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Comparison study of co-encapsulation beads formed by extrusion and emulsion method using a probiotic culture with L-ascorbic acid

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Abstract
Lactobacillus helveticus (V3) culture was co-encapsulated by extrusion and emulsion method with L-ascorbic acid (AA) at different concentrations (60, 80, 100, 120 mg/ml) and evaluated for several parameters. 100 mg of AA showed significantly higher cell viability than other treatments. For extrusion method, it was 9.76±0.039 to 10±0.21 log cfu/g and for emulsion method it was 10.13±0.075 to 10.23±0.013 log cfu/g. Beads from emulsion method showed a higher retention (p<0.05) of ascorbic acid (13.47 mg) than extrusion method (12.65 mg) while beads from emulsion method showed higher (p<0.05) probiotic encapsulation efficiency (EE) % (90.64%) than extrusion method (86.31%) and 100 mg AA addition rate gave significantly good results. In case of emulsion method, EE % of AA (73.93%) was higher than extrusion method (69.61%). Beads from emulsion method had a significantly higher (p<0.05) yield (68.32%) than extrusion method (55.21%) and an increased yield was in proportional with the rate of addition of ascorbic acid. Conclusively from the comparison of the above parameters from extrusion and emulsion method addition rate of 100 mg ascorbic acid/100 ml of coating material, showed optimum results.

Keywords: Vitamin C, synbiotics, microcapsules, encapsulation efficiency, cell viability

1. Introduction
The robustness and survival of the probiotics during manufacturing, storage and their means of access through the gastrointestinal tract (GIT) have to bear significant obstacles. These bacteria need to survive and establish in specific numbers in the gut environment to have a good impact on health (Kailasapathy and Chin, 2000) [9]. Probiotics have been studied for enhancing their vitality and viability in food products along with their transit through GI tract by microencapsulating in hydrocolloid beads (Krasaekoopt *et al*., 2003 [10]; Mandal *et al*., 2006) [12]. Microencapsulation guard cells from harmful environments and low-intensity heat exposure *via* potentially reducing cell damage and ultimately death (Mandal *et al*., 2006) [12].
Probiotics can be effectively protected from deterioration using microencapsulation. Probiotics should be stable throughout storage, secured from severe circumstances in the upper GIT, released into the colon and then encouraged to colonize the mucosal surfaces using an efficient microencapsulation mechanism (Anselmo *et al*., 2016) [3].
The incidence of anemia worldwide has been acknowledged as a public health issue for many years, but it is still unacceptably high. Kids are more susceptible to iron deficiency anaemia as during initial growth period of five years of life iron requirement is more. The causes of anemia are multifaceted. It is commonly accepted that iron shortage contributed for at least half of these occurrences. A thorough analysis revealed that, on a regular basis, in non-malarial locations 38-62% of baseline anaemia observed in preschool children is alerted to iron supplementation (Sachdev and Gera, 2013) [17]. Oral consumption of vitamin C helps the body precisely control tissue and plasma levels. With modest intakes of 30-180 mg of vitamin C on daily basis, around 70-90% is assimilated in body. Ascorbic acid is removed in the urine non-metabolized at doses larger than 1 g/day, though absorption declines to less than 50% of that level. Pharmacokinetic studies revealed that an oral ascorbic acid doses of 1.25 g/day gave mean peak plasma vitamin C concentrations of 135 µmol/L, that is roughly twice which when compared by ingesting 200-300 mg/day of ascorbic acid from vitamin C rich foods (Office of

Dietary Supplements - Vitamin C, 2021) [15].

Probiotic, prebiotics and their preparation as synbiotics confers many health benefits to human and extensive research work have already proven this in the past few decades. Functional foods are ultimately a food supplements that promise to transform, modify and restore the body's natural intestinal flora. It also smooths the progress of various functions of the intestinal environment. By targeting the growth and/or enhancing the metabolism of one or a small number of probiotic bacteria, a mixed preparation *i.e.* synbiotic product positively affects the consumers by stimulating the survival of probiotics and implantation of such live microbial dietary supplements in the gastrointestinal system. The term 'synbiotics' should only be used where the prebiotic compound/s favour the probiotic organism/s specifically because it alludes to synergism (Pandey *et al*., 2015) [16].

2. Materials and Methods

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

The proposed work was focused on encapsulation of lactic acid bacteria and ascorbic acid *via* two methods of encapsulation (Emulsion and Extrusion) wherein coating material Sodium alginate (4%) and Maize starch (2%) (Hi-media) were taken and autoclaved. For core material, 12-hour old lactic culture of *Lactobacillus helveticus* (V3) and L-ascorbic acid (Hi-media) were taken. V3 culture was grown overnight in MRS broth were harvested (5000 g) and washed twice with saline solution (0.85% NaCl). The cell pellet was finally suspended in saline and vortexed for 2 mins.

The ratio of coating to core material was kept at 5:1 ratio (20 ml of coating material to 4 ml of cell suspension). L- ascorbic acid was added in different quantity as 60, 80, 100, 120 mg/ 100 ml of coating material. Both coating and core material were mixed vigorously to get a uniform mixt

2.2 Encapsulation by extrusion method

In this method technique was as described by Krasaekoopt *et al*., (2003) [10] was followed. An insulin syringe (needle thickness of 28G and internal diameter of 0.18 mm) was used. Mixture of the materials (20 ml) were extruded through the needle drop by drop to a 100 ml hardening solution of 0.1 M $CaCl_2$ and kept undisturbed for 1 hour. Afterwards the beads were collected and stored in refrigerator.

2.3 Encapsulation by emulsion method

This method was carried out as described by Mandal *et al*., (2014) [13], where 100 ml of pre-autoclaved soybean oil containing 0.5% Tween 80 as emulsifier was taken (as continuous phase). The coating-core material mixture (20 ml) was then added drop wise to the continuous phase that was magnetically stirred. Within 5 minutes, a uniformly turbid emulsion was obtained into which, 0.1 M calcium chloride (100 ml) that was previously cooled was added quickly to break the emulsion and for hardening of alginate-starch microcapsules. Then it was kept undisturbed for 20 minutes. The capsules were harvested by gentle centrifugation at 350 g for 10 minutes and washed thrice with chilled $CaCl_2$ solution for proper hardening and removal of odour from oil. The beads were separated by filtration and stored in refrigerator. 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.

2.4 Cell viability

The viability of micro-encapsulated probiotic culture was tested after depolymerisation of the capsules followed by plating on MRS agar (Mandal, 2006) [12]. Microcapsules (1 g) were taken in 9 ml of sterilised phosphate buffer solution (pH 7.1 ± 0.2), incubated at 37 °C for 1 hour, and depolymerised by vertexing until a uniform cloudy solution is obtained followed by preparation of suitable dilutions using 9 ml sterile phosphate buffer tubes. Phosphate ions in the solution react with sodium in sodium-alginate and cause de-polymerisation of sodium-alginate, which leads in to the release of encapsulated lactobacilli cells from the alginate-maize capsules. MRS agar was used for enumeration by pour plating and colonies developed were counted after 48 h at 37 °C.

2.5 Ascorbic acid concentration

The ascorbic acid content was determined by the AOAC 967.21 method (1997) [1]. This method is 2,6-dichlorophenolindophenol titration method. It depends upon the stoichiometric reduction of the dye 2, 6-dichlorophenol indophenol to a colorless compound by ascorbic acid. For preparation of the microparticles for ascorbic acid determination, 100 mg of them were dissolved in 50 ml of sterile phosphate buffer and kept for depolymerisation.

Standardized 2, 6-dichlorophenol indophenol (Hi-media) dye (titrated with 1.0 ml of 0.1% ascorbic acid) was taken in a conical flask containing 24 ml water. Noted the amount of dye needed to obtained pink colour end point. 25 ml of cloudy solution from the depolymerized sample was taken in Erlenmeyer flask and 25 ml of 5% TCA was added. Shake well and filtered through Whatman filter paper No. 1. 25 ml of filtrate was taken in conical flask and titrate with the dye to pink color end point. Note the amount of dye used for titration. Calculated vitamin C by using following formulae:

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

Y=Titer value of sample

X= Titer value of standard ascorbic acid

2.6 Efficiency of encapsulation

The enumeration of lactic culture and ascorbic acid concentration was carried out as described earlier and encapsulation efficiency was calculated by the following formulae (Maciel *et al*., 2014) [11]

$$\text{EE of probiotic (\%)} = \left(\frac{N_o}{N_i}\right) \times 100$$

Where, EE: encapsulation efficiency, N_i: the count of initial live cells used for encapsulation (log cfu/g or ml) and N_0: the count of live cells released from capsules (log cfu/g or ml).

Encapsulation efficiency of ascorbic acid was calculated by the following formulae (Fraj *et al*., 2021) [6]

$$\text{EE of Ascorbic acid (\%)} = \left(\frac{AA_o}{AA_i}\right) \times 100$$

Where, EE: encapsulation efficiency, AA_i: the initial amount of ascorbic acid added to the emulsion (mg/100 ml of coating material) and AA_0: total amount of ascorbic acid (mg/100 mg of dried capsules) present in the sample.

2.7 Yield of microcapsules

The microcapsules yield was calculated based on the encapsulated sample that was freeze dried as described by Nizori *et al*. (2020) [14]. This was done by dividing the weight of microcapsules by the total dry weight of the ingredients incorporated into the feed solution and the result was expressed as a percentage. The yield of microcapsules produced was calculated using the following formulae:

$$\text{Yield } (\%) = \left(\frac{W_o}{W_i}\right) \times 100$$

Where,

W_i: initial dry weight of materials (g) and W_o: initial dry weight of microcapsules (g)

2.8 Statistical analysis

Statistical analysis was carried out using statistical design FCRD (Steel and Torrie, 1980) [19]. All the experiments were conducted in required numbers of replications and the results are expressed as mean± standard deviation (SD).

3. Results and Discussion

3.1 Viability test

Cell viability for microcapsules has been shown in Table 1. Comparing the treatment means microcapsules made by emulsion method had a significantly (p<0.05) higher viability (10.25 log cfu/g) with comparison to microcapsules made by extrusion method (9.93 log cfu/g).

Table 1: Comparison of cell viability between two methods of encapsulation at varying ascorbic acid concentrations

Treatment (T)	Cell viability (log cfu/g) at varying ascorbic acid concentration (mg/100 mg) (C)				Treatment mean (T)
	60	80	100	120	
Extrusion	9.76±0.04	9.87±0.04	10.1±0.03	10.00±0.21	9.93^{a}
Emulsion	10.13±0.08	10.21±0.05	10.41±0.03	10.23±0.01	10.25^{b}
Concentration Mean (C)	9.95^{A}	10.04^{A}	10.26^{C}	10.12^{B}	
Source	SEm±		CD(0.05)		CV (%)
T	0.025		0.08		0.891
C	0.036		0.114		
T x C	0.050		0.161		

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

Among the four different concentration rates of AA, the concentration mean values 60 and 80 mg of ascorbic acid concentration rates did not show significantly different viability. 100 and 120 mg of ascorbic acid concentration rates were showing significantly different values for cell viability. In case of 100 mg of ascorbic acid concentration viability was significantly higher. For extrusion method, cell viability ranged from 9.76±0.039 to 10±0.21 log cfu/g and for emulsion method it ranged from 10.13±0.075 - 10.23±0.013 log cfu/g.

A study on the viability of microencapsulated *L. rhamnosus* GG (ATCC 53103) and *L. acidophilus* NCFM in stimulated gastrointestinal circumstances was done by Sohail *et al*. (2011) [18]. Cell viability for extruded microcapsules used in that investigation ranged from 9.0 to 9.30 log cfu/g. In other similar study Ji *et al*. (2019) [8] created alginate-coated gelatin microspheres to encapsulate probiotic *Bifidobacterium adolescentis* 15703T's. The bacterial density in free cell suspension was 10^{9}–10^{10} cfu/g. Alginate microcapsules and microcapsules coated with chitosan each had encapsulation yields of 95 2.5% and 90 3.4%, respectively. This indicates that the cell count after encapsulation was still above 9.5 log cfu/g.

3.2 Ascorbic acid concentration

To determine ascorbic acid concentration 2,6-dichlorophenolindophenol titration method was used. Comparison between two methods of encapsulation in terms of ascorbic acid retention is shown in Table 2.

Treatment wise both methods are significantly different (p<0.05), emulsion method showing a higher retention of ascorbic acid (13.47 mg) than extrusion method (12.65 mg). Concentration wise all the values are significantly different. Highest retention of ascorbic acid was found in case of addition of ascorbic acid at the rate 120 mg/100 ml of coating material. Ascorbic acid retention in extrusion for different ascorbic acid addition was from 7.87±0.14 to 17.22±0.15 mg and for emulsion it was 8.26±0.14 to 18.55±0.36 mg.

Table 2: Comparison of ascorbic acid retention as affected by two methods of encapsulation

Treatment (T)	Ascorbic acid concentration (mg/100 mg)				Treatment mean (T)
	60	80	100	120	
Extrusion	7.87±0.14	10.9±0.22	14.59±0.14	17.22±0.15	12.65^{a}
Emulsion	8.26±0.14	11.45±0.32	15.62±0.5	18.55±0.36	13.47^{b}
Concentration Mean (C)	8.06^{A}	11.17^{B}	15.11^{C}	17.88^{D}	
Source	SEm±		CD (0.05)		
T	0.078		0.23		
C	0.111		0.325		
T x C	0.157		0.46		
CV (%)	2.012				

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

3.3 Efficiency of encapsulation

The encapsulation efficiency (EE %) is the ratio obtained when the concentration of the incorporated material detected in the formulation divided by the initial concentration used to make that formulation. Here efficiency for encapsulation for both probiotic culture and ascorbic acid has been analysed. In Table 3, a comparison of encapsulation efficiency for probiotic viability has been shown between two different methods of encapsulation at different ascorbic acid concentrations.

By comparing treatment means, EE by emulsion method (90.64%) was significantly higher with comparison to extrusion method (86.31%). While comparing mean values for different ascorbic acid concentration, there was no significant difference (p>0.05) of EE values among 60, 80 and 120 mg of ascorbic acid concentration but 100 mg of ascorbic acid concentration showed significantly higher viability that led to a high EE percentage (89.82%). For extrusion method, this efficiency score ranged within 84.99±5.36% to 85.47±2.79 and for emulsion method it ranged from 89.39±0.68 to 90.57±0.67%.

Table 3: Comparison of encapsulation efficiency of probiotic culture between two methods of encapsulation at varying ascorbic acid concentrations

Treatment (T)	Encapsulation efficiency of culture at varying ascorbic acid concentrations (mg/100 mg) (C)				Treatment mean (T)
	60	80	100	120	
Extrusion	85.47±2.79	87.05±3.03	87.73±3.29	84.99±5.36	86.31^{a}
Emulsion	89.39±0.68	90.69±1.39	91.92±1.57	90.57±0.67	90.64^{b}
Concentration Mean (C)	87.43^{A}	88.87^{A}	89.82^{B}	87.78^{A}	
Source	SEm±		CD(0.05)		
T	0.803		1.618		
C	1.135		2.288		
T x C	1.606		3.236		
CV (%)	2.088				
Each observation is mean ± standard deviation of three replicates Mean with different superscripts (a,b) within column and superscripts (A,B) within row differ significantly (p<0.05)					

On the same trend, Gul and Dervisoglu (2017) [7] assessed the impact of rate of additions of sodium alginate made during the experiment on the microencapsulation of the probiotic *Lactobacillus casei* Shirota by extrusion and emulsion methods using the Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) method. Encapsulation efficiency after encapsulation of lactobacillus strain using the extrusion and emulsion methods, was discovered to be in the range of 95.92- 99.75% and 86.71-95.25%, respectively. Chen *et al*. (2017) [4] encapsulated *Lactobacillus bulgaricus* in whey protein isolate by emulsion technique using transglutaminase-induced gelation and alginate as a coating material. Researchers observed the high encapsulation efficiency (95.28 ±2.31%) in trials proved that the compatibility of the probiotic strain with applied materials.

Table 4: Comparison of encapsulation efficiency of ascorbic acid between two methods of encapsulation at varying ascorbic acid concentrations

Treatment (T)	Encapsulation efficiency of ascorbic acid at varying concentrations (mg/ 100 mg) (C)				Treatment mean (T)
	60	80	100	120	
Extrusion	65.61±1.17	68.12±1.39	72.96±0.70	71.74±0.61	69.61^{a}
Emulsion	68.79±1.20	71.56±2.02	78.09±2.38	77.29±1.50	73.93^{b}
Concentration Mean (C)	67.20^{A}	69.84^{B}	75.53^{D}	74.51^{C}	
Source	SEm±		CD(0.05)		
T	0.428		1.189		
C	0.605		1.682		
T x C	0.856		2.378		
CV (%)	1.893				
Each observation is mean ± standard deviation of three replicates Mean with different superscripts (a,b) within column and superscripts (A,B,C,D) within row differ significantly (p<0.05)					

Apart from determining EE % for probiotic culture, EE % has also been determined for ascorbic acid. In Table 4, comparison of encapsulation efficiency for ascorbic acid has been shown between two different methods of encapsulation at different ascorbic acid concentrations.

While comparing treatment means, it has been found that microcapsules made with emulsion method had a higher retention of ascorbic acid leading to significantly higher (p<0.05) EE % of ascorbic acid (73.93%) compared to that of extrusion method (69.61%). By comparing mean values between different concentrations of ascorbic acid, a significant difference was found within those values and 100 mg of ascorbic acid had a significantly higher EE % (75.53%) than other concentrations of ascorbic acid. Efficiency of ascorbic acid for microcapsules prepared with extrusion method ranged from 65.61±1.17 to 71.74±0.61% and for emulsion method 68.79±1.2 to 77.29±1.50%.

A study was conducted by Thangaraj and Seethalakshmi (2014) [20] to develop microcapsules for vitamin C using the extrusion method by sodium alginate (2g) as coating substance. The 100 mg of vitamin C beads showed greater encapsulation efficiency of 74%. Similarly Abbasi *et al*. (2019) [2] studied some formulations to enhance ascorbic acid stability. They microencapsulated six formulations prepared using gum arabic (GA), maltodextrin (MD) and chitosan at different ratios of 4:1:1, 1:4:1, 1:1:4, 2:2:2, 3:2:1, 3:1:2, respectively by freeze drying the coating material solution. Encapsulation efficiency differed depending on composition of coating material and ranged from 40.5% to 80.4%.

3.4 Microcapsule yield

Yield is a ratio which is obtained by dividing the weight of microcapsules by the total dry weight of the ingredients incorporated into the feed solution. It is usually expressed as a percentage.

In Table 5, comparison for microcapsule yield has been shown. Treatment wise, capsules made by emulsion method had a significantly higher ($p<0.05$) yield (68.32%) than capsules made with extrusion method (55.21%). By comparing concentration means a significant increase in yield was observed, as the ascorbic acid concentration was increased. Highest yield (68.33%) was observed in case of 120 mg of addition rate of ascorbic acid and lowest was observed when addition rate was kept at 60 mg (55.7%). In case of extrusion method, the yield ranged from 53.2±4.49 to 57.5±2.23% and for emulsion method yield ranged from 58.2±3.91 to 79.17±2.31%.

Table 5: Comparison of yield of microcapsules between two methods of encapsulation at varying ascorbic acid concentrations

Treatment (T)	Microcapsule yield at varying ascorbic acid concentrations (mg/100 mg) (C)				Treatment mean (T)
	60	80	100	120	
Extrusion	53.2±4.49	53.88±1.47	56.26±1.63	57.5±2.23	55.21[a]
Emulsion	58.2±3.91	63.88±3.59	72.03±4.35	79.17±2.31	68.32[b]
Concentration Mean (C)	55.7[A]	58.88[B]	64.14[C]	68.33[D]	
Source	SEm±		CD(0.05)		
T	0.926		1.513		
C	1.309		2.139		
T x C	1.851		3.026		
CV (%)	2.797				

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

In relatively similar study in 2020, Elida and Saufani [5] sought to maximize the yield and viability from various carrageenan coating material concentrations. *Lactobacillus paracasei* ssp. *paracasei* Ml3 probiotic was encapsulated using the carrageenan extrusion technique at concentrations of 1%, 2%, 3%, and 4%. The results showed that a 3% carrageenan concentration produced the best yield of 43.2%.

4. Conclusion

With the above results, one can conclude that after performing comparison study on certain parameters, emulsion method of encapsulation is comparatively better than extrusion method. In present investigation the addition rate of 100 mg ascorbic acid/100 ml of coating material showed optimum results in terms of better probiotic viability, encapsulation efficiency for both of the core materials *i.e. Lactobacillus helveticus* (V3) and ascorbic acid. Addition of optimum amount of ascorbic acid (100 mg/100 ml) enhances its survivability in certain conditions as well as both of the core materials retain in adequate quantity while co-encapsulated.

5. Conflict of interest

Authors declare no competing interests.

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