

Evaluation of viability of *Lactobacillus bulgaricus* in symbiotic microcapsules: before and after freeze drying

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Abstract

Lactobacillus bulgaricus is known to be tolerant to bile and low pH and has probiotic effects due to multiple mechanisms. Prebiotics act as a substrate for the growth and/or activation of beneficial colonic bacteria. *Ficus carica*, commonly known as “Fig” belongs to Moraceae family. The aim of this study was to develop synbiotic microcapsules using fig powder as prebiotic and *Lactobacillus bulgaricus* as the probiotic strain and evaluate the viability of probiotic strain in synbiotic microcapsules before and after freeze drying. The viability of probiotic culture in MRS medium and MRS medium incorporated of fig powder was found to be $3.6 \pm 0.05 \times 10^9$ CFU/ml and $6.01 \pm 0.05 \times 10^9$ CFU/ml respectively. Increased viability is attributed to presence of resistant starch and fructo- and galacto-oligosaccharides. Probiotics were then encapsulated with and without prebiotic and the microcapsules were then freeze dried. Survivability of probiotics entrapped in alginate and synbiotic microcapsules as well as in freeze dried alginate and synbiotic microcapsules was determined at refrigerated temperature. Probiotics entrapped in freeze dried synbiotic microcapsules showed better viability amongst all others, thus having better storage period.

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Keywords

Prebiotic

Probiotic

Synbiotic

Lactobacillus bulgaricus

Introduction

Prebiotics are known as “Non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson and Wang, 1994). A particular food product can be claimed as a prebiotic if; its hydrolyses or absorption does not take place in the upper part of the digestive system; it acts as a substrate for the growth and/or activation of beneficial colonic bacteria; it improves the composition of colon microflora; and it induces beneficial luminal effects to the health of the host (Miyazato *et al.*, 2010). Various categories of prebiotics include some non-digestible carbohydrates or dietary fibers (oligosaccharides and polysaccharides), some peptides and proteins, and some lipids (Gibson and Roberfroid, 1995). According to the European Food Safety Authority dietary fibers are classified into four groups: Non-starch polysaccharides (cellulose, hemicelluloses, pectin and hydrocolloids), resistant oligosaccharides like galacto-oligosaccharides (GOSs) and fructo-oligosaccharides (FOSs), lignin which usually associates with dietary fibers and resistant starch such as retrograded amylose, raw starch granules,

and chemically modified starches (Westenbrink *et al.*, 2012). Resistant starch may not be digested because of its dense molecular configuration protected by botanical cell wall inhibits the accessibility and action of digestive enzymes. Gelatinization is a process in which starch granules are disrupted when heated under high humidity. These gels after cooling form retrograded starch crystals which are resistant to enzymatic digestion and thus are categorised under resistant starches (Homayouni *et al.*, 2013).

The most significant species belonging to *Ficus* genera found in India are *F. bengalensis*, *F. carica*, *F. racemosa* and *F. elastica*. *Ficus carica*, commonly known as “Fig” belongs to Moraceae family. The fruits and leaves parts have significant level of antioxidant and antimicrobial activity (Kislev *et al.*, 2006; Jeong *et al.*, 2009). The antioxidant properties are attributed to the presence of total phenolics, flavonoids, alkaloids and saponins and other secondary metabolites. Consuming dried fig daily enhanced the antioxidant capacity of plasma (Veberic *et al.*, 2008). Fig also has laxative properties. Dried fig fruit is a good source of carbohydrates and minerals while it has average protein and dietary fiber content with very low amount of fat and is hence considered as the richest nutritional source.

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Encapsulation techniques can be applied to introduce viable probiotic bacteria as the wall material can provide a physical barrier to the encapsulated material against adverse environmental conditions (Boh, 2007; Zuidam and VA, 2010). Commonly used encapsulation methods include emulsion, extrusion, spray drying, freeze drying, and spray-freeze drying (Serna-Cock and Vallejo-Castillo, 2013).

The natural resistant starch from green banana flour and retrograded rice flour could protect the cell and promote survival of *L. bulgaricus* (TISTR 895) (Panuwat *et al.*, 2012). In this study *Lactobacillus bulgaricus* ordered from NCIM, Pune is used as probiotic strain while Ficus carica powder is used as a source of prebiotic. Synbiotic microcapsules were developed and freeze dried and storage studies were conducted before and after freeze drying.

The aim of this research is to increase the viability of probiotic strain under the influence of fig powder as prebiotic in synbiotic encapsulation. Freeze drying technique is used to further increase the shelf-life of synbiotic microcapsules and conduct comparative analysis is thus carried out for probiotic viability before and after freeze drying of synbiotic microcapsules.

Materials and Methods

Materials

Fresh ripened fig fruits were purchased from the local market, of Chennai, India. Freeze dried *Lactobacillus* strain was procured from NCIM-CSIR; National Chemical Laboratory, Pune. MRS broth and MRS agar were used for growing the probiotic. Bile salt, NaCl and pepsin were used for conducting tolerance tests on probiotics. Alginate was used for probiotic encapsulation while sorbitol was used as cryoprotectant.

Preparation of prebiotic powder

Pulp from fresh figs was prepared (without peeling) using grinder. The pulp was lyophilised at a temperature of -40°C and pressure of -3 mbar for 30 hr (Emperatriz *et al.*, 2008). The freeze-dried pulp was then ground and the powder so obtained was stored at room temperature (30°C) in sealed aluminium pouches.

Revival of probiotics

Freeze-dried *Lactobacillus* strains were procured from NCIM-CSIR-National Chemical Laboratory, Pune. The cultures were stored at 4°C until required. DeMan-Rogosa- Sharpe broth (MRS broth) of pH 4 to 5.2 was used to prepare the cell suspensions for

probiotic strains (Ashraf and Shah, 2011). The MRS agar was inoculated with active strains and incubated at 37°C for 24 hr under anaerobic condition (EL-Sayed *et al.*, 2014).

Effect of prebiotic on the viability of probiotic strain

The effect of different prebiotics on viability of different probiotic strains was tested (EL-Sayed *et al.*, 2014). The sterilized prebiotic was added to MRS broth medium by 2% and inoculated by active probiotic strains and incubated at 37°C for 24h under anaerobic condition. Also, MRS broth inoculated with culture was used as control. The viable count was determined using spread plate method after serial dilutions of the respective broths in physiological solutions. 0.1 ml of 10^{-2} dilution sample was inoculated and the plates were incubated at 37°C for 48h under anaerobic condition. All plating was done in triplicates and the result is the average value.

Encapsulation

Extrusion method was adopted and slightly modified (Farnezah *et al.*, 2012). Sodium alginate solution (2.5%) and calcium chloride solution (4%) was prepared. Also, sodium alginate solution was mixed with prebiotic powder in the ratio 1:1. All the above solutions were sterilised and allowed to cool. Probiotic culture was mixed and homogenized in sodium alginate solution and sodium alginate-prebiotic mixture separately. The solution was loaded in syringe without air bubble and dropped in calcium chloride solution drop wise to form beads. Encapsulated and synbiotically encapsulated beads so formed were collected from the base of the vessel and stored as two different sets each. One set of both encapsulated and synbiotically encapsulated beads was stored at refrigeration temperature without any treatment. Other set was dipped in 10% sorbitol solution as cryoprotectant for 10 minutes and freeze dried for 24 hours. Later the freeze dried beads were lyophilized and stored at refrigerated temperature.

Encapsulation efficiency

Encapsulated beads were disintegrated in 0.1M sodium citrate solution homogenized for 5 minutes and was used to make serial dilutions in physiological solution (EL-Sayed *et al.*, 2014). The viable count was determined using spread plate method the plates were incubated at 37°C for 48 h under anaerobic condition. All plating was done on triplicates and encapsulation efficiency (EE), which is a combined measurement of efficacy of entrapment and survival of viable cells during microencapsulation procedure, was calculated as follows:

$$EE = N/N_0 \times 100$$

Where, N= viable number of entrapped cells released from microcapsules and N_0 = free cells added to the biopolymer mix during production of microspheres.

Size and morphological analysis

Particle size, shape surface and morphology of microencapsulated beads of samples were determined by scanning electron microscopy (Mahendrasingh and Vijay, 2011).

Survivability tests

The samples were diluted in solutions simulating gastric/gut and homogenized for 5 min to test tolerance of probiotic strains (Gbassi and Vandamme, 2012). To obtain the viable count, 0.1 ml of the 10⁻² dilution of microbes were plated in MRS agar (EL-Sayed *et al.*, 2014). Plates were incubated at 37°C for 24 hr under anaerobic conditions. Samples were checked at intervals of every two days of storage at room temperature for 1 month. Experiments were performed in triplicates.

Statistical analysis

Analysis of variance (ANOVA) was applied for determination of significant difference ($p < 0.05$) between means of cell counts (Hernández-Carranza *et al.*, 2014).

Results and Discussion

The results were in accordance to the hypothesis of increasing shelf life of microcapsules using prebiotic for the formation of synbiotic microcapsules and freeze drying technique. Freeze drying technique further increased the shelf-life of microcapsules which is observed in the comparative analysis of probiotic viability before and after freeze drying of synbiotic microcapsules.

Revival and survivability tests for free cells

Probiotic strains were successfully revived and were tested for bile and NaCl tolerance. Probiotics should show good tolerance to low pH in gastric environment and should exhibit tolerance to bile released in intestinal environment. The lowest pH recorded has been pH 1.5 (Huang and Adams, 2004; Lin *et al.*, 2006). A good probiotic should withstand at least pH 3.0 (Fernandez *et al.*, 2003).

Microbial counts in the range of 30-300 CFU/ml are considered to be viable in a given specific environment. Free probiotic strains showed a count of

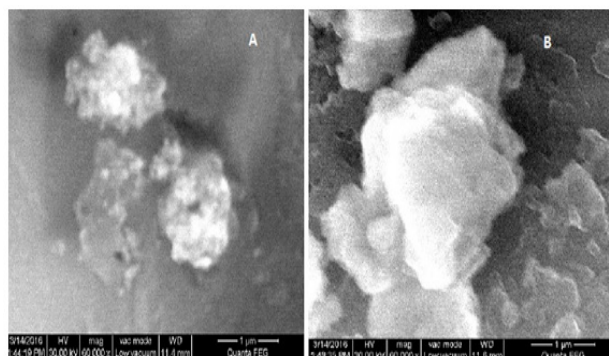


Figure 1 (A). SEM Photomicrograph of Encapsulated Beads (B). SEM Photomicrograph of Synbiotically Encapsulated Bead

11 x 10⁷ CFU/ml in the NaCl environment whereas a count of as 20 x 10⁷ CFU/ml in the bile environment. As their colony count is not in the required range, it suggests most probiotics were killed by this harsh pH. Also, upon exposure to bile acids, cellular homeostasis disruptions cause the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial content and ultimately cell death; thus, necessitating encapsulation.

Effect of prebiotic on probiotic

The initial viable count was found to be $3.6 \pm 0.05 \times 10^9$ CFU/ml. After adding prebiotic to the media, the viable count was found to be $6.01 \pm 0.05 \times 10^9$ CFU/ml. Thus, Prebiotic was found to be more effective for viability of the probiotic strains. This may be attributed to resistant starch content in the sample. The fig fruits have significant level of antioxidant and antimicrobial activity. The antioxidant properties are attributed to the presence of total phenolics, flavonoids, alkaloids and saponins and other secondary metabolites (Kislev *et al.*, 2006; Jeong *et al.*, 2009). The above mentioned properties are added advantage of fig powder for using as prebiotics.

Encapsulation and size and surface morphology

The SEM photomicrographs of encapsulated beads and synbiotically encapsulated beads (Figure 1A, 1B) indicated that the microspheres were roughly spherical in shape having particle size of 11.4 mm and 11.6 mm respectively. The probiotics remained dispersed in the polymer matrix.

Encapsulation efficiency

Encapsulation efficiency for encapsulated and synbiotic microcapsules was found to be $99.5 \pm 0.05\%$ whereas for freeze dried encapsulated and synbiotic microcapsules encapsulation efficiency was found to be $98.01 \pm 0.05\%$.

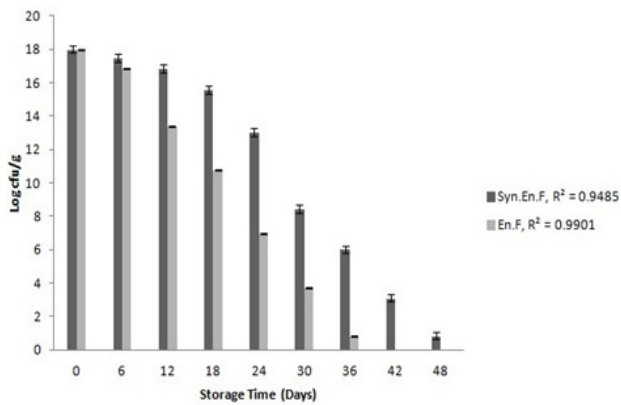


Figure 2. Survival of probiotics in freeze dried alginate microcapsules and synbiotic microcapsules in gastric environment at different time intervals

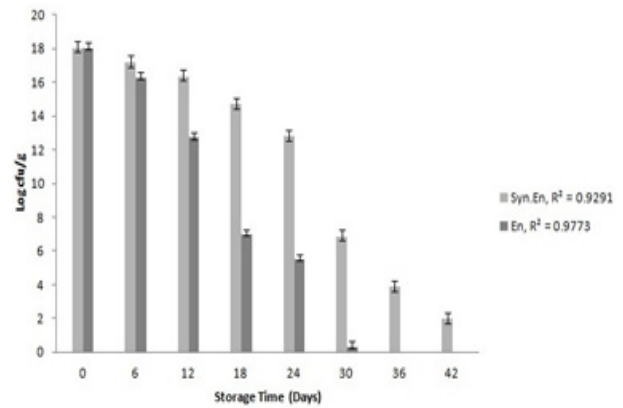


Figure 4. Survival of probiotics in alginate microcapsules and synbiotic microcapsules in gastric environment at different time intervals

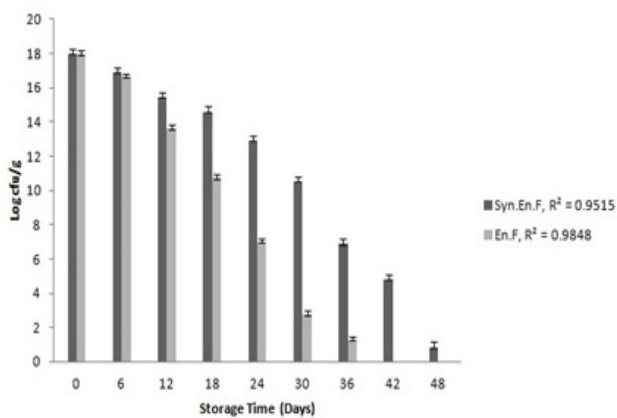


Figure 3. Survival of probiotics in freeze dried alginate microcapsules and synbiotic microcapsules in intestinal environment at different time intervals

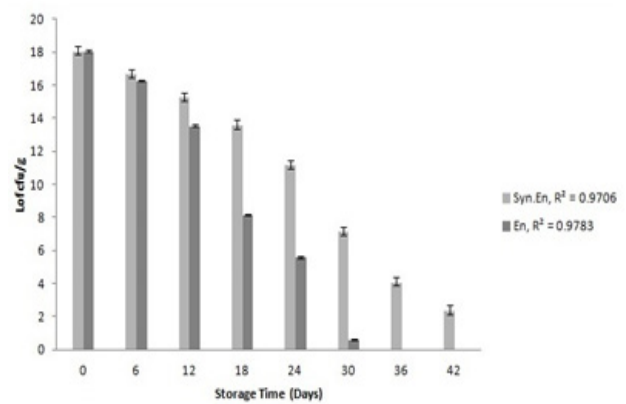


Figure 5. Survival of probiotics in alginate microcapsules and synbiotic microcapsules in intestinal environment at different time intervals

Survivability of microencapsulated probiotics

Survivability for probiotics was assessed in gastric as well as intestinal environment. Survival of probiotics entrapped in freeze dried synbiotic microcapsules was significantly improved over those entrapped in freeze dried alginate microcapsules as shown in Figure 2 and 3. Similarly survival of probiotics entrapped in synbiotic microcapsules was significantly better than those entrapped in alginate microcapsules without freeze-drying also as shown in Figure 4 and 5. Microencapsulation with alginate is able to protect probiotics in food products (Hansen *et al.*, 2002). Increased viability is attributed to presence of resistant starch and fructo- and galactooligosaccharides. Probiotics entrapped in freeze dried synbiotic microcapsules showed better viability amongst all others, thus having better storage period. The present study suggest fig powder as a potential prebiotic and symbiotic microcapsule as a medium for effective delivery of probiotic, *Lactobacillus bulgaricus*.

Conclusion

Using the prebiotic source (fig) and *Lactobacillus bulgaricus* as the probiotic, synbiotic formulation was prepared. Sodium alginate was used as carrier material for encapsulation. An increase in viability of probiotic strain was observed in the presence of fig powder in the growth medium. Blending of prebiotics in the coating materials resulted in better protection for the encapsulated organisms and increased cell viability during storage, relative to the prebiotic free variants and microcapsules without prebiotic. Freeze drying of the microcapsules further increased the storage time for the alginate and synbiotic microcapsules.

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