Study of Bifidobacterium Lactic 300b Survival during Encapsulation, Coating and Freeze Drying Process and the Release in Alkaline Media

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Abstract. The survival of probiotics is of paramount importance because to have their beneficial effects on the host’s health, they must stay alive up to the site of their action. It is known that the resistance of probiotic cells in acidic media, as in the stomach, is very low, that’s why the encapsulation of Bifidobacterium lactis 300B in alginate matrices is needed. This study present the mechanism of cell survival during encapsulation, coating and freeze drying process and release in simulated intestinal media. Microspheres with mean diameter 1100 µm and 66.87% encapsulation rate were obtained and described. In order to increase the protection of Bifidobacterium lactis 300B in the alginate/pullulan based microspheres, three types of dip coating were proposed. Namely, the alginate microspheres were coated in alginate, chitosan or gelatin, using the dip coating method by cross-linking. The survival of the cells was tested after encapsulation, coating application and compared with free and encapsulated cells, using the plate counting method. Also, the survivability in freeze dried microspheres was tested. Although the coated microspheres showed higher survivability compared with free and encapsulated cells, the release in alkaline pH was no possible for the gelatin coated alginate/pullulan based microspheres.

Keywords: probiotic, encapsulation, coating, alginate, pullulan, chitosan, gelatin

INTRODUCTION

Probiotics are active cells with valuable and multiple benefits for human health. FAO/WHO recommends the characterization of probiotics as live microorganisms which, when ingested in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002).

To ensure the sufficient amount of viable cells, estimated to be 10^7 CFU/g product (Brachkova et al., 2010; Ouwehand et al., 1998), the encapsulation of probiotic in all range of formulations was used (Anal et al., 2007; Burgain et al., 2011), alginate being the most used material for entrapment of the living probiotics (Chan et al., 2009). Nevertheless the alginate matrix is not providing enough protection in the acidic media (Sultana et al., 2000).

Pullulan is a biopolymer with a specific characteristic to form strong, oxygen barrier films (Leathers, 2003), which makes it very useful in the entrapment of the anaerobic bacteria. The main challenge in our days is to ensure the survival of these useful microbes during processing, storage, passage through upper gastrointestinal tract and controlled release (Burgain et al., 2011). The encapsulation technique in association with coating application is largely used in order to reach the desired properties in the formed microspheres (Krasaeekoot et al., 2004; Mokarram et al., 2009).

Bifidobacterium is a category of commonly selected probiotics, are indigenous to the human intestine, where they preferentially locate in the colon (Fuller, 1991). Bifidobacterium is a Gram-positive, anaerobic, branched rod-shaped bacterium. In the
intestines, they ferment sugars to produce lactic acid. *Bifidobacterium* are considered as important probiotics and used in the food industry to alleviate and treat many intestinal illnesses. *Bifidobacterium* exert a series of healthful effects, including the control of intestinal microbial equilibrium, the suppression of pathogens and harmful bacteria that colonize and/or infect the gut mucosa, the modulation of local and systemic immune responses, the repression of procarcinogenic enzymatic activities within the microbiota, the outputting of vitamins, and the bioconversion of a number of dietary compounds into bioactive molecules.

The objective of this study was to develop a novel protection system for *Bifidobacterium lactis* 300B based on encapsulation in alginate/pullulan matrix by cross-linking gelation and coating with three different tips of biopolymers. Furthermore, the physico-chemical properties of the resulting fresh and freeze-dry microspheres, as well as their ability to protect probiotic cells during exposure to different temperatures and periods of time, were evaluated.

**MATERIALS AND METHODS**

**Materials**

A commercially available Manguel GMB sodium-alginate was supplied by FMC, Norway. Chitosan and gelatin from Merck (Germany) were used and compared as coatings for alginate/pullulan based microspheres. Calcium chloride, pullulan, E-poly-L-lysine, glutaraldehyde and sodium phosphate were purchased from Merck (Germany), bifidobacterium selective medium (BSM) agar and vegetable peptone from Sigma-Aldrich Chemie GmbH (Germany).

All materials and solutions were sterilized using the autoclave at 121 °C for 15 min, including the CaCl$_2$ solution.

**Probiotic strain**

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

**Preparation of alginate/pullulan microspheres**

The microspheres were prepared aseptically using a Spherisator M, type 2002SP-AE5-D0 at Brace GmbH Germany with a nozzle size of 300 µm, and crosslinked in calcium chloride (40 g/L). Before encapsulation the viscosity of the mixture was measured using a Haake Viscotester VT-2. The standard condition used for encapsulation were: 15 g/L alginate, 75 g/L probiotic powder, and 15 g/L pullulan. Microspheres were hardened for 30 min in calcium chloride solution, and then rinsed with sterile sodium chloride (8.5 g/L).

The entrapment efficiency was determined according to (Sandoval-Castilla et al., 2010) with small changes as follows:

\[
\text{Entrapment efficiency} = \left(\frac{a \times F}{b}\right)100
\]

Where $a$ is cfu/g in the microspheres, $b$ is cfu/g in the mixture before encapsulation and $F$ is the sphere packing factor (Aste et al., 2008), which was considered the dense packing for all calculations 0.70.
**Microspheres characterization**

The theoretical and the corrected diameter of the microspheres was determined using (Chan *et al.*, 2011b) method. The bead shape was quantified using the sphericity factor (SF), which is given by the following equation:

\[
\text{Sphericity factor} \ (\text{SF}) = \frac{d_{\text{max}} - d_{\text{min}}}{d_{\text{max}} + d_{\text{min}}}
\]

where \(d_{\text{max}}\) is the largest diameter and \(d_{\text{min}}\) is the smallest diameter perpendicular to \(d_{\text{max}}\).

For each determination of the \(d_{\text{max}}\) and \(d_{\text{min}}\) ten beads were used and the average was calculated. The \(d_{\text{max}}\) and \(d_{\text{min}}\) was obtained using an optical microscope fitted with a calibrated micrometer scale.

**Coating application**

Three types of dip coatings were applied to the wet alginate/pullulan based microspheres. After the microspheres were obtained, were rinsed with sterile 8.5 g/L NaCl for 5 minutes.

For the first coating 0.8 g/L E-poly-L-lysine and 1 g/L alginate solutions were used. Fresh rinsed microspheres were immersed in E-poly-L-lysine solution under continuous stirring for 30 minutes, after that the microspheres were separated from the solution and wash with sterile NaCl. The alginate coating was applied by stirring the microspheres for another 30 minute in the 1 g/L alginate solution. Finally were washed as described above.

Chitosan coating was the second coating applied. For these, the fresh rinsed microspheres were immersed under continuous stirring in 1g/L chitosan solution for 30 minutes, and washed with sterile NaCl.

For the third type of coating, with gelatin, glutaraldehyde 5 g/L was used to form a chemical cross linked. The fresh alginate/pullulan microspheres were stirred for 60 minutes at 32°C in 100 g/L gelatin solution in 1:1.5 proportions. Then were aseptically separated from the gelatin solution and mixed 2 minutes in the glutaraldehyde solution, finishing with NaCl wash.

**Freeze drying process**

The fresh obtained microspheres were shock freeze at -18°C in isopropanol before freeze dried. The microspheres were freeze dried at -50°C and 5x10^-2 mbar for 24h using a VaCo 5 freeze dryer from Zirbus (Germany). The freeze dried material was collected in sterile recipients and analyzed immediately the process was complete.

**Cell viability**

The enumeration of viable probiotic strain was conducted in triplicates before/after encapsulation, coating and freeze drying. The entrapped cells were release from the microspheres using phosphate buffer with 7.4±0.2 pH. After that 10-fold dilutions were made in peptone water (casein peptone 1g/L, sodium chloride 5g/L and Tween 80 ml/L). Aliquots 1 ml from the last three dilutions was used in the plate counting method on BSM agar for the colony forming units (CFU) determination. After 72h of incubation in anaerobic jars at 37°C the number of CFU was counted and converted to \(\log_{10}\) CFU. All determinations were done in duplicate.

**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 analyze the cell counts. Significant differences among individual means were determined using Turkey test. The statistical evaluation was
RESULTS AND DISCUSSIONS

Characterization of microencapsulation process
Throughput and processing conditions of the alginate/pullulan based microspheres were according to studies described before (Brandau, 2002).

The production in this trial was at laboratory scale recording an average production of 11.56 g/min material, characteristic influenced by mixture viscosity and parameters used in the process, like pressure and frequency (Chan et al., 2009).

Using the Brace Encapsulators the scale up for small industrial production is possible with slight modifications in the process, like adding a multiplate nozzle, and it is suitable for large industrial production (Brandau, 2002).

Microspheres characterization. Entrapment efficiency
The obtained microspheres were characterized in terms of size and surface using an optical microscope.

Table 1 shows size results for the four types of microspheres and encapsulation yields of the alginate/pullulan microspheres. The mean diameter of the coated microspheres were significantly (p<0.05) higher that the uncoated microspheres. As is showed in Figure 1 the shape and roundness of the alginate/pullulan based microspheres is influenced by the coating application.

Tab. 1

<table>
<thead>
<tr>
<th>Capsule type</th>
<th>Capsule size (µm) (n=10)</th>
<th>Encapsulation yield (%) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate/Pullulan</td>
<td>1110.5±12.7^a</td>
<td>66.87±0.28</td>
</tr>
<tr>
<td>Coating 1</td>
<td>1269.5±14.4^bd</td>
<td>-</td>
</tr>
<tr>
<td>Coating 2</td>
<td>1245±9.2^c</td>
<td>-</td>
</tr>
<tr>
<td>Coating 3</td>
<td>1275±5.9^d</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: Means with different letters in a column are significantly different (p<0.05).

Fig. 1. Optical microscope image of the four types of microspheres a) alginate/pullulan; b) alginate-coated alginate/pullulan microspheres; c) chitosan-coated alginate/pullulan microspheres; d) gelatin-coated alginate/pullulan microspheres
The entrapment in calcium alginate based microspheres can be influenced by the capsule size, the hardening time in calcium chloride, the concentration of cells and alginate (Chavarri et al., 2010). The encapsulation yield, in the alginate/pullulan based microspheres was significantly higher that the alginate/chitosan based microspheres mentioned in the literature (Chavarri et al., 2010), but lower that the alginate based microspheres (Mokarram et al., 2009).

**Comparative survival of probiotic cells**

The release from the gelatin-coated alginate/pullulan microspheres was not possible. It can be observe that encapsulation and respectively coating process did not significantly influence the cell viability. The alginate coating proved the highest viability.

![Comparison of the viability of probiotic cells after encapsulation and coating process](image)

Fig. 2. Comparison of the viability of probiotic cells after encapsulation and coating process (microspheres – alginate/pullulan; coating 1 – alginate-coating alginate/pullulan microspheres; coating 2 – chitosan-coating alginate/pullulan microspheres; coating 3 – gelatin-coating alginate/pullulan microspheres). Bars represent the SD of the means.

Survival of Bifidobacterium is affected by various factors such as pH, nature of the dissolution media, presence of nutrients and incubating conditions (Özera et al., 2009). The survival of *Bifidobacterium lactis* 300B loaded in alginate/pullulan based microspheres, respectively in the coated microspheres is shown in Figure 2. Freeze-dried *Bifidobacterium*-loaded will contain both unharmed and damaged cells. Under suitable conditions the injured cells may repair and become viable, i.e. capable of colony formation on suitable media (Cui et al., 2000).
Effect of freeze drying process in probiotic survivability during time

![Graphs showing stability of probiotic cells in freeze-dried microspheres.](image)

Fig. 3. Stability in freeze dried microspheres during 3, 6, 9 and 15 days at a) room temperature and b) 4°C (●) alginate/pullulan microspheres, (■) alginate-coated alginate/pullulan microspheres, (▲) chitosan-coated alginate/pullulan microspheres.

Stability of *Bifidobacterium lactis* 300B loaded in alginate/pullulan based microspheres, respectively in the coated microspheres at room temperature and 4°C is shown in Figure 3. The survival was maintained at about 10⁹ CFU/g after approximately 4 weeks storage even at the room temperature. The storage at refrigeration demonstrated higher protection than the storage at room temperature, result confirmed by literature (Cui et al., 2000; Saarela et al., 2011). At room temperature the coated microspheres provide higher protection for the entrapped probiotic cells that the uncoated ones. This results are confirmed by the literature (Chavarri et al., 2010; Mokarram et al., 2009), where is showed that encapsulation of probiotic bacteria in coated chitosan microspheres respectively coated alginate microspheres improved the survival of cells.

The release behavior in simulated intestinal media

The intestinal media was mimed by the phosphate buffer used for the release of the entrapped cells in order to determinate the cell viability inside the microspheres. The first trial was with a 4±0.2 pH, but the release did not happen. To mime the lower gastrointestinal tract the pH of the buffer was modified using sodium hydroxide to 7.4±0.2. The alginate/pullulan microspheres and the alginate-coating alginate microspheres and chitosan-coating alginate microspheres have showed complete degradation after 20 minute of intense shaking. This degradation is due to phosphate ions chelate calcium, because of that, the alginate and chitosan coating becomes weaker and releases the entrapped cells. The microspheres coated with gelatin – coating 3, showed no release either in the acid or basic pH, even after 24 h.

CONCLUSIONS

This research tracks the survival of *Bifidobacterium lactis* 300B in alginate/pullulan microspheres, in alginate-coated, chitosan-coated and gelatin-coated alginate/pullulan microspheres in the encapsulation and freeze drying process and during storage.

There appears to be much potential for using the probiotic strains with alginate and pullulan during encapsulation since it does potentiate the survival of the probiotic bacteria. Our study has indicated that the survival of alginate/pullulan immobilized cells is higher that
the therapeutically minimum \((10^7 \text{ CFU/g})\) before and after freeze drying. Among the three types of coatings applied in this research the viability from the gelatin-coating alginate/pullulan microspheres could not be tested, due to the fact that the release did not happen. Further studies need to be carried out in order to monitor the behavior of the microspheres in gastrointestinal juice that contains specific enzymes. Research of a proper release method from gelatin-coated alginate/pullulan microspheres is also needed.

In conclusion, the encapsulation of \textit{Bifidobacterium lactis 300B} with alginate and pullulan, in addition with coating application offers an effective protection during freeze drying process and through storage at room and refrigeration temperature.

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**REFERENCES**


