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Research Article

Preservation of *L. Rhamnosus* with Calcium Alginate and It's of Survival Under Gastric Condition and in Yogurt

Marwah Ali Oudah, K Rajyalakshmi, Syed Shabana and A Krishna Satya*

Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India

***Corresponding Author:** A Krishna Satya, Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India.

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Abstract

Potential strain isolated from mother milk sample, *L. rhamnosus* was selected from previous probiotic property studies is co –encapsulated with complementary Hi-Maize starch (a prebiotic) improved encapsulation of viable bacteria as compared to when the bacteria were encapsulated without starch. A preliminary study was carried out in order to monitor the effects of encapsulation on the survival of *Lactobacillus rhamnosus* in yogurt for storage and sensory evaluation over 8 week's storage. This study showed that the survival of encapsulated cultures of selected probiotic showed a decline in viable count of about 0.5 log over a period of 8 weeks while there was a decline of about 1 log in cultures which were incorporated as free cells in yoghurt. Addition of probiotic bacteria (free or encapsulated form) reduces acid development in yogurt during storage studies. Post acidification in yogurt with encapsulated probiotic bacteria was slower compared to yogurt with free probiotic bacteria.

Keywords: *L. rhamnosus*, Encapsulation, Survival Studies, Yogurt and Sensory Evaluation.

Introduction

Microencapsulation have been successfully used to enhance and improve the viability of dairy fermentation for the production of concentrated lactic acid producing bacteria and to increase the survival rate in dairy products and mayonnaise [1,2]. Encapsulation with calcium alginate beads has been significantly widely used for immobilization of probiotic bacteria such that it was ease of handling, non toxic and low cost [3]. This encapsulation procedure is the best method to preserve the bacteria from environmental factors such as low pH and high bile concentration [4,5], and also in case of anaerobic bacteria, bacteriophages and in chemical agents and in antimicrobial agents.

Encapsulation method has been implemented to enhance the survival and delivery of the probiotic culture. Several researches have shown successful microencapsulation and coating of bacteria using various materials and methods. Several model studies are available where alginates have been used for the microencapsulation of probiotic bacteria for fermentation purpose or for incorporation into products [6-10]. The main objective of the present research were to optimize the encapsulation conditions of the probiotic bacteria and to evaluate the survival of encapsulated culture under stimulated gastric conditions and in yogurt over a period of 8 weeks during the storage studies and sensory evaluation.

Methodology

Encapsulation

The promising probiotic isolate screened were further investigated for encapsulation by a slight modification method of [11] was used, bacterial strains were cultivated in appropriate broth for 24 h at 37 °C. After that the cells were harvested by centrifugation at 2500 × g for 10 min at 4 °C. The cells were washed twice be-

fore re suspending them in 5 mL normal saline. It served as the inoculums of free cells to prepare microencapsulated cells and for the survival studies. Now bacterial suspension in a saline solution re-suspended in aqueous alginate solutions with different ratios (1:2, 1:4, 1:6, 2:2, 2:4, 2:6, 3:2, 3:4, 3:6 likewise) at different alginate concentrations (1%, 2%, 3% and 4% w/v) (Sigma, Sigma Aldrich,) to achieve 10^8 $CFUmL^{-1}$ (final concentration) and to study the efficiency and viability of capsulated beads. Then, the mixture of alginate and cells were added drop wise using 5ml or 10ml syringe with a needle attached at the end into different 40mL calcium chloride solutions containing 0.5%, 1%, 2% and 3% Ca Cl_2 (w/v). This solution was constantly homogenized using a magnetic stirrer situated at the bottom of the vessel, in order to prevent the beads from sticking together. A dropping height of 7 - 10 cm was used to ensure that spherical droplets were formed. Capsules were maintained in the calcium chloride solution for 30min and then transferred to a saline solution.

Efficiency of bacterial encapsulation:

To determine the viable count of entrapped bacteria under different alginate and calcium chloride concentrations according to the methods of [12]. One gram of beads was released from calcium alginate capsules by sequestering calcium ions with a 0.1M phosphate buffer solution (at pH-7). Then serial dilution was performed in a saline solution fallowed by bacterial count determination by plating. Bacterial counts contained in alginate capsules were expressed as $CFUcapsule^{-1}$.

The efficiency of encapsulation was expressed as a percentage calculated by dividing microcapsule bacterial contents by the bacterial concentration of equal volumes of alginate suspension.

Survival of encapsulated beads at different storage temperatures

The encapsulated beads were divided into two batches and maintained at 4 and 22^0C. The numbers of cultural probiotic cells were determined at different time intervals (3, 6, 9, 20 and 30 days) by releasing bacterial contents and plating on appropriate media. A total of 10 capsules were analyzed each time and three independent experiments (i.e. three replications) were performed at each temperature.

Survival of free and encapsulated strains in pH and bile

Tolerance to pH and bile was evaluated for free and encapsulated *L. rhamnosus* strain. Freshly prepared microcapsules (1 g) or 1 mL (8 log cfu/mL) of free cell suspensions placed separately in test tubes containing 10 mL of appropriate medium adjusted to pH 2.0 and 3.0 were incubated at 37 _C for 24 h. The cells were harvested, washed and immediately used for enumeration of viable cells at 0 h and 24 h by plating in appropriate media at 37 _C for 24 h after de-polymerization of the capsules in 10 mL phosphate buffer [13].

Tolerance of microencapsulated strains to various bile salt concentrations were carried out as similar to above said method. Briefly, 1 g of microcapsules or 1 mL of free cell suspension was transferred in test tubes containing 10 mL of appropriate medium with 0.3, 0.6 and 0.8 g/100 mL bile salt (Merck, Darmstadt, Germany) concentration and incubated at 37 _C. The enumeration of viable cells was carried out at 0 h and 24 h (modified [14,13].

Yogurt production

Three batches of set yoghurt including a control (without probiotic cultures) were made with probiotic bacterial cultures incorporated into the product in different states: free and encapsulated. The probiotic cultures were incorporated at the same time as the yoghurt cultures. Homogenized and pasteurized milk was heated to 45 1C and skim milk powder (SMP) was added with high-speed stirring, to make 180 g/l total solids in yoghurt. Heating was continued to 80 - 85 1C, and the mixture was held at this temperature for 20 min. It was then cooled to 45 1C and the yoghurt starter culture was added. The probiotic cultures were added as free or encapsulated cultures. The yoghurt mix was distributed in 500 ml plastic cups. Incubation was carried out at 42 - 43 1C until a pH of 4.5 was reached at which time the yoghurt was cooled in an ice water-bath and stored at 4 1C for 7 weeks. The '0 day' analysis were carried out after overnight cold storage of samples, and week 3, 5 and 7 analysis were carried out after 21, 35 and 49 days of storage, respectively.

Determination of pH

The pH of yoghurt samples was determined using a Digital pH meter (Denver Instruments, USA). The pH meter was standardized using reference pH 4.0 and pH 7.0 buffer solutions. The yoghurt sample was stirred with a little distilled water before pH measurement.

Sensory evaluation of yogurts

Sensory evaluation of yogurts was carried out after 8 weeks of storage at 4 1C. A panel consisting of 6 members, (Members of As-

sistant professors in Department of Food and Nutrition, Acharya Nagarjuna University) evaluated the yoghurt samples presented in coded cups in individual booths at room temperature. The process used a series of horizontal lines marked with degrees of intensity of yoghurt attributes such as appearance and colour, body and texture, acidity, flavour, after taste and overall liking. Scoring was performed on a hedonic scale of 1 - 15 with 1 being most desirable [15].

Results and Discussion

Morphological identification of beads

The size and shape of the probiotic encapsulated beads was identified using light microscopy. The size of the bead obtained in the present study is 0.5 - 1mm, and only small portion of the encapsulated bead is considered as the size range < 500µm. Size difference is carried out using 1mm, 500µm and 150 µm sieve size. And the shape of the bead size I normally spherical sometimes due to height variance drop shape or elliptical shaped capsules was observed. The prebiotic Hi-Maize starch gives the much potential to the bead as in the shape, texture, viability and low pH and high bile concentrations.

Survival of strains in pH and bile and storage condition

The main criteria factor that effects the survival and growth of probiotic bacteria id low pH condition, and the results indicates that no significant decrease in viable count was identified in encapsulated bacteria. Survival rate is 75 - 80% under low pH condition with prebiotic Hi-Maize starch. And under bile concentration the survival rate was 75 - 85% when compared to the free probiotic bacteria. (Table-1) The viability of bacteria on storage analysis was identified as much higher than the free probiotic bacterial cells. Viability of the encapsulated bacteria is compared with the previous results [13,14,16-18]. In these results they concluded that most advantage of encapsulation is the high survival rate under low pH and high Bile concentration. Another research reported that immobilization of bacterial cells *Bifidobacterium bifidi* and *Lactobacillus acidophilus* in calcium alginate was not much accuracy in protecting the beads under high concentration of bile (2 and 4%) [19]. Prebiotic used in the present study aids the increase of survival rate under pH and bile conditions. These end products of fermentation exert significant positive health effects of the host [20]. On storage analysis under room and freeze condition the viability of the probiotic did not significantly alter the count of the bacterial cell (Table 2).

Treatment	Initial mean	Acid concentration			Bile concentration (%)		
		pH2	pH3	pH6.5	0.3	0.5	0.8
Free cells	8.82 ± 0.13	7.23 ± 0.35	8.68 ± 0.30	8.81 ± 0.16	6.54 ± 0.45	8.23 ± 0.10	6.44 ± 0.21
Encapsulated	9.71 ± 0.18	8.73 ± 0.30	8.67 ± 0.34	9.18 ± 0.39	9.16 ± 0.35	8.16 ± 0.17	9.27 ± 0.52

Table 1: Survival of Encapsulated *L. rhamnosus* under gastric condition.

Storage temperature	Moisture content	0 days	15 days	30 days	60 days	90 days	Survival %
Beads at 4°C	4.31 ± 0.28	8.37 ± 0.32	8.27 ± 0.43	7.11 ± 0.88	6.69 ± 0.23	6.53 ± 0.17	77%
Beads at 25°C	4.21 ± 0.53	9.15 ± 0.18	8.44 ± 0.16	7.32 ± 0.18	7.05 ± 0.52	6.89 ± 0.24	74%

Table 2: Storage analysis of Encapsulation and survival rate.

pH changes during yoghurt storage

The pH changes in the control and experimental yoghurts during storage at 4°C for a period of 8 weeks is shown in (Table 3). The control yoghurt with the traditional yoghurt starter cultures showed the lowest pH. The final pH (at end of 8 week storage) of yoghurt with encapsulated probiotic bacteria was greater than the yoghurts inoculated with free probiotic bacteria. Probiotic bacteria are slow acid producers [21]. Sevaral research reports suggest that there was much difference in probiotic strains with respect to survival under acid condition. The acid production may increase on storage which was sensitivity to probiotic culture and this process is indicated as over acidification. A comparative studies between the capability of probiotic bacteria strain to survive over a short period of time (2 - 3 hours in stomach) and capability to survive over a long term period on storage in fermented such as yogurt is not recorded. The results suggest that on storage analysis of acidification of yogurt with encapsulated form was slower when compared to both control and free probiotic.

Storage (weeks)	Control	Yogurt with free *L.rhamnosus*	Yogurt with Encapsulated form
1	4.59	4.62	4.68
2	4.21	4.51	4.60
3	4.09	4.40	4.52
4	4.0	4.35	4.48
5	3.98	4.31	4.45
6	3.97	4.29	4.40
7	3.95	4.25	4.37
8	3.94	4.24	4.35

Table 3: pH of yohurt on storage analysis.

Sensory evaluation

The average sensory scores of all panel lists are shown in table 3. The results showed that there were no significant differences in the appearance and color of the yogurt samples. Expected that addition of alginate capsules to yogurt mix could slightly color the yoghurts, but the panelist could not identify the differences in the appearance and color between yoghurts with encapsulated cultures from the other two treatments. The body and texture including smoothness of the yoghurt samples, however, showed significant differences between the yoghurts containing free probiotic bacteria and encapsulated bacteria. The yogurt gel is not a tight matrix but formed as a loose structure with fractal characteristics. This phenomenon was reported in yogurts made with encapsulated ropy and non ropy yoghurt cultures during storage [22]. The panel lists also could not differentiate the three types of yoghurts in flavor or after taste attributes. The mean overall liking scores were not significantly different between the three types of yoghurts (Table 4).

Treatment	Appearance and color	Body and texture	Acidity	Flavor	After taste	Overall liking
Control	4.43	5.31	8.54	8.11	7.55	8.0
free probiotic yogurt	4.78	5.65	9.11	9.32	8.12	7.81
Encapsulated yogurt	5.11	9.05	8.32	7.19	7.22	7.78

Table 4: Mean values of sensory evaluation of 6 panel members: (1-most desirable, 15-least desirable).

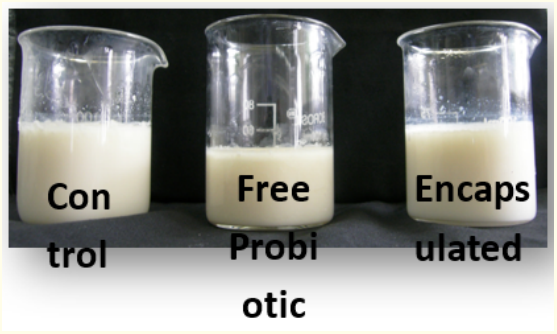

Figure 8: Illustration of control, free probiotic, and encapsulated yogurt.

Conclusion

The present research indicates that effect of probiotic *Lactobacillus rhamnosus* on encapsulation with calcium alginate beads with potential prebiotic Hi-maize starch give high survival rate under Gut condition (low pH and high bile concentration) and increase viability on storage conditions. On yogurt preparation with free and encapsulated form slows down the post- acidification on storage analysis when compared to the traditional yogurt (control). Usage of prebiotic Hi-maize starch helps to maintain the structure of yogurt texture. Sensory evaluation of the yogurt by panel member's results that addition of encapsulated probiotic did not much alter the appearance, color, acidity, flavor and taste of the yogurt. However slight variation in texture properties of yogurt and grittiness was observed in yogurt with encapsulated form. This research work suggests that the usage of *Lactobacillus rhamnosus* as potential probiotic by encapsulating with suitable prebiotic.

Conflicts of Interest

All authors have none to declare.

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