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Microencapsulation of probiotic Lactobacillus rhamnosus by extrusion technique with the selected coating material

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Abstract: Probiotics are defined as essential live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Survival and stability in the gastrointestinal tract are being debated by scientists. To overcome these limitations, microencapsulation techniques are receiving considerable attention. Microencapsulation is a physicochemical or mechanical process to entrap a substance in material to produce particles with diameters of a few micrometers to a few millimeters. In the present investigation, an indigenous probiotic strain Lactobacillus rhamnosus MTCC 5462 was microencapsulated through the extrusion process using chitosan and κ- carrageenan as coating polymers. Based on cell viability and bead size, the microencapsulation process was optimized. Probiotic L. rhamnosus MTCC 5462 was activated in MRS broth, harvested and the biomass was adjusted to 10^{11} to 10^{12} cfu/ml in sterile saline and then cell concentrate was added to sodium alginate and encapsulation was done by spraying in calcium chloride solution. The extrusion process with concentration (1, 2, and 3 %) of chitosan or κ-carrageenan showed a significant effect on cell viability and bead size. The chitosan as coating material gave maximum cell viability and minimum bead size.

1. Introduction

Probiotic bacteria are defined by the World Health Organization (WHO) as "live microorganisms which, when administered in adequate amounts, confer health benefits on the host". The therapeutic applications of functional live probiotics are received due to attention by scientists and health professionals. The probiotics have proven beneficial effects on gastrointestinal disorders, lactose intolerance, lowering the concentration of several metabolic products and exerts several immune-modulating on the host.

The incorporation of probiotics into food products for functional food development is well proven. However, some reports have demonstrated that during storage the viability reduced below the minimum recommended dose of 10^6 cfu/g. The low level of the cell's stability and survival in the gastrointestinal tract is also debatable and is a major challenge for functional probiotic food development. To overcome these technological challenges, new techniques, such as microencapsulation is currently receiving considerable attention to protect active substances during manufacturing, storage, and passage to GIT or any other extreme conditions and delivering functional active ingredients to the GIT[1]. Encapsulation surrounds probiotics by a compatible coating material forming a microcapsule *i.e.*, a small sphere with a uniform wall around. The internal material of the microcapsule is referred to as the core/ internal phase or fills while the wall is called a shell, coating, or membrane. Most microcapsules have diameters between a few micrometers to few millimeters.

The encapsulation of bioactive functional ingredients can be done using co-extrusion, spray drying, spray chilling, bead processing, etc. These methods apply to food ingredients as well as to other materials. The materials used include fats, waxes, glyceride derivatives, sugars, starches, and modified starches, dextrin, vegetable gums, gelatins, proteins, cellulose derivatives, caseinates, non-fat milk, alginates, carrageenan, guar gum, and others. It is possible to form the coating, which will release active material under conditions of the target site.

L. rhamnosus MTCC 5462 (earlier Lactobacillus acidophilus LBKI4) is a well-studied probiotic gastrointestinal strain isolated from infant fecal material. It can tolerate bile, phenol, and salt concentrations up to 2.4%, 0.4%, and 4%, respectively, and had antibacterial activity against milk spoilage bacteria and pathogens [2]. L. rhamnosus MTCC 5462 has been used for the production of dahi, lassi, fermented milk, and functional dairy products.

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In the present study, microencapsulation process extrusion optimization was carried out with selected carrier matrices for probiotic strain L. rhamnosus MTCC 5462 to improve survivability.

2. Materials and methods

2.0 Materials

Probiotic strain L. rhamnosus MTCC 5462 was obtained from the Dairy cultures collection of Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand. The strain was isolated from healthy human intestine Lactobacillus strains and preserved and well-characterized at the Department of Dairy Microbiology, AAU, Anand. Lactobacillus de Man Rogosa Sharpe (MRS) broth and agar, Sodium alginate (AR Grade), chitosan, and κ-carrageenan of food-grade were procured from Himedia Laboratories Pvt. Ltd, Mumbai. The Oxy bile, Hydrochloric acid and Acetic acid of food-grade were procured from Sigma Aldrich, Mumbai. Sodium citrate and Calcium chloride were purchased from Fisher Scientific. Vegetable oil meeting quality standards were procured from the local market. Unless otherwise specified, all other chemicals used were of analytical grade.

Methods

2.1 Preparation of L. rhamnosus suspension

For preparing the MRS medium, the quantity of media suggested by the supplier was suspended in 1000 ml distilled water. The contents were heated to boiling to dissolve the medium completely and made up to one liter. The medium was cooled and the pH of the medium was adjusted to 6.5 ± 0.2. The medium was then filled in conical flasks/ test tubes and sterilized at 121 °C for 15 min. This was used for the activation and preservation of L. rhamnosus strain.

2.2 Characterization of Probiotic Culture

MRS broth and agar slants were prepared as per the guideline provided by the supplier. To maintain the probiotic culture in an active stage, they were subcultured for three consecutive days in MRS agar slants at 37 °C for 24 h. The active culture was transferred in 1000 ml MRS broth and incubated at 37 °C for 24 h to get active cell biomass for further studies.

2.3 Morphological properties of L. rhamnosus

The activated culture was examined for Grams staining and Catalase reaction. The active culture was incubated in MRS broth having a pH of 3.0, 4.0, and 5.0 at 37°C for 24 h. The pH was adjusted with 0.5 N hydrochloric acid. The culture was also incubated in MRS broth at 37°C for 24 h having bile salts at the rate of 2% and 4%. Media containing bile salts were prepared by dissolving Oxi bile at the rate of 2g and 4g in 100 ml MRS broth respectively and adjusting the pH to 8.0 with 0.1 N NaOH.

2.4 Preparation of L. rhamnosus suspension

L. rhamnosus was reactivated 3 times before use. The culture was inoculated nto sterilized MRS (Hi-media, India) broth and incubated at 37 °C for 24 h. The cell biomass was harvested using a laboratory tabletop centrifuge (Make: Remi Laboratory Instruments, Mumbai) at 6000 rpm for 20 min and washed twice with the sterilized saline (0.85% NaCl) solution. Subsequently, the cell pellet was dispersed into a saline solution. The cell concentration was adjusted to 10^{11} to 10^{12} cfu/ml by spectrophotometer (Make: Shimadzu) readings and enumeration on MRS agar using a standard protocol to study the morphological characterization.

Microencapsulation

2.5 Microencapsulation of L. rhamnosus

L. rhamnosus MTCC 5462 containing alginate microcapsules were prepared as per the method previously reported by author Krasaekoopt, et al., [3] with minor modification. Sodium alginate solution was prepared by dissolving 2 g (w/v) of sodium alginate powder with 100 ml distilled water in 250 ml sterilized beaker. Afterward, it was heated until boiling for proper mixing. Calcium chloride solution was prepared by dissolving 5 g (w/v) calcium chloride granules in 500 ml beaker with simultaneous gentle stirring. Both the reagents were sterilized before use.

The cell biomass was added to a 2.0 % (w/v) strength sodium alginate solution with gentle stirring. Under aseptic conditions, the bacterial alginate suspension was packed into the sanitized container of spray gun (make: Pilot spray gun P-5) having nozzle size of 1.6 mm and this was connected to a laboratory air compressor (range: 14-58 psi). The filled alginate cell biomass mix was then sprayed into prepared 1% (w/v) calcium chloride solution. This allowed the formation of microcapsules and these beads were allowed to harden for 5 min. The probiotic ca-alginate beads were then harvested with the help of sanitized nylon sieve (size< 100 µm) and were air-dried at the normal temperature.

2.6 Coating of microcapsules

Coating of probiotic microcapsules was carried out using chitosan and κ- carrageenan solutions with 1%, 2% and 3% concentration as per the method suggested by Koo et al, [4], with minor changes. For preparing chitosan solution, the required amount (w/v) of chitosan flakes was dissolved in 100 ml of distilled water and then 2 ml 0.3 M acetic acid was added into the solution. It was heated on a hot plate until a clear solution was obtained. The solution was then filtered (Whatman filter paper No. 22) and used for the coating of microcapsules. For preparing κ- carrageenan solution, the required amount (w/v) of κ- carrageenan powder was dissolved in 100 ml of distilled water and then it was heated on a hot plate till the clear solution was obtained. The solution was then filtered (Whatman filter paper No. 22) and used for the coating of microcapsules. The probiotic microcapsules obtained were immersed in these solutions at a normal temperature for five minutes. Microcapsules that were obtained after coating were washed in sterile saline, harvested with 100 µm sanitized nylon sieve, air-dried, packed in glass vials, and stored under refrigeration at 7 ± 2 °C for further analysis.

3. Morphological Observation of Probiotic Encapsulated Beads

3.1 Size

The diameters (µm) of 20 randomly selected beads from each treatment were measured using an optical micrometer mounted on an optical microscope (make: Labomed).

3.2 Viability

The 10 g microencapsulated beads were accurately weighed and added in a 250 ml conical flask containing 90 ml of sterilized 2 % sodium citrate solution. This solution was constantly stirred for 60 min for the dissolution of beads and the release of entrapped probiotic bacteria. The homogeneous solution obtained was serially diluted to get appropriate concentrations and they were plated on MRS agar by pour plate technique. The plates were incubated for 48 h at 37 °C and the encapsulated bacteria were enumerated with the help of the colony counter (make: Nova scientific).

3.3 Microencapsulation efficiency

The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as:

$EY = (N / N_o) \times 100$

Where, N is the number of viable entrapped cells released from the microspheres, and N_o is the number of free cells added to the biopolymer mix during the production of the microspheres. This will be further used to express the viability as percent viability.

3.4 Statistical analysis

Statistical analysis was performed using Completely Randomized Design (CRD) where the extrusion process, type, and strength of chemicals were taken as independent variables. Probiotic microbial viability and bead size were taken as dependent variables.

4. Results and discussions

4.1 Morphological Characteristics of L. rhamnosus MTCC 5462

The probiotic culture L. rhamnosus procured from Dept. of Dairy Microbiology, SMC College of Dairy Science was extensively studied and proved as probiotic due to their acid and bile tolerance. Morphological features of the strain were observed and found gram-positive and catalase-negative (Fig. 1). Colony characteristics on MRS agar showed irregular shaped round colonies with varying sizes and rough surfaces. The results are summarized in Table1. The culture showed growth in MRS broth at pH 3.0, 4.0, and 5.0 proving acid resistance characteristics. The culture was also found active in broth containing bile salt at the rate of 2% and 4%, hence showed resistance to bile salts up to 4%. These results confirmed the probiotic properties of L. rhamnosus MTCC 5462.

Table 1: Physiological and morphological characteristics of L. rhamnosus MTCC 5462.

S. No.	Characteristics	Observations
1	Gram reaction	Positive
2	Morphology	Thin, long slender rods occurring singly, in pairs and short chains
3	Catalase test	Negative
4	Colony characteristics on MRS agar	Irregular shaped round colonies with varying size, rough surface.

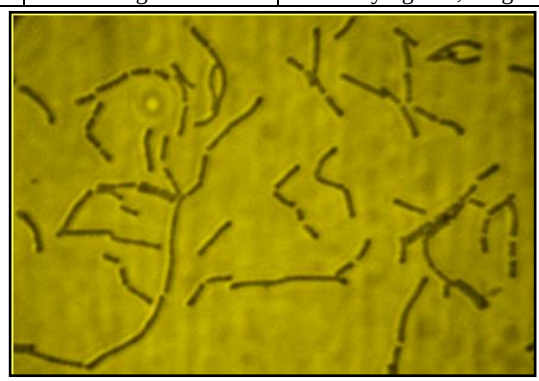

Fig. 1: Microscopic view of L. rhamnosus MTCC 5462

4.2 Standardization of Cell Concentration

Probiotic culture Lactobacillus rhamnosus MTCC 5462 was activated by sub-culturing three consecutive days in MRS broth at 37 °C for 24 h. Before proceeding for experimentation, probiotic cells were harvested by centrifugation to get cell biomass. The pellet was dissolved in saline to get cell biomass. The cell biomass of different concentrations was checked for OD at 640 nm in a spectrophotometer. Standardization of cell concentration was done by plotting the graph of cell concentration and optical density (Fig. 2). The relation between OD_{640} nm and log cfu/ml was evaluated by enumerating different concentrations of cell mass in MRS agar. As per the results obtained, the desired probiotic count of 10^{11} was obtained at 0.88 absorbances and this cell concentration was taken for all the further study.

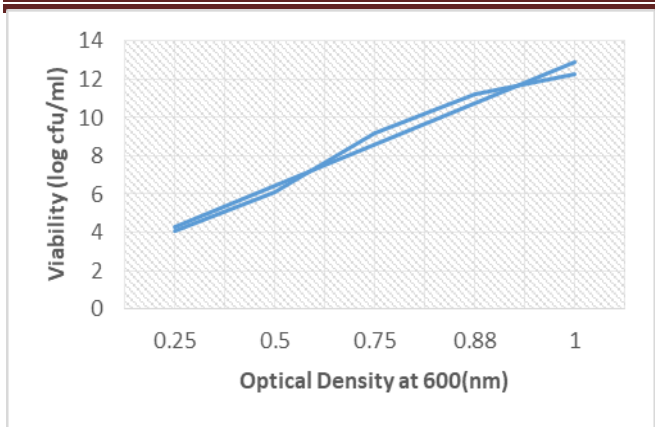

Fig. 2: Standardization of cell concentration of Lactobacillus rhamnosus MTCC 5462

4.3 Effect of chitosan and K-carrageenan and it's concentration on the viability of encapsulated cells and size of beads during the extrusion process

The effect of coating material i.e., chitosan and K-carrageenan and concentration of coating material on the viability of encapsulated cells and size of beads during the extrusion process is presented in figure 3 and 4 respectively.

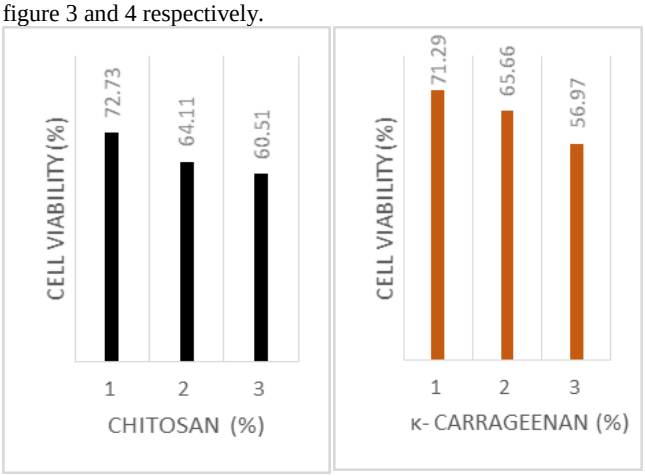

Fig. 3: Effect of chitosan and k-carrageenan in the extrusion process on cell viability

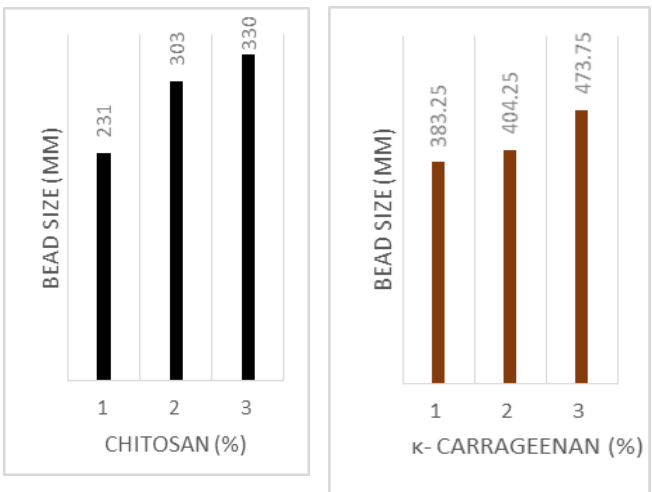

Fig. 4 Effect of chitosan and k-carrageenan in the extrusion process on bead size

The encapsulation matrix used primarily was sodium alginate (2%) because of its biocompatibility, nontoxicity, mildness at gelation, and low immunogenicity. Alginate beads are sensitive to the acidic environment and may not be compatible with resistance in the stomach conditions. The beads obtained were very porous and probiotic cells may not be protected from the stomach environment. To overcome these problems, the coating of alginate beads with chitosan and κ- carrageenan was done. The present findings are following the work done by Cirone et al., [5]; Urbanska et al., [6] and Mortazavian et al.[7].

The microencapsulation efficiency was calculated as average viability (%) and presented in Fig. 3. The highest cell viability of 72.73% was noticed in the extrusion process at 1% chitosan as coating material and the lowest cell viability of 56.97% was noticed in the extrusion process at 3% κ- carrageenan as a coating material. Overall, the chitosan was found more effective in protecting the viability of cells as coating material compared to k-carrageenan in the extrusion process.

It was observed that at 3% chitosan concentration, the mean viability was 60.51% in an extrusion process, whereas at 1% chitosan concentration it was found to be 72.73%. There was a decrease in the microbial cell population as the concentration of chitosan was increased. The viability was low at higher concentrations during the extrusion process.

The effect of chitosan concentration was found significant ($P<0.05$) on the probiotic L. rhamnosus viability. As the strength of the chitosan increased, the viability of the probiotic cells decreased (Fig 3). The present observations are in accordance with Zhou et al.,[8] wherein at a lower concentration of coating materials, the viability of cells was better. Groboillot et al.[9] reported that a secondary layer of chitosan polymer around the alginate capsules improves physical and chemical stability.

It was observed that as the concentration of chitosan increased from 1 to 3%, the viability of the microbial cells decreased which might be due to antimicrobial activity of chitosan on lactic acid bacteria. Sudarshan et al., [10] reported that the antimicrobial property of chitosan limits its use as a coating material in the process of encapsulation. The present observation shows that the strength of coating material has a significant effect on the viability of probiotic cells.

In present investigation κ- carrageenan was used as another coating material. It was observed that at 3% κ- carrageenan, the mean cell viability was 56.96% in the extrusion process, whereas at 1% κ- carrageenan concentration it was found to be 71.28%. As the concentration of κ- carrageenan increased, there was a decrease in the microbial cell population (Fig 4).

The effect of κ- carrageenan concentration has a significant effect at a 5% level on the probiotic cell viability. As the strength of the κ- carrageenan increased, the viability of the probiotic bacteria decreased (Fig 3). The higher concentration of κ- carrageenan might be inhibitory to lactic acid bacteria. In a similar experimental study, Audet et al.[11] reported an inhibitory effect on some lactic acid bacteria such as Lactobacillus delbrueckii ssp. bulgaricus (yogurt bacteria).

The bead size of the microcapsules coated with 1% chitosan was 230.50 µm while with 1% κ- carrageenan it was 238.35 µm in the extrusion process. As the concentration of chitosan and κ- carrageenan increased, the size of the beads also increased (Fig. 4). Similar findings were reported by Jankowski et al.[12] and Krasaekoopt et al., [3]. Bead diameter was affected by the concentration and viscosity of the chemicals used in the process of encapsulation.

4.4 Optimized process parameter for microencapsulation of L.rhamnosus

Optimization of the microencapsulation process was done to get minimum bead size and maximum viability of the probiotic microorganisms. The minimum bead size in the extrusion process was 233 µm, at 1% chitosan concentration. Similarly, the maximum percent viability in the extrusion process was 73.95 at 1% chitosan concentration.

4.5 Morphological studies of encapsulated probiotic beads

Gram staining and catalase test were performed and found results similar to without encapsulated cells. These observations showed no cross-contamination during the encapsulation process.

5. Conclusions

From the present investigation, it is concluded that chitosan is more effective as protective material than k-carrageenan for use in the extrusion process for microencapsulating probiotic Lactobacillus rhamnosus MTCC 5462 with 1 % concentration. These encapsulated probiotic beads can be used for the development of various functional food products for the effective ingestion of probiotics.

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