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Influence of Prebiotic Composition on Probiotic Survivability in Calcium Alginate Coated Synbiotic Microcapsules at Thermal Incubation

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Abstract: The aim of this study is to improve the thermal resistivity of prebiotic on probiotics, using various combinations of high resistance maize starch and inulin in the form of synbiotic microcapsules. *Lactobacillus casei* MTCC 1423 was used as probiotic strain. Oil emulsion technique was used for the microencapsulation of *Lactobacillus casei* with prebiotic (high resistance maize starch and inulin) as thermal protective agent and calcium alginate as coating material. The freeze drying process produced agglomerated powder of synbiotic microcapsules. Eight types of synbiotic microcapsules were exposed to 45, 50, 55, 60, 65 and 70°C for 15, 30, 45 and 60 min which revealed that the effect of prebiotic composition on the thermal resistance property of prebiotics on probiotics was significant ($p<0.05$) as analyzed using ANOVA. Multiple linear regression proved that inulin provides higher heat resistance to probiotics than that of high resistance maize starch when used in combination. Synbiotic microcapsule type H2I3 was found with highest probiotic survivability 40.36% during thermal incubation. The rate of probiotic death (k) and heat resistance property (D) were found to be 0.056 day^{-1} and 17.67839 min, respectively at 70°C when exposed for 60 min.

Key words: Microencapsulation, probiotic, prebiotic, synbiotic, *Lactobacillus casei*, India

INTRODUCTION

Probiotics are the live microbial feed supplements that beneficially affect the host health by improving its intestinal microbial balance. The whole concept of probiotics is not new and in fact, they have been consumed by human beings in the form of fermented foods for thousands of years (Kopp-Hoolihan, 2001; Ranadheera *et al*., 2010).

Probiotic bacteria cannot thrive well in digestive tract without of its prebiotic (Cruz *et al*., 2010). The combination of probiotic with its prebiotic gives synergistic effect on host health (Morelli *et al*., 2003). It has been suggested that adding non-digestible food ingredients known as prebiotics to certain foods may increase the viability of bacteria passing through the gastrointestinal tract and thus exert a beneficial effect on human health (Chow, 2002; Fooks *et al*., 1999; Iyer and Kailasapathy, 2005; Khalf *et al*., 2010) combination of both prebiotic and probiotic termed as synbiotic.

A number of intrinsic and extrinsic factors influence the survival of probiotics in foods during processing and storage. It is important to consider these factors at all stages from additions of the probiotic in the food to delivery of the probiotic to the gut of the consumer. These include manufacturing processes, food formulations and matrices, packaging materials and environmental conditions in the supply chain and storage.

Higher levels of viable probiotic micro-organisms 10^6-10^7 cfu g^{-1} of the product are recommended in probiotic foods for better efficacy in regulating beneficial effects. It is a great challenge for the food processers to maintain probiotic viability during processing as well as storage. During processing of food temperatures >45°C are detrimental for survival of free probiotic cells. The higher temperature and shorter time period ranging from minutes to hours at 45-55°C leads to reduction of the probiotic viability (Doleyres and Lacroix, 2005).

Microencapsulation is a newest emerging technology and most efficient method of chemical and mechanical process for packaging of prebiotic and probiotic as synbiotic microcapsules which isolates the probiotic cells from the adverse environmental conditions (Lee *et al*., 2004; Shah and Ravula, 2000; Gardiner *et al*., 2000; Heidebach *et al*., 2010) and reduces the cell losses. Microencapsulation provides the physical protection to probiotic bacteria by prebiotic against detrimental conditions during processing of food (Anal and Singh, 2007). Physical protection of probiotic bacteria in the form of microcapsules is a new concept that does not give any detrimental effects on organoleptic property of food (Homayouni *et al*., 2007; O'Regan and Mulvihill,

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2010; Kim *et al*., 1996). Many techniques of microencapsulation are available but in this study, oil emulsion technique was used. It is a suitable method for encapsulation of probiotic bacteria with smaller in size <100 μm (Crittenden *et al*., 2006; Vidhyalakshmi *et al*., 2009). Inulin and high resistance maize starch are legally classified as prebiotic food or food ingredients in all countries (Kolida *et al*., 2002; Coussement, 1999).

Aim of this study was to investigate the effect of prebiotic contents on probiotic viability in synbiotic microcapsules at various time and temperature combination. It is expected to be useful for process designing in the development of thermally processed probiotic products.

MATERIALS AND METHODS

In this study, *Lactobacillus casei* MTCC 1423 was used as probiotic strain supplied by Microbial Type Culture Collection and Gene Bank Chandigarh, India in freeze dried form. All microbiological media were collected from Himedia and Sodium alginate collected from SRL, India. The sun flower oil was procured from the local market. Inulin and high resistance maize starch used as prebiotic were obtained from SD fine Chem. Ltd., India.

Bacterial growth and harvesting: Freeze dried pure culture of *Lactobacillus casei* was activated by inoculating a loop of culture in the MRS-broth at pH 6.8 and incubated at 37°C for 72 h. The probiotic cells in late-log phase were harvested by centrifugation at 4500 rpm for 10 min and the supernatant was discarded. Collected pellet was washed three times in sterile, 0.9% saline solution and again centrifuged. Finally, pellet was collected and used for microencapsulation process (Chen *et al*., 2006).

Microencapsulation: Oil emulsion technique was used for the production of synbiotic microcapsules (Sheu and Marshall, 1993; Homayouni *et al*., 2007; Moayednia *et al*., 2009). All the solutions and glassware required for microencapsulation process were sterilized at 121°C for 15 min. A mixture of 3% (w/w) sodium alginate and prebiotics, i.e., high resistance maize starch and inulin (Table 1) was prepared in double distilled water followed by heating of the mixture at 70°C for 20 min and allowed to cool at room temperature (Olivas *et al*., 2007). On the other hand, 0.1% suspension of washed pellet of *Lactobacillus casei* was prepared in double distilled water. About 10 mL suspension of *L. casei* was added in already prepared 100 g mixture of sodium alginate and prebiotic at room temperature. It was mixed using magnetic stirrer for 10 min for equal distribution of probiotic cells into the mixture. It was lead to formation of synbiotic mixture.

It was added to presterilized 200 mL of sun flower oil containing 0.02% tween 80 and stirred at 400 rpm using magnetic stirrer for 20 min till it was emulsified and appeared creamy. It was followed by addition of 200 mL of 0.1 M calcium chloride solution quickly along the side of the beaker then cross linkage between sodium alginate and calcium chloride occur which formed calcium alginate coated synbiotic microcapsules. Calcium alginate coated synbiotic microcapsules soon settled down to the bottom of the beaker in chloride solution. Complete process of cross linking took 45-50 min.

The oil phase was removed from the top with aspirator and microcapsules were harvested from chloride solution by gentle centrifugation at 200 rpm for 5 min with the removal of supernatant. The microcapsules were washed with 0.9% of saline solution containing 5% glycerol and again centrifuged at same condition. Thus, slurry of calcium alginate coated synbiotic microcapsules was obtained. This was stored at 4°C for 24 h.

Freeze drying of synbiotic microcapsules slurry: The freeze drying process was performed by manifold freeze dryer (Lyodel, LYO1550, India) (Higl *et al*., 2008). Aqueous slurry of calcium alginate coated synbiotic microcapsules were transferred to 100 mL round bottom flask and pre-freezed to -40°C in the pre freezer bath at atmospheric pressure for 2 h.

It was allowed to freeze drying for 6 h with reduction of pressure on the round bottom flask to 10^{-3} m bar and raising the shelf temperature of sample +10°C. After completion of freeze drying, dry agglomerates of synbiotic microcapsules were obtained.

Table 1: Composition of prebiotic in synbiotic microcapsules for microencapsulation and notation

Composition of synbiotic microcapsules	H2I0	H3I0	H0I2	H0I3	H2I2	H2I3	H3I2	H3I3
High resistance maize starch (%)	2	3	0	0	2	2	3	3
Inulin (%)	0	0	2	3	2	3	2	3
Sodium alginate (%)	3	3	3	3	2	3	3	3
Probiotic cells pellet (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Calcium chloride 0.1 M (mL)	200	200	200	200	200	200	200	200
Vegetable oil (mL)	200	200	200	200	200	200	200	200

H: High resistance maize starch; I: Inulin

Thermal incubation of synbiotic microcapsules: Thermal incubation of synbiotic microcapsules was done in 15 mL test tube. A suspension of peptone water and microcapsules was prepared. Total 1 g of freeze dried synbiotic microcapsules was added to each test tubes containing, 9 mL of 0.1% (w/v) sterilized peptone water and suspension was agitated for 15 sec using high speed cyclomixer. It was incubated in water bath at 45, 50, 55, 60, 65 and 70°C temperature for 15, 30, 45 and 60 min.

Enumeration of thermal incubated probiotic bacteria: After cooling of thermal incubated suspension containing tubes, 1 mL suspension was removed after 15 sec of cyclomixing and added to sterilized 9 mL of 0.02 M citric acid solution. It was allowed to cyclomixing at high speed to break up coated layers of microcapsules. Several dilutions were prepared by using 0.1% peptone water 0.02 M citric acid solution was used as solvent of calcium alginate which releases probiotic cells from calcium alginate coated synbiotic microcapsules without affecting their viability (Denish *et al*., 1995). About 1 mL of the samples from each dilution tube was inoculated in sterilized Petriplates poured with 12 mL of MRS agar medium at 45°C. Selected plates had 25-250 colonies as described by Houghtby *et al*. (1992) after 48 h of incubation at 37°C. The colonies were enumerated using cubic colony counter and calculated as colony forming units (cfu) per gram of synbiotic microcapsules.

Survivability of probiotic bacteria: Survivability of *Lactobacillus casei* expressed in percentage was determined as initial probiotic count (cfu g^{-1}) of synbiotic microcapsules before thermal incubation and probiotic count (cfu g^{-1}) after thermal incubation:

$$\text{Survivability (\%)} = (N_2 / N_1) \times 100$$

Where:

N_1 = Probiotic viability (cfu g^{-1}) of synbiotic microcapsules before thermal incubation

N_2 = Probiotic (cfu g^{-1}) of synbiotic microcapsules after thermal incubation

Statistical analysis: All the experiments were performed in duplicates. The multiple linear regression was used to estimate average relationship between time and temperature to predict viability of probiotic in calcium alginate coated synbiotic microcapsules. Two way ANOVA tests were performed to signify the effect of the time, temperature and prebiotic composition factor on the viability of probiotic after heat treatment of synbiotic microcapsules. MINITAB software (Version 15) was used for statistical analysis.

RESULTS AND DISCUSSION

The different combinations of prebiotic yielded various amounts of synbiotic microcapsules after freeze drying of the slurry. Higher composition of prebiotic incorporation during microencapsulation gave higher yield of synbiotic microcapsules. Initial probiotic count (cfu g^{-1}) of freeze dried synbiotic microcapsule was varying (Table 2).

Microencapsulation of *Lactobacillus paracasei* and *Bifidobacterium lactis* improves the survivability after incubation at low pH (Heidebach *et al*., 2010). Viability of encapsulated probiotic cells, depend on the physio-chemical properties of the capsules. In fact, the type and the concentration of the coating material and initial cell numbers and bacterial strains are some basic parameters which are important to maintain the probiotic viability (Chen and Chen, 2007).

Viability of probiotic decreased with increase in temperature and/or time of the thermal incubation. Therefore, the mortality rate was different in particular types of microcapsules. Difference in probiotic viability after thermal incubation was different in specific types of synbiotic microcapsules. In this study, high resistance maize starch 2% and inulin 3% proved to be a good combination of prebiotics to provide thermal resistance to probiotic.

Two factor ANOVA showing significant effect of time and temperature on the probiotic survivability (%) in eight different types of synbiotic microcapsules at $p<0.05$. Multiple linear regression revealed the effect of time and temperature on the probiotic survivability as follows:

$$Y_1 = 215 - 2.04\,X_1 - 0.775\,X_2$$

where, Y_1 is probiotic survivability (%) at X_1 time and X_2 temperature combination of thermal incubation of eight types of synbiotic microcapsules.

Regression analysis of probiotic survivability versus high resistance maize starch and inulin had a significant effect for survivability of probiotic during thermal incubation as follows:

Table 2: Yield of synbiotic microcapsules and initial probiotic count in Synbiotic Microcapsules (SM) (cfu g^{-1})

Microcapsule type	Yield after freeze drying (g)	Initial cfu g^{-1} of SM	Log cfu g^{-1} of SM
H2I0	6.03	4.8×10^9	9.674612
H3I0	6.97	4.81×10^9	9.682884
H0I2	5.23	5.9×10^9	9.771522
H0I3	6.72	5.9×10^9	9.771522
H2I2	7.02	5.1×10^9	9.710656
H2I3	9.41	4.9×10^9	9.695005
H3I2	8.23	4.6×10^9	9.666178
H3I3	8.50	4.81×10^9	9.682884

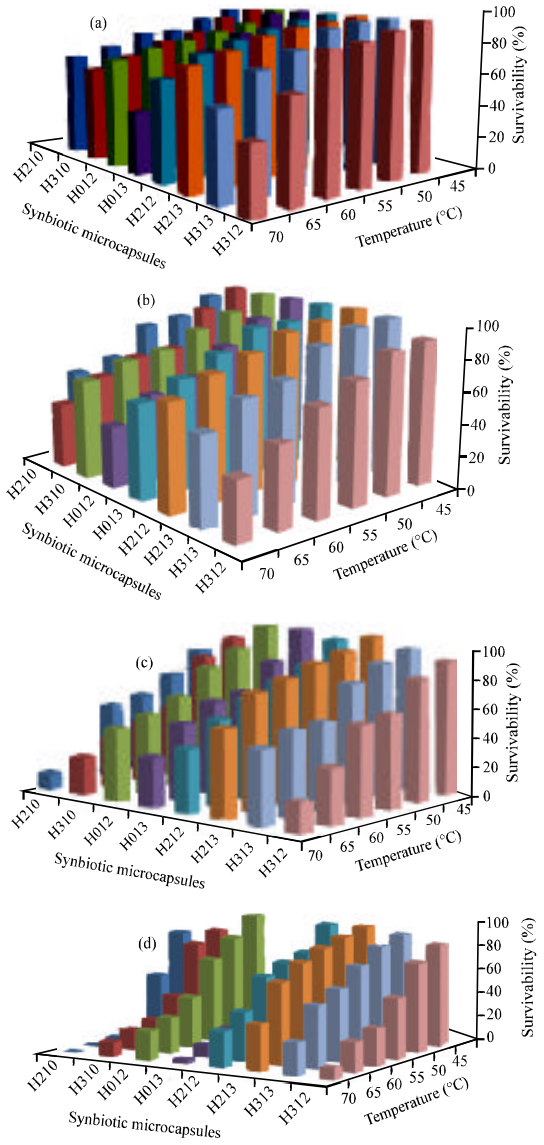

Fig. 1: Survivability of probiotic (*Lactobacillus casei*) in eight different types of synbiotic microcapsule, i.e., H2I0, H3I0, H0I2, H0I3, H2I2, H3I2, H3I3 and H2I3 for 45, 50, 55, 60, 65, 70°C temperature and for time; a) 15; b) 30; c) 45 and d) 60 min

$$Y_2 = 51.6+4.96X_3+3.86X_4$$

Where:

Y_2 = Probiotic survivability (%)
X_3 = Inulin content (%)
X_4 = High resistance maize starch (%) (Fig. 1)

The death of microbial populations exposed to lethal temperatures follows the kinetics of first order reactions (Esty and Meyer, 1922; Ball and Olson, 1957; Stumbo *et al*., 1975; Pflug, 1987; Casolari, 1988). According to equation of exponential decay:

Table 3: Rate of probiotic death (K) min^{-1} of H2I3 synbiotic microcapsules under thermal incubation with given time and temperature combination

Temperature (°C)	Time of thermal incubation (min)			
	15	30	45	60
45	0.200000053	0.100000	0.066667	0.050480
50	0.200808091	0.100680	0.067307	0.050697
55	0.201639336	0.101104	0.067693	0.050995
60	0.202786137	0.102613	0.068409	0.051549
65	0.205225721	0.103945	0.069067	0.052610
70	0.207543196	0.106206	0.071656	0.056566

Table 4: Heat resistance property of probiotic H2I3 synbiotic microcapsules in terms of decimal reduction time (D min) at given time and temperature of combination

Temperature (°C)	Time of thermal incubation (min)			
	15	30	45	60
45	4.999998685	9.999997	15.00000	19.80965
50	4.979879022	9.932479	14.85724	19.72521
55	4.959349798	9.890852	14.77250	19.60974
60	4.931303568	9.745367	14.61805	19.39899
65	4.872683573	9.620475	14.47873	19.00771
70	4.818274071	9.415697	13.95559	17.67839

$$N_t = N_0 \times e^{k*t}$$

Where:

N_t = The cfu g^{-1} of synbiotic microcapsules after thermal incubation at specific temperature for time (t)
N_0 = The initial cfu g^{-1} of probiotic
k = The death rate (min^{-1})

The Decimal reduction time (D) is widely used to heat resistance property of microorganisms exposed to higher temperature. It is expressed in min:

$$D_t = \frac{1}{K} = \frac{t}{(\text{Log } N_0 - \text{Log } N_t)}$$

Synbiotic microcapsules type H2I3 provided highest the probiotic survivability after the thermal incubation of 70°C and 60 min time temperature combination. Rate of probiotic inactivation (k) was found least and heat resistance property was found highest on the basis of D value at various time temperature combination of thermal incubation (Table 3 and 4).

CONCLUSION

Microencapsulation process provides a way to develop thermal resistance property in probiotics by prebiotics. These types of prebiotics helped to develop

thermal resistant synbiotic microcapsules. H2I3 was the highest thermal resistant synbiotic microcapsules. Highest probiotic survivability was maintained at 70°C for 60 min of thermal incubation and proved 2% high resistance maize starch and 3% inulin is the best combination of prebiotics to maintain probiotic survivability during thermal processing. Therefore, inulin alone is not effective as thermal protective agent for probiotic.

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