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Microencapsulation of *Lactobacillus sp.* Using two Different Materials and Comparison for Encapsulation Efficiency

V. Padhmavathi and Radhakrishnan Preetha*

Department of Food Process Engineering, SRM University, Kattankulathur - 603203, India.

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Probiotics are microorganisms which, when taken orally, provide beneficial effects on human gut health. Microencapsulation of probiotics is a technique that is currently receiving considerable interest as it helps probiotics to survive against adverse environmental conditions in the human body. In this project , a study to determine the stronger encapsulating material is done by encapsulating *Lactobacillus sp.* using alginate and skim milk alginate. The encapsulation was done by extruding into 100mM $CaCl_2$ solution. Viability test, bile salt tolerance test and storage stability was performed. On analyzing the results it was found that, the skim milk alginate beads had the efficiency of 96.48%. The bacteria encapsulated using skim milk alginate was more viable and more tolerant towards bile salt. The storage stability test was carried out for a period of 28 days and it was found that the probiotic encapsulated using skim milk alginate were more viable. From the results it was concluded that skim milk alginate was a stronger encapsulating material than alginate because of the strong network forming nature of milk proteins.

Keywords: Probiotics, *Lactobacillus sp.*,microencapsulation, alginate, skim milk.

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host"[1]. When consumed in adequate amounts, probiotics are live microorganisms that provide a health benefit to the host[2]. Interest in the consumption of probiotic food products is increasing and a many functional foods have been developed[3]. They confer many health benefits such as suppressing growth of pathogens, preventing diarrhoea, constipation, and food allergies, synthesizing nutrients and enhancing their bioavailability, and anti-neoplastic activity[4, 5]. The survivability of the probiotics is to be maintained during manufacture, storage, and delivery to gastrointestinal tract to provide their health benefit[3]. The most common probiotic-containing foods are fermented dairy products that contain lactic acid bacteria (LAB). Probiotics are highly stable in dairy products than in non-dairy products. Various solutions to this problem, such as durable strain selection in adverse environments[6] and addition of prebiotics[7] have been evaluated.

Encapsulation has been investigated for protecting probiotics in gastrointestinal tract[8]. The advantages of encapsulation are prevention of interfacial inactivation, stimulation of production and excretion of secondary metabolites, and continuous utilization. During fermentation it also enhances microbial survival and operating efficiency[9]. Severalstudies have shown successful microencapsulation and coating ofbacteria using various encapsulating materials and methods[10-14].

* To whom all correspondence should be addressed.
Tel.: 9176610351;
E-mail: preetha.r@ktr.srmuniv.ac.in

A process in which the cells are retainedwithin an encapsulating matrix or membrane is known as microencapsulation.

Extrusion technique of microencapsulation has been used exclusively for encapsulating volatile and unstable ûavors in glassy carbohydrate matrices and probiotic microorganisms[15-21]. The main advantage of this process is that, the matrix is strong enough and protects the microbes in very low pH conditions too.

Alginates are natural anionic polysaccharides made up of D-mannuronic and L-guluronic acid residues[22]. Alginate, a polymer extracted from seaweed, is a commonly usedencapsulation agent because it is non-toxic, biocompatible,and inexpensive. The additional benefits of using alginate is the ease of solubilizing alginategel (by Ca++sequestration) and its release of entrapped cellswithin the human intestine[23,24]. There are few studies on the effect of whey protein and skim milk powder on encapsulation efficiency of probiotic microorganisms[25]. Milk proteins have good immobilization properties.

Strains of Lactobacillus and Bifidobacterium species , which are lactic acid bacteria, are the most common microbes employed as probiotics. Other species considered as probiotic may include lactococci, some enterococci and some streptococci. Curd and other dairy products are a very good source for Lactobacillus species.

MATERIALS AND METHODS

Subculturing and maintenance

The probiotic organism,*Lactobacillus sp,* was obtained from MTCC, Chandigarh. The lyophilised powder was sub-cultured by inoculating into MRS broth and incubating at 37°C for 24 hours. After 24hours, the culture from the broth was further sub-cultured by streak plating onto MRS agar and incubated for 24hours at 37°C. These plates were then stored at 4°C for a period of one month before sub-culturing again.

Microencapsulation of probiotic

Microencapsulation of the organism was done using two materials mentioned below by extrusion technique.

Alginate

Alginate solution was sterilised at 121°Cfor 15min. 14 ml sodium alginate was added to a beaker.1 ml culture was added to the beaker. This mixture was extruded in 100mM $CaCl_2$ solution and stirred for 30 min at 100 rpm. The beads obtained were washed with distilled water and sealed in sterile conical flasks.

Skim milk-alginate

Alginate solution was sterilised at 121°Cfor 15min. Skim milk was sterilised at 110°C for 15 min. 13 ml sodium alginate was added to a beaker along with 6 ml skim milk. 1 ml culture was added to the beaker. This mixture was extruded in 100mM $CaCl_2$ solution and stirred for 30 min. The beads obtained were washed with distilled water and sealed in sterile conical flasks.

Encapsulation efficiency

The microencapsulated bacterial samplewas solubilized in sodium citrate solution. The sample was serially diluted upto 10 fold and plated onto MRS agar by spread plating. It was incubated at 37°C for 24 hours. Number of colonies were counted and the encapsulation efficiency (EE) was calculated as described by[26].

$EE = N / No \times 100$

Where, N – Number of colonies from beads

No- Number of colonies from free cell suspension**.**

Bile salt tolerance test of free and encapsulated probiotic

Free cell suspension (0.5ml) was added to 4.5ml of bile salt solution (2%) and kept at 37°C for 1 hour and 2 hours. Solution from each flask was then serially diluted upto 10 fold using saline solution. 1ml from last test tube was plated onto MRS agar plates by spread plate method. These plates were incubated at 37°C for 24 hours and number of colonies were counted.

0.5g beads were added to 4.5ml of bile salt solution (2%) and kept at 37°C for 1 hour and 2 hours. The capsules were removed from bile salt solution at specified time intervals and added to 4.5ml of sodium citrate solution to be solubilised. Once released completely these solutions were serially diluted upto 10 fold using saline solution. 1ml from last test tube was plated onto MRS agar plates by spread plate method. These plates were incubated at 37°C for 24 hours and number of colonies were counted.

Storage stability of free and encapsulated cells

Encapsulated beads and free cell suspension are stored at 4°C for a period of 1 month.At regular intervals such as 1, 3, 5, 7, 14, 21, 28 days the encapsulated cultures were revived by using sodium citrate solution and serially diluted. These were then plated onto MRS agar. While, the free cells were serially diluted upto 10 fold and plated onto MRS agar. The colonies were counted to determine the survivability.

Statistical analysis

All the experiments were repeatedthree times. The data was subjected to analysis of variance (ANOVA) and the significance of difference between means was determined by Duncan's multiple range tests ($p < 0.05$). The results were presented in mean value ±standard deviation (SD).

RESULTS AND DISCUSSION

Size and Encapsulation efficiency

Extrusion method is the most commonly used method for encapsulating probiotic.In extrusion method the size and encapsulation yield of microspheres is affected by several factors such as nozzle size, polymer concentration and composition [27]. Their results showed thatdiameters of alginate milk microspheres, prepared using nozzle 0.45 and 0.20 mm were 830 ± 10 and 381 ± 8 µm (ûgures not shown), respectively.The size of microspheres should be below 100 µm to avoid negative sensory impact in food products [28]. Due to the limitation of nozzle size (minimum nozzle size provided was 0.20mm) ,the minimum size of microspheres obtained by Voo et al., 2011 was around 381 ± 8 µm.

In this study it was seen that polymer composition influenced the slight variation in diameter of microspheres. Alginate and skim milk alginate were the two different compositions used. The microspheres were prepared with a 0.20mm nozzle. Alginate microspheres had a diameter of 321 ± 10 µm and skim milk alginate microspheres had a diameter of 367 ± 6 µm.

Studies show that high encapsulation efficiency (close to 100%) were easily obtained through extrusion method [29,30]. In the present study, 94.44% efficiency was obtained when alginate was used as encapsulating material. On the other hand, a significantly different ($p<0.05$) efficiency of 96.48 % was obtained when skim milk alginate was used. The encapsulation efficiency of skim milk-alginate was higher than that of alginate.

Bile salt tolerance of free and encapsulated cells

In this study, as result of action of bile salts , there was deterioration in cell wall intergrity and free *Lactobacillus sp* totally lost its viability in bile salt. Many references mentioned probiotics were sensitive to bile salt solution. There are observations that a decrease of 5 logCFU/ml in viable cell counts of *Biûdobacterium adolescentis* (B. adolescentis) occurs in 2% bile salt solution at 37C after 12 h incubation[31]. It has been found that *B.adolescentis* reduced by about 2 logCFU/mL after 2 h incubation in 0.5% bile salt at 37C[28]. However, the results shown in **Table 1** clearly indicate that encapsulated microspheres could provide a good protection against the damage of the bile salt solution compared to free *Lactobacillus sp* cells and there was a significant difference ($p<0.05$) in the results.

Different researchers have used different concentrations and sources of bile salts. So,

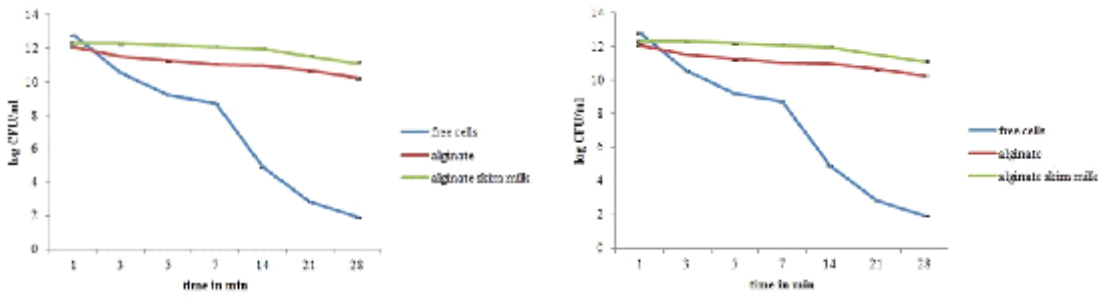

Fig. 1. Storage stability of free and encapsulated cells

making a comparison was difficult. It was also found that encapsulated probiotic bacteria could survived better than free probiotic cells in 1–3% bile salts solution[32, 26].

Storage stability of free and encapsulated cells

In the present study it was seen that the number of viable cells reduced drastically when left without encapsulation. Encapsulated cells were protected and hence the cells were viable and survived a storage period of 28 days. Special treatments, suchas coating of beads with polymers, blending with other polymers,improves the storage stability of probiotic[33]. The protective effectby whey protein encapsulation was evaluated at 5C on storing for 180 days [34]. **Fig 1**, below, clearly indicates that microencapsulation using skim milk alginate, improved the viability of cells on storage.

CONCLUSION

Lactobacillus sp. was successfully encapsulated in alginate and skim milk- alginate microspheres prepared by extrusion method. The cells of *Lactobacillus sp*.encapsulated in microspheres showed better survival ability than that of free cells in high bile salt concentrations (2.0%) and long time storage (28 days). It was also found that skim milk-alginate was a stronger material when compared to alginate as all results indicated more viability in skim milk- alginate microspheres. Encapsulation has once again proved to be a good method to protect probiotics in gastrointestinal environments. Skim milk-alginate microspheres show the potential as a new encapsulating material for preserving the viability of probiotics during oral administration. Further studies are to be carried out in gastro-intestinal environments.

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