The effect of Immobilization of Lactic acid Bacteria in Alginate coated with Chitosan on their Resistance to Laboratory Model of Human Gut and on Antibacterial Activity

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Abstract: ***Probiotics have been incorporated into many products due to their promising preclinical and clinical results. However, there is poor survival of free LABs exposed to gastric acid and bile salts. The present study aimed to immobilize LAB in alginate using chitosan as coating material. Successful immobilization of LAB were accomplished and high viable counts (· 10^8 cfu/g) in the gel beads were obtained. Model conditions of digestion were provided and resistance of immobilized LAB were investigated. The high resistance of the immobilized cells to low and neutral pH values and the presence of pepsin and high concentrations of bile salts were demonstrated. The immobilized cells of studied probiotic LABs showed a significantly higher resistance when compared to free growing cells. The antibacterial activity of immobilized LAB were observed against several pathogenic bacteria. Thus, these immobilized isolates could be considered as candidate for future application in probiotic preparation***.

Keywords: Immobilization, Chitosan, Pepsin

I. INTRODUCTION:

Lactic acid bacteria (LAB) have received increasing attention in recent research due to their highly valued physiological effects on the functioning of the intestines and, hence, on human and animal health [1]. Probiotics are helpful live micro-organisms administered as food supplements in order to improve host intestinal microbial balance and thus digestive system. Most probiotic belong to strains of lactic acid bacteria (LAB) because benefits of LAB include : inhibiting infection by pathogens, improve digestion, lowering blood cholesterol, antimutagenic properties, anti-diarrheal properties, improvement in inflammatory bowel disease, reducing risk of colon cancer. In order to serve beneficial effects of probiotics microorganisms, they must have to reach at intestine in active form and in high concentrations. Inactivation of LAB during food processing also limits the health benefits. Most of free LAB are very sensitive to environmental conditions, such as acidic pH, various denaturant factors such as bile acids, oxygen or antimicrobial compounds present in certain food. So, there is a compelling need to develop new protective technological conditions to keep LABs alive and active during processing and storage. Immobilization of micro- organisms by entrapment in gel beads was considered as a good method to preserve their viability for nutraceutical formulations. Most commonly alginate is used as biomaterial for probiotics encapsulation. Although the Ca-alginate gel possesses no toxicity against cells, it is known to be chemically unstable in the presence of calcium chelators, such as phosphate, lactate or citrate, and of cations, such as sodium and magnesium which are able to displace calcium. Still, however, the chemical stability of alginate beads can be coated with polycations, such as chitosan, gelatin, and poly-L-lysine, to improve bead stability [1]. Chitosan is a linear polysaccharide, the main component of the crab shells, some species of shrimp and sepia, cuticle of insects. Chitosan inhibits the growth not only of viruses and bacteriophages, but also of Gram-positive bacteria, such as *Staphylococcus aureus* and *Listeria monocytogenes*, and some of Gram-negative bacteria such as *Pseudomonas aeruginosa*. The aim of this study was to check the effect of immobilization of probiotic LAB in alginate coated with chitosan on their resistance to model conditions of digestion and on antibacterial activity and develop probiotic preparations.

II. MATERIALS AND METHODS:

(A) Materials: Chitosan powder – 40 MESH was purchased from Everest scientific laboratory, Bengaluru. Pepsin (IP 1:3000) was purchased from Anthem Biosciences. Sodium alginate, $CaCl_2$ and Bile salts were purchased from Finar Ltd. Mueller-Hinton agar medium was purchased from Hi-Media, Mumbai.

(B) Collection of samples: As milk and milk products are good source of Lactic acid bacteria raw milk, homemade curd and ferment product such as Idli batter were taken as samples. Total 9 samples were collected from different areas of District Valsad and Navsari in clean small container and stored at 4 C.

(C) Isolation and Identification of Lactic Acid bacteria: Under aseptic condition, loopful of sample was directly streaked on sterile MRS agar plates and they were incubated at room temperature for 24 to 48 h anaerobically in anaerobic jar. Total 8 isolates were studied for colony characteristics, Gram's reaction, MR test, Oxidase activity, Catalase activity and Hemolytic activity. All isolates were purified on MRS agar medium and were maintained by monthly sub-culturing from 48 h MRS broth.

(D) Immobilization of isolates in alginate-chitosan gel matrix: Isolates were cultured at 37˚ C for 48 h followed by centrifugation for 20 min. at 3000 rpm. Then 2 ml ofcell suspension was added in 8 ml of 3% w/v sodium alginate solution and mixture was taken into the syringe (23 gauge). Beads were formed by dropping the solution drop wise into 50 ml of chilled 1 M Calcium chloride solution and $CaCl_2$ solution refrigerated for 2 h. Formed beads were recovered by filtration using Whatman filter paper no.1 and were dried in natural condition. Alginate beads were immersed in 50 ml of 1% chitosan-acetate solution (1 g chitosan was mixed with 98 ml distilled water and 0.8 ml 5% acetic acid and stirred at boiling temperature until the solution lighten) and were put on rotary shaker at 150 rpm for 15 minutes for coating. Afterwards beads were rinsed with sterile distilled water to remove excess chitosan, and then dried.

(E) Growth of the isolates in alginate-chitosan gel matrix: A modified method Denkova *et al*., 2007 was used. After immobilization, around 2g of beads were washed with saline solution and transferred in 25ml fresh nutrient LAPTg10 medium. After inoculation, tubes were incubated at 37°C for 24-48 h for further development in cell density in the gel matrix.

(F) Determination of the viable cell counts in the gel matrix: A modified method Denkova *et al*., 2007 was used. Physical and Mechanical destruction of beads was carried out by both vortexing and pressing the beads with sterile rod and the obtained suspension was diluted using Peptone water. After that suspension was spread on LAPTg10 agar plates, which were incubated for 24-48 h at 37°C.

(G) Titrable acidity: For determination of the acid forming ability of the immobilized isolates, the titrable acidity was determined by titration method. 10 ml of sample was taken in flask and 2-3 drops of phenolphthalein indicator were added, mixture was titrated against 0.02 N NaOH. Endpoint was confirmed by development of pink colour. Calculations were carried out to determine acidity of sample.

(H) Determination of the resistance of isolates to low and neutral pH values and in presence of pepsin: The inoculum of isolates were incubated for 16 h in MRS-broth and then centrifuged at 3000 rpm for 20 min. Pellets were washed with 0.2 M phosphate buffer, pH 7.0 and resuspended to the original volume with the same buffer. Buffer with pH 2.0 and pH 7.0, containing 2 g/l NaCl and 3.2 g pepsin were incubated with 0.5 ml of the bacterial suspension. The resistance of the immobilized cells to low and neutral values of pH in the presence of pepsin was determined after the incubation of 2 g of gel beads in the respective buffers. The buffers were incubated at 37°C and samples were taken on the 0, 2h, 4h

and 24 h of incubation. Proper dilutions were made in peptone water and spreaded on LAPTg10 agar plates which were incubated at 37ºC for 24-48h.

(I) *Determination of Bile tolerance of isolates:* Steps were the same as mentioned above in the method for determination of acidity resistance until the bacterial suspension was obtained. 20 ml of MRS-broth, containing different concentrations of bile salts (0%; 0.3%; 0.6%; 1.0%) was incubated with 0.5 ml of the cell suspension or 2 g of gel beads. The MRS broth containing free and immobilized cells of the lactic acid bacteria were cultivated at 37ºC and the viable cell counts were determined at 0 h, 3 h, 6 h, and 24 h respectively.

(J) *Evaluation of survival of free and immobilized cells under the heat treatment:* The viability of free and immobilized cells dispersed in 1% normal saline solution was exposed to heat treatment. Free cells and immobilized cells of isolates were placed in test tubes containing 9 mL of normal saline solution. The test tubes were further incubated in a water bath at general pasteurization temperatures (63 and 72 C) for 2 min. Aliquots were collected at different intervals of time after incubation (20s, 40s, 60s, 80s, 100s). The samples were cooled down to room temperature. To check the viability, proper dilutions were made in peptone water and spreaded on LAPTg10 agar plates. The plates were incubated at 37ºC for 24-48h.

(K) *Antibacterial activity*: The antibacterial activity of chitosan-acetate solution and

isolates before and after immobilization in chitosan coated alginate beads were determined on MH agar plates by well diffusion method against pathogenic test organisms – *Staphylococcus aureus, Bacillus cereus, Salmonella typhi* and *Escherichia coli*. First, MH agar plates were swabbed on the surface with test bacterial cultures. Then, 6 mm diameter wells were prepared with cup borer and 20 µl chitosan-acetate solution, 20 µl cell-free supernatants of free and immobilized cells of isolates were loaded in the respective wells. After 24-h incubation at 37◦C, inhibition zones were measured.

(L) *Preparation of starter cultures and Fermentation of milk:* A modified method of Akabanda *et al*., (2014) was used to prepare inoculum. The LAB isolates to be used as inoculum were prepared by transferring a loopful of an overnight culture from MRS agar into 15 ml MRS broth and incubated at 37° C for 24h. 100 µL of the 24h old culture was transferred into 15 ml MRS broth and incubated at 37 C for 16h. After incubation, cells were harvested by centrifugation at 2500 rpm for 15 minutes; washed three times with sterile normal saline and finally suspended in 5 ml of sterile diluent(normal saline), and these served as the isolate inoculum. Clean containers containing 50 mL of fresh pasteurized milk were inoculated with 1 ml of inoculum or 1 g of beads packed in manjarpat cloth at room temperature (30-32° C) for 24h.

III. RESULTS AND DISCUSSION :

Isolation and probiotic characteristics of LAB:

In this study, 9 different samples were used for the screening of bacteria on MRS (deMan, Rogosa and Sharpe) agar plates. Among the 9 samples, 7 samples were screened positive while 2 were negative on the basis of colour changes observed due bacterial growth on MRS agar plates. Total 8 isolates were obtained from 7 samples tested for lactic acid bacteria. Obtained isolates were subjected further for Gram staining in which all the 8 isolates were found to be Gram positive in nature. Isolates were then selected for further studies on the basis of their ability to fulfil probiotic characteristic that is catalase activity and hemolytic activity in which S3 and S5 were selected as they were catalase negative and nonhemolytic.

Immobilization of isolates in alginate coated with chitosan:

Immobilization of the cells S3 and S5 in alginate gel coated with chitosan was successfully achieved. During the cultivation of immobilized S3 and S5 cells in LAPTg10 broth for 48 hours, the increase of the viable cell counts in alginate- chitosan gel structures aspire the opportunity for the application of alginate and chitosan in the immobilization of LAB. Variation in titrable acidity as shown in Table - 1 indicates that the cells have preserved their physiological activity even after immobilization

Table – 1 Growth of the S3 and S5 cells in the alginate-chitosan gel matrix

Organism	0 hour		24 hours		48 hours	
	Log N	TA (%)	Log N	TA (%)	Log N	TA (%)
S3	7.53	20	8.28	29.27	8.97	52.24
S5	7.57	21.3	8.50	31.52	9.22	58.55

Resistance of immobilized cells to model conditions of digestion:

The free cells of both isolates were very sensitive at low pH and in presence of pepsin, reduced their viability with 2-3 orders whereas immobilized cells were found resistance and only decreased with 1 order after 4 hours of incubation that is ideal time period of food to stay in stomach. At a same time, both free and immobilized cells of both isolates were less sensitive towards media with neutral pH and in presence of pepsin (Figure-1). Optimum pH values of pepsin is pH-1.2-2.0 which indicates that action of pepsin is inhibited in neutral media, thus investigated both isolates remained highly viable even in the free form at neutral pH values.

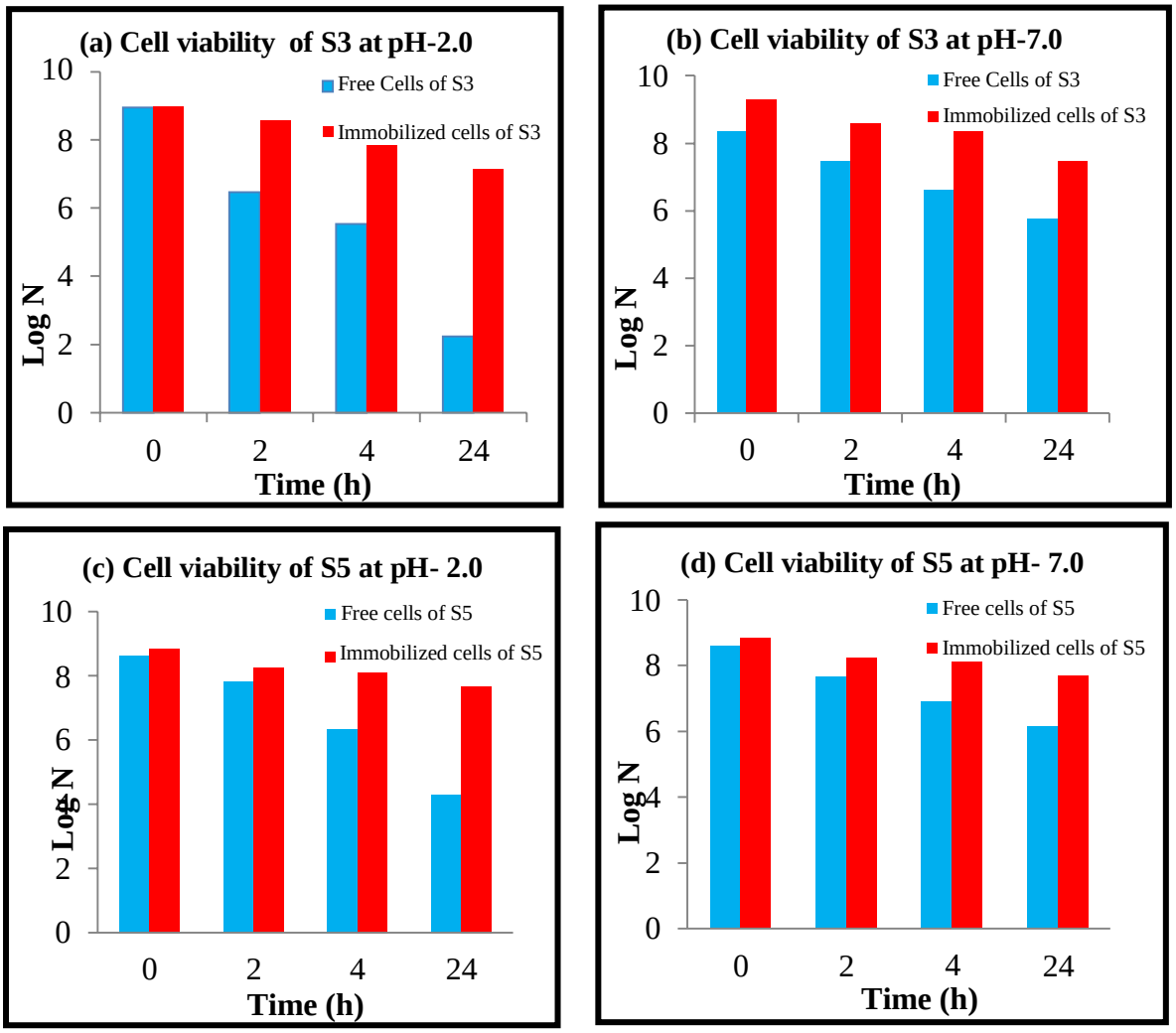

Figure – 1 Cell viability of (a) S3 at pH-2.0 (b) S3 at pH-7.0 (c) S5 at pH-2.0 (d) S5 at ph-7.0In the of bile salts into the

medium, viability of free and immobilized cells of both isolates increased whereas survival rate of cells was decreased at increased bile salts concentrations (Figure-2). Free cells of both isolates were sensitive to the bile salts concentrations their viability was drastically decreased with 3, 4 and 5 orders at 0.3%, 0.6% and 1.0% respectively after 24 h of incubation. On the contrary, the immobilized cells of both isolates were more resistant. At 0.3% bile salts in the medium for 24h of cultivation they kept high viable cell counts of 10^7cfu/g, and at 1.0% bile salts the number of viable cells diminished with 3 orders only after 24h of incubation.

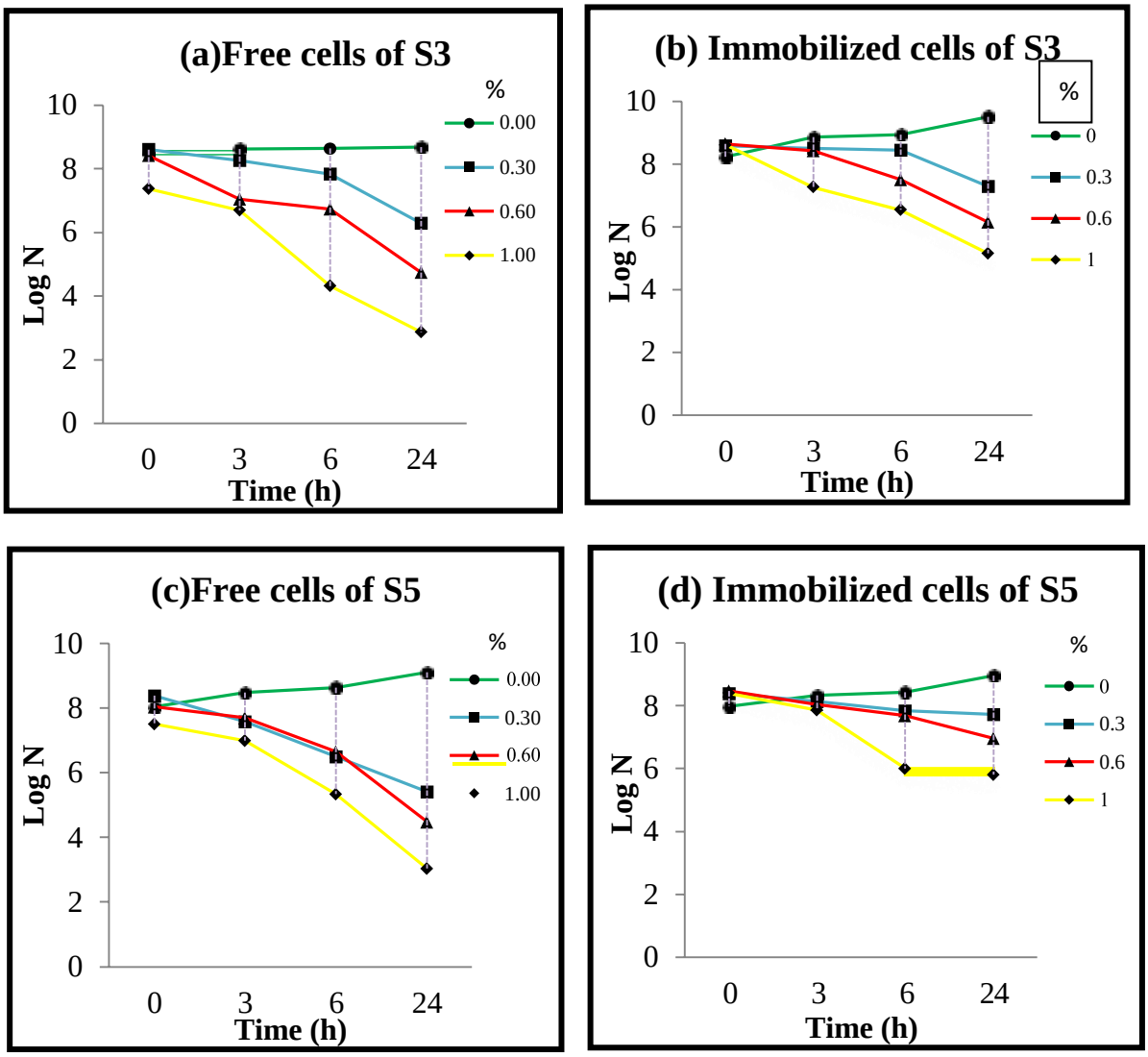

Figure-2 Variation in the concentration of viable (a) free cells of S3 (b) immobilized cells of S3 (c) free cells of S5 (d) immobilized cells of S5 during cultivation in the presence of different bile salt concentrations in the medium

The cytoplasmic membrane is made up of phospholipids that contain fatty acids. They form inclusion substances after interacting with bile salts that may disturbs the transport of nutrients through the membrane. Mostly young growing cells are more susceptible while cells in stationary phase may overcome this stress and form colonies on solid nutrient medium's surface. From above results, it can be stated that lower concentrations of bile salts permit the growth of survived cells to develop and the higher ones inhibit their growth partially or completely.

Viability of free and immobilized cells under heat treatment:

Free cells of both S3 and S5 were decreased with 5 and 4 orders respectively after 100s of incubation at 63 C whereas immobilized cells of S3 and S5 in alginate-chitosan gel beads remained with their good viable cell counts $1.8*10^6$ and $4.5*10^6$ respectively after 100s of incubation at 63 C as shown in Figure- 3 (a), (b).

Almost Free cells of S3 and S5 were killed during incubation of 100 s at 72 C whereas immobilized cells of S3 and S5 managed to maintain their good viability with survival rate of 10^5 and 10^6 cfu/ml respectively as shown

in Figure-3 (c), (d).

Overall, free and immobilized cells of S5 are more resistant to heat treatment in compared to free and immobilized cells of S3. Above results confirmed the presence of immobilized S5 bacterial cells at minimum levels of 10^6CFU/ml, which can recommended to use in functional foods.

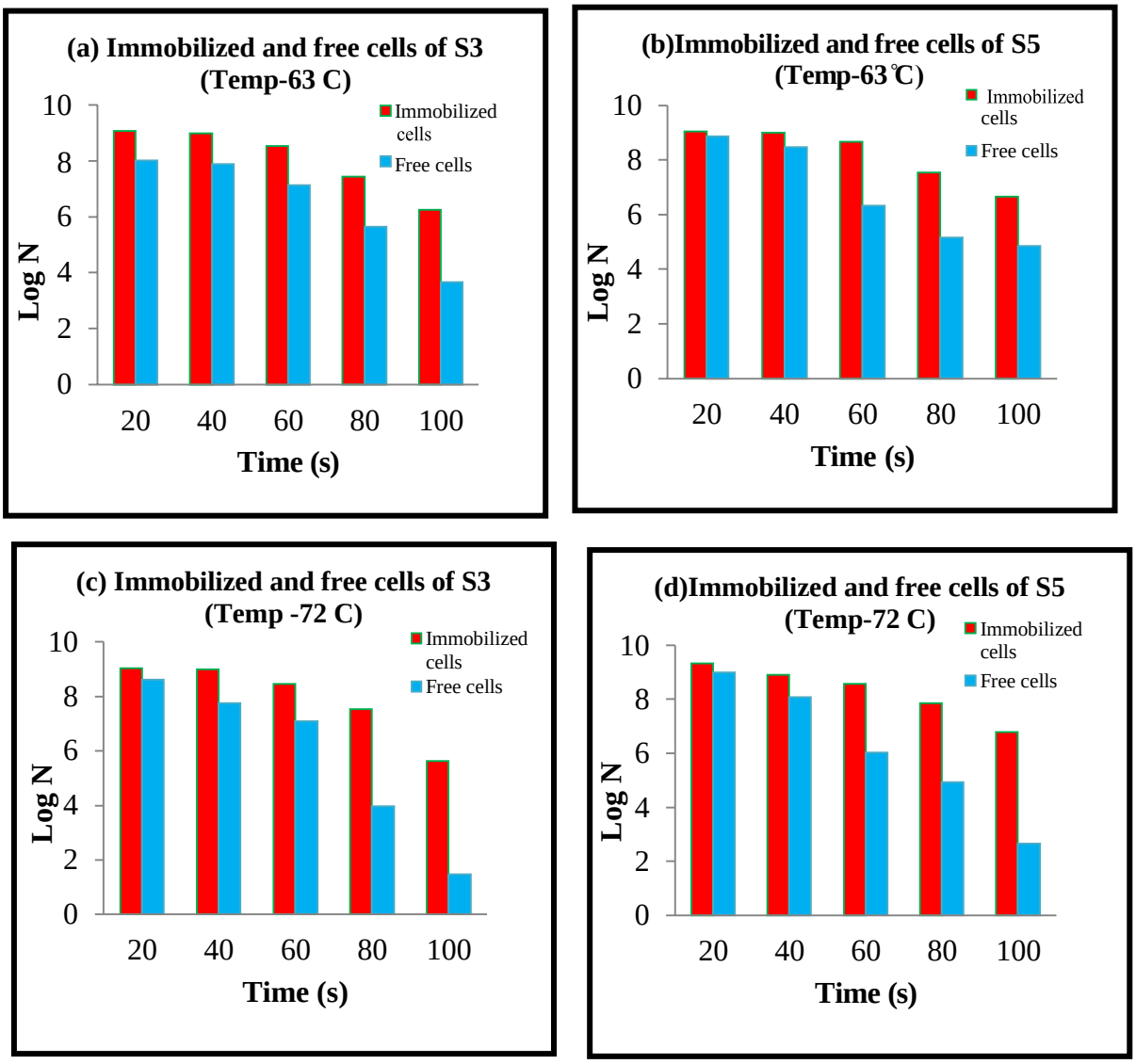

Figure- 3 Survival of free and immobilized cells of (a) S3 under heat treatment at 63 C (b) S5under heat treatment at 63 C (c) S3 under heat treatment at 72 C (d) S5 under heat treatment at 72 C for different time interval.

Antibacterial activity before and after immobilization:

Chitosan-acetate solution which was used to coat alginate beads displayed greatest activity against *S. aureus* with 21 mm inhibition zone, followed by uncleared 19 mm inhibition zone against *B. cereus.*

Figure- 4 Antibacterial activity of chitosan-acetate mixture against test organisms- *S.aureus*, *B. cereus*, *S. typhi B* and *E. coli*.

Table – 2 Results of Antibacterial activity of free and immobilized cells of S3 and S5

Cell type / Test organisms	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus cereus*	*Salmonella typhi B*
S3	-	-	10 mm	10 mm
S3B*	13 mm	16 mm	15 mm	15 mm
S5	12 mm	13 mm	14 mm	10 mm
S5B*	14 mm	21 mm	19 mm	10 mm

B*= immobilized cells

From the above results, it can be stated that free and immobilized cells of S3 and S5 were less effective against *E. coli* and *S. typhi* whereas much more effective against *S. aureus* followed by *B. cereus*. The immobilization of cells in chitosan coated alginate beads led to proliferation of S3 and S5 cells and that may increase the secretion of antimicrobial compounds which indicates that chitosan as a coating material is more beneficial and it can protect viable and hence biologically active cells in the beads.

Fermentation of Milk :

Free cells as starter culture: After 18 hours of incubation at room temperature, free cells of S3 formed thin and mild yoghurt whereas free cells of S5 formed thick yogurt with good odor, taste and firm texture.

Immobilized cells in chitosan coated alginate beads as a starter culture: After 16 hours of incubation, it was observed that process was started and mild yogurt formation was seen and whole process was completed in 24 hours. Immobilized cells of S3 formed lumpy yogurt in

which watery liquid oozed to the surface whereas immobilized cells of S5 formed smooth and firm yogurt. However, typical smell of alginate and chitosan was observed that made unable to taste the product. To overcome this problem, there is a need to try making fruit based yogurt. After the whole process was completed, found that added beads were not broken which indicates that they remained stable in the fermentation medium during the incubation period. From these recovered beads, viable cell counts of S3 and S5 1.01 x 10^8 and 1.62 x 10^8 cfu/g respectively were found.

IV. CONCLUSION

High concentrations of viable cells were achieved by the immobilization of the cells of S3 and S5 in alginate beads coated with chitosan. The results revealed that model conditions of digestion such as low and neutral values ofpH, presence of enzyme and high concentration of bile salts had no diminishing effects on immobilized cells. Even immobilized cells survived longer relative to free cells of S3 and S5 subjected to heat treatment. Findings of the experiment indicated that immobilized cells of S3 and S5 in chitosan coated alginate beads exhibited great antibacterial activity against *S.aureus* followed by *B. cereus*. Yogurt of good quality was also formed by using immobilized S5 cells and the problem of undesirable typical smell can be solved by making fruit based yogurt. Thus, by using this strategy in the composition of probiotics will improve their quality by supplying the organisms with high concentrations of viable cells. After the yogurt formation, viability of immobilized cell is reduced negligible which indicates that this yogurt contains sufficient concentration of LAB when it is consumed by the host.

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