Effect of Glycerol, as Cryoprotectant in the Encapsulation and Freeze Drying of Microspheres Containing Probiotic Cells

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ABSTRACT

It is reported that probiotics provide several health benefits as they help in maintaining a good balance and composition of intestinal flora, and increase the resistance against invasion of pathogens. Ensuring adequate dosages of probiotics at the time of consumption is a challenge, because several factors during processing and storage affect the viability of probiotic organisms. Major emphasis has been given to protect the microorganisms with the help of encapsulation technique, by addition of different protectants. In this study, probiotic cells (*Bifidobacterium lactis* 300B) were entrapped in alginate/pullulan microspheres. In the encapsulation formula glycerol was used as cryoprotectant in the freeze drying process for long time storage. It was observed that the survival of *Bifidobacterium lactis* 300B when encapsulated without cryoprotectant was higher than the formula with glycerol in the fresh obtained microspheres. The addition of glycerol was in order to reduce the deep freezing and freeze drying damages. In the chosen formulations, glycerol did not proved protection for the entrapped probiotic cells in the freeze drying process, for which the use of glycerol as cryoprotectant for alginate/pullulan *Bifidobacterium lactis* 300B entrapment is not recommended.

Keywords: *probiotic, encapsulation, freeze- drying, cryoprotectants, glycerol.*

INTRODUCTION

Probiotics are defined as live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002; Makinen *et al.*, 2012). The health benefit results from the improved microbial balance in the intestine (Teitelbaum and Walker, 2002). Probiotic cells are commonly available as culture concentrates in dried or deep-freeze form to be added to a food for manufacturing or home uses. These may be consumed either as food products (fermented or non-fermented) or as dietary supplements (products in powder, capsule or tablet forms) (Makinen *et al.*, 2012; Tripathi and Giri, 2014). The encapsulation of living cells and valuable molecules is widely studied, fact that can be seen in the large amount of publications and patents in the field (Anal and Singh, 2007; Augustin and Sanguansri, 2003; Benita, 2006; Burgain *et al.*, 2011; Cook *et al.*, 2012; de Vos *et al.*, 2010; Manojlovic *et al.*, 2010; Nedovic *et al.*, 2011; Shahidi and Han, 1993; Tripathi and Giri, 2014). The encapsulation is applied mainly due to the fact that many of this probiotic cells reports low survival to various factors such as industrial processing and storage or gastrointestinal passage (Cook *et al.*, 2012; Makinen *et al.*, 2012). The most common used probiotics are *Lactobacillus* and *Bifidobacteria* (Martin-Dejardin *et al.*, 2013; Rokka and Rantamäki, 2010), leading to intensive studies. The International Dairy Federation (IFD) recommends the presence of at least 10^7 CFU/g probiotic in dairy products until the end of their shelf life. Lee et al. (Lee and Salminen, 1995) underline the same amount 10^7 CFU/g, of product present this time at the point of delivery. In order to achieve this goal the encapsulation was proposed, as a technique able to provide protection for the entrapped probiotic cells.

Despite the fact that alginate is the most used polymer for the encapsulation of the probiotics (Anal and Singh, 2007) there are a considerable number of papers that describe methods and formulas in order to increase the stability and viability of the probiotic cells in the entrapped systems. The use of various encapsulation techniques (Chan and Zhang, 2005; Song *et al.*, 2014), same techniques but different combinations of polymers (Ding and Shah, 2009), the utilization of prebiotics (Chavarri *et al.*, 2010; Krasaekoopt and Watcharapoka, 2014; Sathyabama *et al.*, 2014) and cryoprotectants (Capela *et al.*, 2006; Fang *et al.*, 2012) are just some of the systems applied in order to improve the encapsulation of probiotic cells.

A cryoprotectant is a substance that is used to protect biological tissue from damage caused by freezing. In the encapsulation of probiotics, cryoprotectants are added in order to maintain the viability of the cells during freeze drying process (Amine *et al.*, 2014; Capela *et al.*, 2006).

The aim of this study was to investigate the effect of glycerol, as cryoprotectant, in different concentrations, in the process of freeze drying of alginate/pullulan microspheres containing *Bifidobacterium lactis* 300B.

MATERIALS AND METHODS

A commercially available Manguel GMB sodium-alginate was supplied by FMC, (Norway), glycerol from Sigma (Germany) and pullulan from Hayashibara (Japan) were used in alginate based microspheres. Calcium chloride was purchased from Brenntag (Germany) sodium phosphate from Merck (Germany), bifidus selective medium agar (BSM) and vegetable peptone from Sigma-Aldrich Chemie GmbH (Germany).

All materials and solutions, including the $CaCl_2$ solution were sterilized using the autoclave at 121 °C for 15 min.

Probiotic strain

The strain used in the trial, *Bifidobacterium lactis* 300B was purchased as lyophilized probiotics powder from Howaru (USA). The probiotic was used as received from the supplier. A viability test was performed before each trial.

Preparation of alginate/pullulan and alginate/pullulan/glycerol microspheres

Lyophilized probiotic, 75 g×L^{-1}, was encapsulated by addition to a mixture consisting of 15 g×L^{-1} alginate and 15 g×L^{-1} pullulan, using a Spherisator M, type 2002SP-AE5-D0 from Brace GmbH Germany, and crosslinked in calcium chloride (40 g×L^{-1}).

The capsules were hardened for 30 min, and then rinsed with sterile sodium chloride (8.5 g×L^{-1}). The obtained microspheres were used as control. The same formulation was used for the samples with glycerol. The percentage of used glycerol varied from 10 to 40%.

The entrapment efficiency of the fresh microspheres was determined according to (Sandoval-Castilla *et al.*, 2010) with a slight change as follows:

$$\text{Entrapment efficiency} = a \times F/b \qquad (1)$$

Where "a" means CFU×g^{-1}of microspheres and "b" represents CFU×g^{-1} in the biopolymer mix before production and F is the sphere packing factor (Aste and Weaire, 2008). We considered the dense packing for all calculations 0.70.

Tab. 1. Alginate/pullulan/glycerol microspheres containing *Bifidobacterium lactis* 300B encapsulation formulation

Trial		Codification
Microspheres prepared with sodium alginate 1.5% (w/v) 1.5% (w/v) pullulan (Sample AP) and the different glycerol proportions	No glycerol	AP
	10% (w/v) Glycerol	G10
	20% (w/v) Glycerol	G20
	30% (w/v) Glycerol	G30
	40% (w/v) Glycerol	G40

The alginate/pullulan filler was used for encapsulation of the probiotic powder with added glycerol, the codification being shown in the Tab 1.

Probiotic cells viability

Non-encapsulated and encapsulated *Bifidobacterium lactis* 300B were enumerated immediately after the encapsulation respectively freeze drying process, using the plate counting method, on BSM agar. The microspheres were dissolved in sodium citrate (20 g×L^{-1}) with an adjusted pH of 7.3, before enumeration of viable cells. Ten fold dilution was performed in peptone wather (1 g×L^{-1} peptone, 5 g×L^{-1} NaCl and 1 ml×L^{-1} Tween 80). From the last three dilutions, 1 ml of the dilution was filled inside the Petri dish and then poured nutrient agar medium into it. The operation was repeated three times for each dilution. After 72 h incubation at 37°C in an anaerobic jar the number of colony forming units (CFU) was counted. Colonies of bacteria were calculated and converted to $\log_{10}$CFU.

The number of surviving bacteria in each freeze dried samples was determined using the mathematical formula:

$$survival = (n/n_0) \quad (2)$$

where "n_0" is the number of bacteria per gram of wet microspheres before freeze drying, and "n" is the number of bacteia per gram in the freeze dried microspheres immediately after freeze drying (Simpson *et al.*, 2005). All determinations were carried out in duplicate.

Freeze drying of microspheres

The fresh obtained microspheress were deep frozen at -18°C, in isopropanol, and immediately connected to a VaCo 5 freeze dryer from Zirbus (Germany) and freeze dried at -50°C and 5×10^{-2} mbar for 24h. The freeze dried material was collected in sterile recipients. Samples were analysed immediately.

Statistical analyses

The mean of two individual determinations was used to calculate cell counts. A one way ANOVA and Student's t-test was used to analyse the cell counts. Significant differences among individual means were determined using Turkey test. The statistical evaluation was carried out using Graph Prism Version5.0 (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

The preparation of microspheres containing 40% glycerol, as cryoprotectant, was not possible due to high viscosity of the obtained solution. Difficulties arose in working with the solution containing 20 and 30% glycerol too, based the same consideration. The entrapment efficiency and the size of the obtained microspheres are compared in Tab 2. The same size nozzle (700 μm) was used for all the samples.

Fresh obtained microspheres

Tab. 2. Alginate/pullulan/glycerol microspheres containing *Bifidobacterium lactis* 300B size and encapsulation efficiency

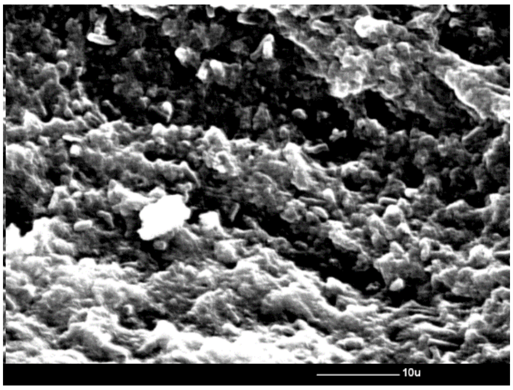
Sample	Microsphere size (μm) (n=10)	Entrapment efficiency (%) (n=10)
AP	2560±1.7[a]	65.87±0.28
G10	2675±2.4[bd]	62.17±0.08
G20	2759±9.3[c]	60.17±0.15
G30	2836±.4.7[d]	61.17±0.22
G40	-	-

The size of the obtained alginate/pullulan microspheres was influenced by the size of the nozzle, 700 μm in our study, the viscosity of the solution and the distance between the drop and the hardening bath. The viscosity of the polymer solution increased with the increase in glycerol concentration.

Consistent with a previous study (Pop *et al.*, 2012) the size of the alginate/pullulan microspheres ranged between 1400 and 1600 μm, and the encapsulation rate between 64,94 and 68,8%. The microspheres with glycerol in their composition had a size 20% higher compared with alginate/pullulan microspheres. In Fig 1 the presence of *Bifidobacterium lactis* 300B can be observed in the alginate/pullulan matrices.

Viability in the fresh microspheres

After the encapsulation process, the viability of the probiotic cells was determined in order to compare the four samples (Fig. 2). It can be observed that the survival of *Bifidobacterium lactis* 300B when encapsulated without cryoprotectant was higher than the formula with 10% glycerol, but not statistically significant higher.



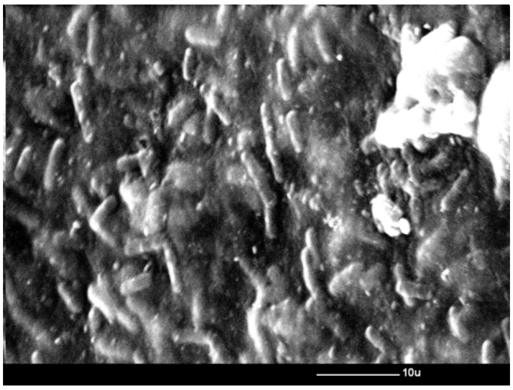


Fig. 1. Scanning electron microscope image of alginate/pullulan microsphere containing *Bifidobacterium lactis* (left 1000X, right 2000x).

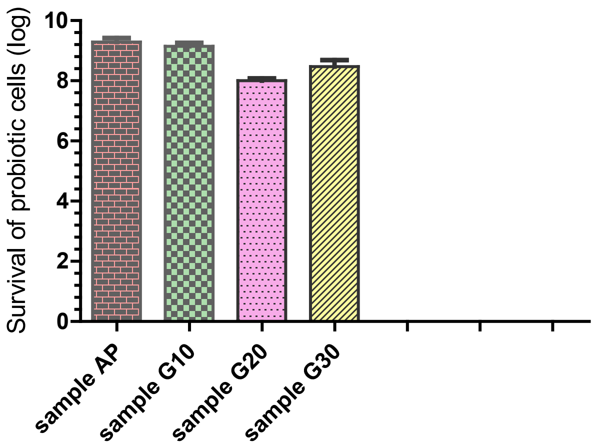


Fig. 2. Survival of *Bifidobacterium lactis* 300B after encapsulation process. Sample AP: *B. lactis* 300B in alginate-pullulan microspheres; Sample G10: *B. lactis* 300B in alginate-pullulan/10% glycerol microspheres; Sample G20: *B. lactis* 300B in alginate-pullulan/20% glycerol microspheres; Sample G30: *B. lactis* 300B in alginate-pullulan/30% glycerol microspheres. Means (n=3) ± SD.

A significant difference can be observed between sample AP, sample G10 and the other two samples, sample G20 and sample G30.

The addition of glycerol was in order to reduce the deep freezing and freeze drying damages. It should be noted that cryoprotectants are chemicals (in the essentially protective concentrations) not normally encountered by living cells, same as glycerol. A noticeable toxicity (osmotic and chemical) can be detected if the exposure to cryoprotectants is not optimised (Fuller, 2004). The osmotic toxicity can be quickly understood when added to cells in the required concentrations (1 mol/L upwards) cross biological membranes only relatively slowly compared to water, so there is a well-documented rapid water efflux from the cells, with associated volume collapse.

Cells can only tolerate moderate excursions in cell volume without significant damage.

Effect of cryoprotectant, on survival of probiotic cells in the freeze dried microspheres

The effect of freeze drying shows different behavior on the viability of *B. lactis* 300 B entrapped in the four types of microspheres. It can be observed in Fig. 3, that the survival of *Bifidobacterium lactis* 300B when freeze dried without cryoprotectant, was higher than in the samples containing glycerol.

Although main damaging role has been assigned to ice formation during cryopreservation

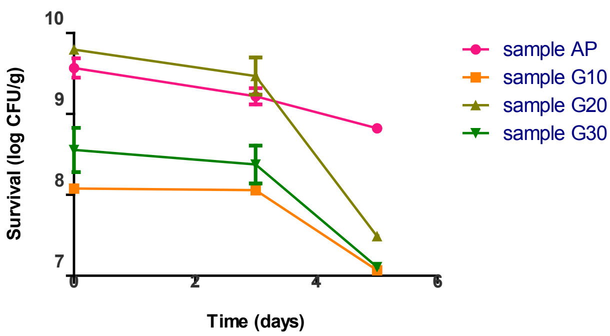


Fig. 3. Survival of *Bifidobacterium lactis* 300B in the four types of microspheres, at time 0, 3 and 5 days after freeze drying. Sample AP: *B. lactis* 300B in alginate-pullulan microspheres; Sample G10: *B. lactis* 300B in alginate-pullulan/10% glycerol microspheres; Sample G20: *B. lactis* 300B in alginate-pullulan/20% glycerol microspheres; Sample G30: *B. lactis* 300B in alginate-pullulan/30% glycerol microspheres. Means (n=3) ± SD.

(whether it be the total quantity of ice formed, the presence of ice inside cells or the relationships between ice and high densities of cells in fixed geometries in tissues), the mayn reason for the drop in viability may be linked to the osmotic toxicity instead than the ice formation. In prior studies we demonstrated that even without the addition of a cryoprotectant the drop in viability of the probiotic cells can be reduced by deep freezing (Pop, 2012). Fuller (2004) discussed the first serious attempts to achieve the freezing in biological systems, applying the concept of cooling sufficiently quickly to avoid ice crystal formation on a kinetic basis, until such low temperatures were reached so that ice crystals would not grow.

CONCLUSION

Application of low temperature technology, as freeze drying, progressed significantly since the early years and plays an important role in many modern scientific efforts. While much has been learned about the role of cryoprotectants and their mode of action, there still remain significant gaps in our understanding about their molecular interactions with cell components and potential toxicities. In order to preserve the probiotic (*Bifidobacterium lactis* 300B) viability, the addition of glycerol as cryoprotectat was tested in our experiment, with no positive results.

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