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Evaluation of thermal tolerance of co-encapsulated beads incorporated with probiotic culture and L-ascorbic acid

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Abstract
The proposed work was focused on the thermo tolerance of co-encapsulated beads formed with lactic acid bacteria and ascorbic acid. The beads were prepared by emulsion and extrusion method of encapsulation wherein coating material sodium alginate (4 %) and maize starch (2 %) were used. Microcapsules thus prepared by these two methods were freeze dried at -72˚C under vacuum. Thermo tolerance of the co-encapsulated beads were analyzed to check their stability under different temperatures viz. 45, 55, 65 and 75°C for 20 minutes using distilled water as suspending medium. In case of beads made from extrusion method, treatment and periodic mean value showed a significant difference from initial mean of 10.01 log to 8.61 log. For ascorbic acid a significant reduction was observed in the treatment mean values. In periodic mean value also, a significant difference was observed from initial mean of 14.39 mg to 10.24 mg. For each of the temperatures applied, the lowest reduction was observed at 45˚C (1.63 mg) and highest reduction was observed at 75˚C (5.97 mg). In case of beads made from emulsion method, a significant reduction was observed in the treatment as well as periodic mean values. For each of the temperatures applied, a significantly lowest and highest reduction was observed at 45˚C (0.13 log) and 75˚C (1.83 log), respectively. Similarly. a significant reduction was observed in the treatment mean values and periodic mean values for ascorbic acid and significantly lowest reduction was noted at 45˚C (1.08 mg) and highest at 75˚C (6.8 mg).

Keywords: Probiotics, microencapsulation, co-encapsulation, ascorbic acid, thermo-tolerance

Introduction
Major challenges are associated with the probiotic's survival and vigor throughout production, storage, and passage through the gastrointestinal tract. For these bacteria to positively affect health, they must endure and proliferate in the gut environment in a certain quantity (Kailasapathy and Chin, 2000) [8].

Probiotics have been investigated for improving their viability and vitality in food products as well as their passage through the gastrointestinal tract using microencapsulation in hydrocolloid beads (Krasaekoopt *et al*., 2003; Mandal *et al*., 2006; Annan *et al*., 2008) [9,11,3]. According to Mandal *et al*. (2006) [11] and Ding and Shah (2009) [7], microencapsulation protects cells from damaging conditions and low-intensity heat exposure by possibly lowering cell damage and eventual death.

Microencapsulation is an efficient way to prevent probiotics from degrading. According to Tripathi *et al*. (2013) [17] and Anselmo *et al*. (2016) [4], probiotics should be stable during storage, protected from extreme conditions in the upper gastrointestinal tract, released into the colon, and then stimulated to colonize the mucosal surfaces utilizing an effective microencapsulation technique.

Numerous studies conducted over the past few decades have demonstrated various health benefits that probiotics, prebiotics, and their formulation as synbiotics offer to people. According to Pandey *et al*. (2015) [13], functional foods are essential dietary supplements that claim to alter, change, and replenish the body's natural gut flora. Environmental factors such as temperature, pH, oxygen, metal ions, UV, and X-rays affects the stability of ascorbic acid. Because ascorbic acid is usually unstable and readily broken down during processing, it may impart yellowish tinge.

Moreover, ascorbic acid may interact with other ingredients to cause undesirable alterations in the flavor and color of food product (Wilson and Shah, 2007; Moon *et al*., 2020) [20, 12]. Foods that contain stable vitamin C may benefit from their inclusion (Wilson and Shah, 2007) [20]. To overcome some of its disadvantages, ascorbic acid is encapsulated using the microencapsulation technique. In addition to mask the acidic taste, the microencapsulated ascorbic acid prevents color changes and maintains the product's stability. According to Uddin *et al*. (2001) [18], microencapsulation reduces the possibility of any interaction between ascorbic acid and other ingredients in food products.

Since ascorbic acid is sensitive to temperature, water, and oxygen, it is challenging to incorporate it into many food products. The naturally high ascorbic acid content of many fruits and vegetables rapidly degrades when they are kept or exposed to specific post-harvest processes. Ascorbic acid oxidizes rapidly in solution but is generally stable in air when dried. Additionally, exposure to heat, moisture, and light may cause it to darken. Cooking typically destroys ascorbic acid by accelerating the oxidation process. Packaging and encapsulation can help to reduce some of these losses, but will add to the cost of the product (Teucher *et al*., 2004) [16].

The instability of ascorbic acid during storage, heat processing, and cooking are the main causes of unwanted sensory changes in liquid products. Thermotolerance is one of the criteria which can indicate the proper passage of co-encapsulated microcapsule in the form of food through gut. Here, present investigation was conducted to determine whether micro-encapsulated beads tolerate and protect probiotic bacteria and ascorbic acid from temperature abuse which is a main concern during manufacturing of dairy products.

Materials and methods

Probiotic culture *Lactobacillus helveticus* MTCC 5463 (V3) encapsulated with ascorbic acid by two conventional methods *i.e.* extrusion and emulsion method.

Encapsulation of lactic acid bacteria cells and ascorbic acid using suitable medium

The suggested work focused on encapsulating lactic acid bacteria and ascorbic acid using two different encapsulation techniques (emulsion and extrusion). The coating materials such as sodium alginate (4%) and maize starch (2%) (Hi-media) were autoclaved and used as encapsulating materials. L-ascorbic acid (Hi-media) and a 12-hour-old lactic culture were used as core material. After an overnight growth period, a cell suspension was made. The coating to core material ratio was maintained at a 5:1 ratio (20 ml of coating material to 4 ml of cell suspension). 100 ml of coating material was mixed with 100 ml of L-ascorbic acid. To create a homogenous mixture, the coating and core materials were thoroughly mixed (Das and Gawai, 2024) [6].

Encapsulation by extrusion method

An insulin syringe with an internal diameter of 0.18 mm and a needle thickness of 28G was used in accordance with the procedure outlined by Krasaekoopt *et al*. (2003) [9]. Drop by drop, the mixture (20 ml) was extruded through the needle into a 100 ml 0.1 M $CaCl_2$ hardening solution, and it was left undisturbed for an hour. The beads were then gathered and kept in a refrigerator.

Encapsulation by emulsion method

This procedure was followed as outlined by Mandal *et al*. (2014)[10], using 100 ml of pre-autoclaved soybean oil with 0.5% Tween 80 added as an emulsifier (which serves as a continuous phase). Next, drop by drop, the coating-core material mixture (20 ml) was added to the magnetically stirred continuous phase. After 5 minutes, a uniformly turbid emulsion was produced, into which 100 ml of previously cooled 0.1 M $CaCl_2$ was rapidly injected to break up the emulsion and harden the alginate-starch microcapsules. After that, it was left undisturbed for 20 minutes. For adequate hardness and elimination of oil odor, the capsules were harvested by gentle centrifugation at 350 g for 10 minutes and then rinsed three times with cooled $CaCl_2$ solution. The beads were separated by filtration and stored in refrigerator (Das and Gawai, 2024) [6]. Microcapsules formed by these two methods were freeze dried in a bench top freeze drier at -72˚C under vacuum and analysed for the following parameters.

Cell viability

The micro-encapsulated probiotic culture's vitality was assessed following the capsule's depolymerization and plating on MRS agar (Mandal, 2006) [11]. One gram of microcapsule was placed in 9 ml of sterile phosphate buffer solution (pH 7.1 ± 0.2), incubated for an hour at 37°C, and then depolymerized by vertexing until a uniformly cloudy solution was formed. Appropriate dilutions were then prepared using 9 ml of sterile phosphate buffer tubes. Encapsulated lactobacilli cells are released from the alginate-maize capsules as a result of the de-polymerization of sodium-alginate caused by phosphate ions in the solution reacting with sodium in the alginate. Colonies formed after 48 hours at 37°C were enumerated using MRS agar by pour plating.

Ascorbic acid concentration

The 2,6-dichlorophenolindophenol titration method of AOAC 967.21 technique AOAC (1997) [1] was used to determine the ascorbic acid concentration. The process relies on ascorbic acid's stoichiometric reduction of the dye 2, 6-dichlorophenol indophenol to a colorless substance. 100 mg of the microparticles were dissolved in 50 ml of sterile phosphate buffer and left to depolymerize in order to prepare them for the ascorbic acid assay.

In a conical flask with 24 ml of water, standardized 2, 6-dichlorophenol indophenol (Hi-media) dye was taken and titrated with 1.0 ml of 0.1% ascorbic acid and noted the quantity of dye required to reach the desired pink color. 25 ml of the depolymerized sample's hazy solution were transferred to an Erlenmeyer flask, and 25 ml of 5% TCA were then added. It was shaken well and filtered using Whatman filter paper No. 1. To get the pink color end point, 25 ml of filtrate were placed in a conical flask and titrated with the dye. Noted the quantity of dye used in the titration. Vitamin C was calculated using the following formulas:

$$\text{Ascorbic acid (mg/100mg microcapsules)} = \frac{8Y}{X}$$

Y=Titer value of sample
X= Titer value of standard ascorbic acid

Thermo tolerance

Tolerance of encapsulated probiotic culture to heat treatment was carried out by the method described by Mandal (2006) [11]. It consists of treating sample at different temperatures *viz*. 45, 55, 65 and 75°C for 20 minutes using distilled water as suspending medium. One gram of microcapsules or 1 ml of the free cell suspension was transferred in test tubes containing 10 ml of distilled water. After the heat treatments described earlier, the content was cooled to room temperature (30°C) and viable cells were enumerated.

Statistical analysis

Statistical analysis was carried out using statistical design FCRD (Steel and Torrie, 1980) [15]. All the experiments were conducted in required numbers of replications and the results are expressed as mean± standard deviation (SD).

Results and discussion

Microcapsules made from both of the methods *i.e.* extrusion and emulsion method were selected for further analysis.

Thermo tolerance of probiotic culture in encapsulated beads

Thermo tolerance of the co-encapsulated beads were analysed to check their stability under four temperature conditions *viz*. 45, 55, 65 and 75°C for 20 minutes using distilled water as suspending medium.

In Table 1, thermo tolerance of probiotic culture encapsulated by extrusion method is shown. A significant (p<0.05) reduction was observed in the treatment mean values. At 45˚C the mean log value was highest and at 75˚C it got reduced by 1.62 logs. In the periodic mean value also, a significant (p<0.05) difference was observed from initial mean of 10.01 log to 8.61 log.

Table 1: Thermo tolerance of probiotic culture encapsulated by extrusion method

Temperature (T, ˚C)	Probiotic count (log cfu/g) at varying heating periods (P, min)		Temperature mean (T)
	0	20	
45	10.31±0.02	10.06±0.10	10.19^{d}
55	10.11±0.07	8.76±0.35	9.44^{c}
65	9.97±0.23	8.11±0.28	9.04^{b}
75	9.62±0.31	7.52±0.04	8.57^{a}
Period Mean (P)	10.01^{B}	8.61^{A}	
Source	SEm±	CD(0.05)	CV (%)
T	0.087	0.263	2.323
P	0.062	0.186	
T x P	0.123	0.372	

Each observation is mean ± standard deviation of three replicates
Mean with different superscripts (a,b,c,d) within column and superscripts (A,B) within row differ significantly (p<0.05)

For each of the temperatures applied for this experiment, there was a significant reduction observed. Lowest reduction was observed at 45˚C (0.25 log) and highest reduction was observed at 75˚C (2.1 log).

Similar experiment was done for microcapsules prepared by emulsion method. Results of thermo tolerance of probiotic culture encapsulated by emulsion method are shown in Table 2.

Analyzed data showed a significant (p<0.05) reduction in the treatment mean values. At 45˚C, the mean log value was highest and at 75˚C it got reduced by 1.24 logs. In periodic mean value also, a significant (p<0.05) difference was observed from initial mean of 10.22 log to 8.98 log. For each of the temperatures applied, there was a significant (p<0.05) reduction wherein lowest reduction was observed at 45˚C (0.13 log) and highest reduction was observed at 75˚C (1.83 log).

When comparison was done between thermo stability of probiotic culture prepared by these two methods, emulsion method of encapsulation was a better choice as the log reduction in emulsion method was lower than extrusion method and also it had a higher viability as compared to extrusion method.

Table 2: Thermo tolerance of probiotic culture encapsulated by emulsion method

Temperature (T, ˚C)	Probiotic count (log cfu/g) at varying heating periods (P, min)		Temperature mean (T)
	0	20	
45	10.28±0.04	10.15±0.2	10.22^{d}
55	10.31±0.06	8.95±0.08	9.63^{c}
65	10.02±0.33	8.51±0.03	9.27^{b}
75	9.90±0.1	8.07±0.06	8.98^{a}
Period Mean (P)	10.13^{B}	8.92^{A}	
Source	SEm±	CD(0.05)	CV (%)
T	0.059	0.152	1.285
P	0.042	0.107	
T x P	0.084	0.214	

Each observation is mean ± standard deviation of three replicates
Mean with different superscripts (a,b,c,d) within column and superscripts (A,B) within row differ significantly (p<0.05)

Mandal *et al*. (2014) [10] added the resistant-maize starch in alginate for micro-encapsulation and coated the microcapsules with poly-L-lysine, stearic acid, and bees wax. They studied the effect of these on the viability of encapsulated *Lactobacillus casei* NCDC 298 at 65°C for 20 min. When heated at 65°C for 20 min observed that the free *L. casei* NCDC 298 cells in distilled water (9.40 log cfu /ml) were significantly (p<0.05) decreased to 4.92 log cfu/ml. They summarised that lactobacilli survived for longer time when it was enclosed in an alginate matrix (4%), with added resistant starch and covering the capsules beads with stearic acid. This might be due to the interaction of alginate polymers and resistant starch which increased the total solid content of the capsules and decreased the hot water molecules ability to diffuse through the capsule's matrix.

Pupa *et al.* (2021) [14] developed a double-encapsulated coating using alginate (1.5%) and chitosan (0.5%). The *Lactobacillus plantarum* strains 31F, 25F, 22F, *Pediococcus pentosaceus* 77F, and *P. acidilactici* 72N were used as cultures. They prepared beads by extrusion, emulsion, and spray drying techniques. Following the encapsulation of these organisms, thermotolerance tests were conducted for varied durations: 60°C for 30, 45, and 60 minutes; 70°C for 15 and 30 minutes; 80°C for 1 minute; and 100°C for 10 and 30 seconds. After 60°C for 60 min and 70°C for 30 min for all three techniques, cell viability decreased by about 2.5 log CFU/g, compared to around 1.5 log CFU/g after 80°C for 5 min. and 100°C for 30 s.

Thermo tolerance of ascorbic acid in encapsulated beads

Along with the probiotic culture, ascorbic acid was also co-encapsulated and its thermal stability was determined in mg of ascorbic acid retained after thermal treatment. Results of thermo tolerance of ascorbic acid encapsulated by extrusion method are shown in Table 3.

Table 3: Thermo tolerance of ascorbic acid encapsulated by extrusion method

Temperature (T, ˚C)	Ascorbic acid concentration at varying (mg/ 100 mg) heating periods (P, min)		Temperature mean (T)
	0	20	
45	14.67±0.22	13.04±0.07	13.85c
55	14.44±0.41	10.16±0.80	12.30b
65	14.32±0.04	9.56±0.15	11.94b
75	14.16±0.13	8.19±0.07	11.17a
Period Mean (P)	14.39B	10.24A	
Source	SEm±	CD (0.05)	CV (%)
T	0.137	0.415	2.725
P	0.097	0.293	
T x P	0.194	0.587	

Each observation is mean ± standard deviation of three replicates Mean with different superscripts (a,b,c) within column and superscripts (A,B) within row differ significantly ($p<0.05$)

As mentioned in table 3, a significant ($p<0.05$) reduction was observed in the treatment mean values. At 45˚C, the mean mg of ascorbic acid was the highest and at 75˚C it got reduced by 2.68 mg. In periodic mean value also a significant ($p<0.05$) difference was observed from initial mean of 14.39 mg to 10.24 mg. For each of the temperatures applied, a significant reduction was observed. Lowest reduction was observed at 45˚C (1.63 mg) and highest reduction was observed at 75˚C (5.97 mg).

Results for thermo tolerance of ascorbic acid prepared by emulsion method are shown in table 4. A significant ($p<0.05$) reduction was observed in the treatment mean values. At 45˚C, the mean mg of ascorbic acid was the highest and at 75˚C it got reduced by 3.63 mg. In periodic mean value also, a significant ($p<0.05$) difference was observed from initial mean of 15.45 mg to 11.25 mg. For each of the temperatures applied, a significant reduction was observed. Lowest reduction was observed at 45˚C (1.08 mg) and highest reduction was observed at 75˚C (6.8 mg).

Table 4: Thermo tolerance of ascorbic acid encapsulated by emulsion method

Temperature (T, ˚C)	Ascorbic acid concentration (mg/ 100 mg) at varying heating periods (P, min)		Temperature mean (T)
	0	20	
45	15.90±0.12	14.82±0.08	15.36d
55	15.55±0.28	11.35±0.06	13.45c
65	15.24±0.11	10.50±0.16	12.87b
75	15.13±0.08	8.33±0.17	11.73a
Period Mean (P)	15.45B	11.25A	
Source	SEm±	CD(0.05)	CV (%)
T	0.066	0.199	1.207
P	0.046	0.141	
T x P	0.093	0.281	

Each observation is mean ± standard deviation of three replicates Mean with different superscripts (a,b,c,d) within column and superscripts (A,B) within row differ significantly ($p<0.05$)

Wills and Silalahi (1990)[19] investigated the loss of vitamin C over a 12 week period at 2, 20, 30, and 50°C. At 2°C, there was little loss of vitamin C, but as the temperature increased, there was an exponential rise in the rate of loss of vitamin C. It was noted that at 20°C, 50% of the vitamin had lost by 6 weeks, whereas at 50°C, 50% lost was recorded after 3 weeks.

By using microencapsulation, Abbasi *et al.* (2019) [2] investigated improving the ascorbic acid's stability under various processing circumstances. Six microcapsule formulations with varying amounts of chitosan, maltodextrin, and gum arabic were used. Analyses were done to know the changes in ascorbic acid retention under diverse circumstances. However, ascorbic acid was retained significantly more (almost twice) in capsule samples (63.8, 54.1, and 46.8% at 50, 70, and 90˚C, respectively). Maximum amount of the ascorbic acid was susceptible to heat and got destroyed in free form at 70˚C and 90˚C.

In another study, microencapsulation of ascorbic acid was conducted by complex coacervation technique and observed that ascorbic acid in its free form was completely degraded after 15 and 30 days of storage at temperatures 37°C and 20°C, respectively. These results highlighted the instability of ascorbic acid in solution during storage (Comunian *et al.*, 2013) [5]. Cited research works indicated sensitive nature of ascorbic acid and probiotic cultures in relation to temperature. Hence, microencapsulation can act as a better medium to deliver ascorbic acid and probiotic culture in food system.

Conclusions

The comparison study on thermotolerance of probiotic culture and ascorbic acid revealed that emulsion method of encapsulation is comparatively better than extrusion method. In present investigation, beads from extrusion method showed a significant reduction of cell viability and ascorbic acid concentration of 1.62 logs and 2.68 mg at 75˚C in comparison to values obtained at 45˚C. Similarly, beads from emulsion method showed a significant reduction of cell viability and ascorbic acid concentration of 1.24 logs and 3.63 mg at 75˚C when compared to values obtained at 45˚C. Importantly maximum cell viability and ascorbic acid retention was observed for beads encapsulated by emulsion method than extrusion method. Conclusively emulsion method is better in providing stability to co-encapsulation of probiotic culture and ascorbic acid.

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