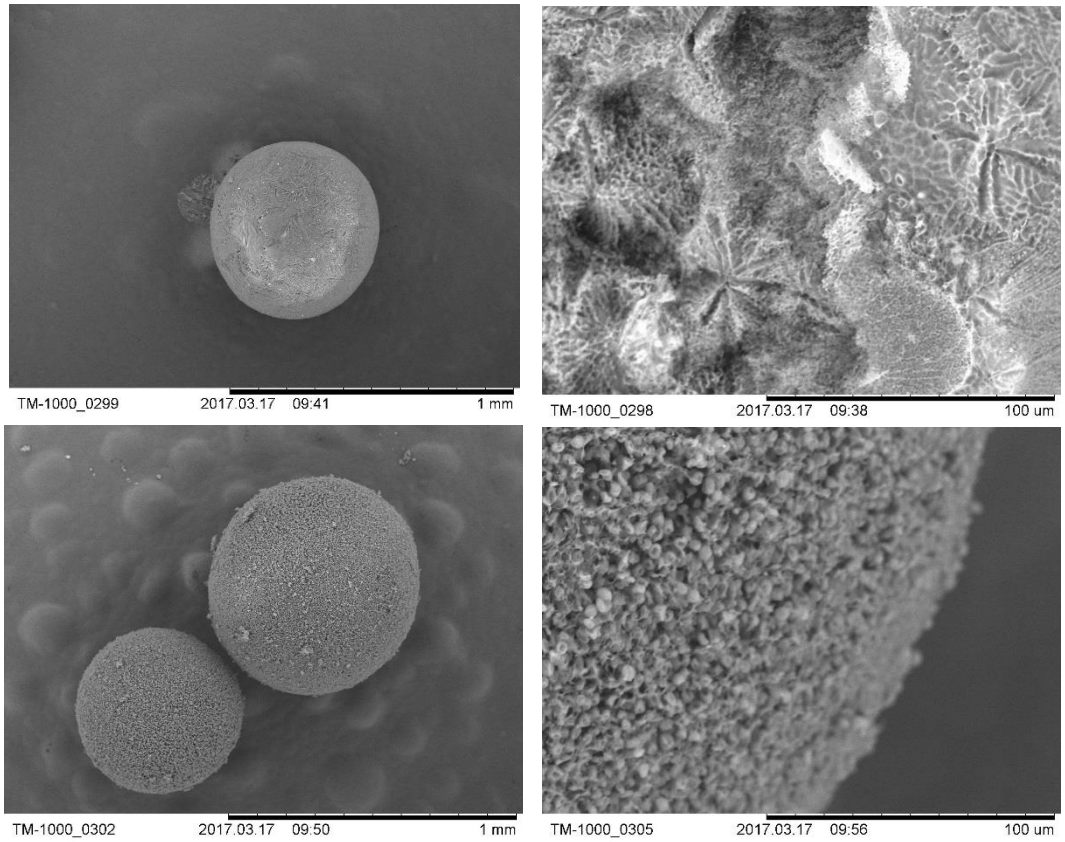


Figure 3: Structure comparison of freeze dryed beads with (bottom) and without (top) yeasts.

When subjected to freeze drying, biological systems can be damaged due to changes in the physical state of the lipids in the membrane or due to changes in the structure of some proteins[3,9]. In order to verify yeast viability, the yeasts were rehydrated, diluted and incubated on YPD-agar plate at 28°C for 24 hours. Figure 4 confirms literature reports showing that despite a loss of viability, yeast can still grow after lyophylisation[2,4,6,10].

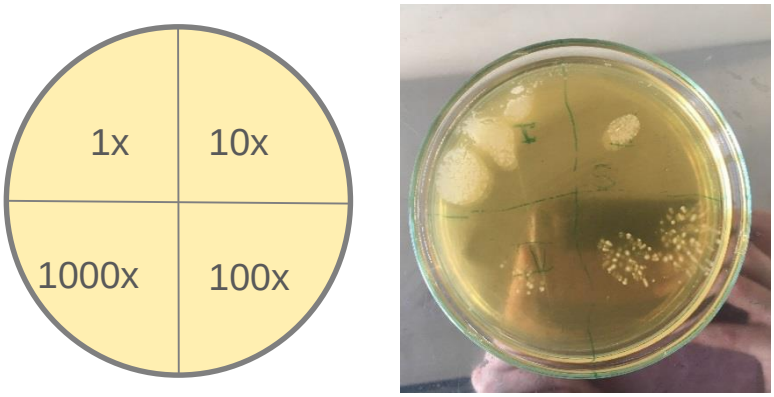


Figure 4: Yeast viability on agar plate after 24h incubation at 28°C

5. Conclusion

Yeast beads could easily be produced with the Encapsulator B-390 and freeze dryed using the Lyovapor™ L-200. Beads of 700 µm and 1500 µm diameter were obtained using the B-390 with the 300 µm and 1000 µm nozzle, respectively. No change in size and shape of the beads were observed after freeze drying. The granules had a good flowability and dosage was easily done, moreover the particles were rapidly dissolved when mixed with water. The microorganisms still kept a good viability during storage after lyophylisation and could successfully be grown again after rehydration.
The combination of granulation and freeze drying in this Application Note showed promising results. It can open new possibilities in fields such as fermentation processes and food transformation to produce culture starters that can easily be dosed and reconstituted or in the field of probiotics and food supplements to obtain a dust free, free flowing powder with homogeneous particle size and particle composition.

6. References

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