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Encapsulation of synbiotics & assessment of survival ability to various physiological stresses

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ABSTRACT

Important criteria to determine the efficacy and the success of the product containing the probiotics are the acceptance of the probiotic product by the consumer, which is possible only when sufficient survival occurs during its production, processing & also passage through the gut to the intestine without the loss of viability. To enhance the growth of probiotics, prebiotic supplements are administered. But, sometimes these prebiotics fails to promote the growth of only beneficial bacteria; they may also give rise to the improved growth of unwanted bacteria residing in the gut. Encapsulation is a useful technique in providing a favorable environment for the synbiotics (combination of probiotic & prebiotics) to sustain & remain viabile under all the physiological stresses. In the present study two probiotics namely Lactobacillus casei and Lactobacillus rhamnosus were taken as probiotic strains, crude orange juice as the prebiotic component, the probiotic strains are checked for survival ability under different physiological conditions of pH(1,3,5,6), temperature (4^0C,45^0C,55^0C,65^0C) & oxygen tolerance(aerobic, facultative anaerobic & microaerophilic), the same parameters were also assessed for probiotic strains with orange juice as prebiotic component & later with cultured probiotics along with the prebiotic that is, synbiotics in an sodium alginate encapsulation matrix. Survival ability was assessed through (colony forming unit) cfu/ml & lactate dehydrogenase assay. From our study, all the encapsulated synbiotics were grown in different pH levels.Interestingly, the microorganisms showed the increased viability in all the pH levels, that is, at pH1(49CFU/mL), pH3(CFU/mL), pH5(121CFU/mL) and pH6(109CFU/mL compared to probiotic or symbiotic alone. Similarly, survival of encapsulated cells was reported at low at 4^0C was 69CFU/mL& high temperature 65^0C 41cfu/ml. Encapsulation also provided sufficient protection against oxygen tension, increasing the survival ability of the synbiotics.

Keywords: *Synbiotic , encapsulation, probiotic, survival, physiological stress.*

Introduction

The popularity of functional foods is increasing steadily over the period of time. Functional food offers personal health benefits due to bioactive microbial cells, as probioticimprove in maintaining gut microbiota, harnessing prevention or curing of illness[1]. A vast population of micro organisms thrives in human gastro intestinal tract, forming gut microbiota. Approximately 10^{10}-10^{12} live in one gram of human colon, constituting both good and bad bacteria[2].Generally these gut bacteria ferment on human non-digestible dietary carbohydrate fructo-oligosaccharides, galacto-oligosaccharides, and trans-galacto-oligosaccharides , forming the most common prebiotics. Some of these are produced in an industrial scale through enzyme technology while others are extracted from natural sources[3].

Potential health benefits of probiotics to the host is gained only if these micro organisms make a successful transit from the stomach to the gastrointestinal tract, thrive & multiply utilizing non- digestible dietary carbohydrates. Minimum recommended level of probiotics (10^6–10^7) cfu/mL product is desirable for ascertaining health benefits[4]. Essentially, probiotics are considered to be anaerobic, slightly tolerant to acid & bile concentration. However, there are exceptions to these.*Lactobacillus acidophilus* is sensitive to acidic pH 2; fasting can sometimes make the stomach pH as low as 1-1.5, oxygen and bile concentration can also vary, affecting the viability of probiotics. Even the probiotic product processing, oxygen present in it, type of prebiotic used may affect the delay or survival ability of the probiotic[5]. Invitro viability assessment is carried out through cfu/mL and survival ability is analyzed through release of lactate dehydrogenase from the damaged cell, reflecting extent of cyto damage[6]. Various methods of encapsulation helps to overcome the problem of viability & survivability.Several polymers are used; ideally should be inert without any antimicrobial activity, such as alginate, chitosan, gelatin or plant mucilages, whey proteins, gums[7].

Co-encapsulation of probiotics with purified prebiotics, such as inulin, resistant starch[8], fructo-oligosaccharide[9], and arabinoxylan[10], seems to be promising in releasing the probiotic to the target host colon, but the prebiotic preparation in these cases are expensive & involves extensive cost in purification.

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Material & Methods

Effect of pH on survivability

The method used to understand the viability of the cells under acidic stress in this study was adapted from [11]. The study were carried out in three conditions;

Effect of pH on selected probiotic strain

Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for different pH conditions by using MRS broth adjusted to (pH 1,pH3,pH5,pH 6) using 1M Hcl, cells were incubated for 2Hr at 37^{0}C in different pH &then plated out on MRS agar medium for checking viability in the form of cfu/mL after 48hr at 37^{0}C. Similarly, the MRS broth containing probiotic strains with different pH were also checked for the LDH assay test to understand the extent of cell damage imposed due pH.

Effect of pH on symbiotic: Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for different pH conditions by using orange adjusted to (pH 1,pH3,pH5,pH 6) using 1M Hcl, cells were incubated for 2Hr at 37^{0}c in different pH bearing prebiotic preparation & then plated out on MRS agar medium for checking viability in the form of cfu/ml after 48hr at 37^{0}c. Similarly the prebiotic orange juice adjusted for different pH containing probiotic strains were also check for LDH assay test to understand extent of cell damage imposed due pH.

Effect of pH on encapsulated symbiotic

For encapsulation,1.5g of sodium alginate was dissolved in 25ml of orange juice, and homogenized. 1ml of cultured cells are mixed with the solution properly 2% of CaCl2 solution was prepared and kept in 4'C for 10minutes. Sodium alginate solution containing microbial cells were added drop wise to the chilled CaCl2 solution with the help of a pipette. Beads are formed. Filter the beads with the help of normal filter paper. After filtration wash the beads 2-3 times with sterile distilled water.Then the beads are subjected to test the effect of pH in the similar manner. Viability is determined through cfu/ml & survivability is assessed through LDH test.

For LDH assay, 100µl of culture subjected to different pH is taken, to this 100µl of reagent solution is added from the kit, incubated for 20minutes at 37'C, 50µl of stop solution is added. OD is checked at 490nm

Effect of temperature

The method used to evaluate the viability of the cells under different temperature stress in this study was adapted from [12,13]. The study was carried out in three conditions ;

Effect of temperature on selected probiotic strain

Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for sensitivity to different temperature conditions by using MRS broth , incubated at temperatures 4^{0}C, 45^{0}C, 55^{0}C and 65^{0}C for 2 hours & then plated out on MRS agar medium for checking viability in the form of cfu/ml after 48hr at 37^{0}C. Similarly the MRS broth containing probiotic strains with different temperatures was also checked for the LDH assay test to understand the extent of cell damage imposed due to low & high temperatures.

Effect of temperature on symbiotic

Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for sensitivity to different temperature conditions in orange adjusted to different temperature 4^{0}C, 45^{0}C, 55^{0}C and 65^{0}C and incubated for 2 hours& then plated out on MRS agar medium for checking viability in the form of cfu/ml after 48hours at 37^{0}C. Similarly, the MRS broth containing probiotic strains with different temperature were also checked for the LDH assay test to understand extent the of cell damage imposed due low & high temperatures.

Effect of pH on encapsulated symbiotic

For encapsulation,1.5g of sodium alginate was dissolved in 25ml of orange juice, and homogenized.1ml of cultured cells are mixed with the solution properly 2% of CaCl2 solution was prepared and kept in 4^{0}C for 10minutes. Sodium alginate solution containing microbial cells were added drop wise to the chilled CaCl2 solution with the help of a pipette. Beads are formed. Filter the beads with the help of normal filter paper. After filtration wash the beads 2-3 times with sterile distilled water.Then the beads are subjected to test the effect of temperature in the similar manner. Viability is determined through CFU/mL& survivability is assessed through LDH test.

Effect of oxygen

The method used to understand oxygen sensitivity in this study was adapted from[14]. The study was carried out in three conditions, simulating different oxygen preferences:

Effect of oxygen on selected probiotic strain

Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for sensitivity to different oxygen preferences by using MRS broth, plugged with cotton very tightly, simulating microaerophilic; loosely, simulating aerophilic & slight tightly simulating facultative anaerobic incubated at 37^{0}C for nearly 3 hours& then plated out on MRS agar medium for checking viability in the form of cfu/ml after 48hours at 37^{0}C. Similarly, the MRS broth containing probiotic strains with different oxygen conditions was also checked for the LDH assay test to understand the extent of cell damage imposed due to variable oxygen.

Effect of oxygen on symbiotic

Here the pure cultures of *Lactobacillus casei and Lactobacillus rhamnosus* were evaluated for sensitivity to different oxygen preferences in orange by incubating pure cultures in orange juice plugged with cotton very tightly, simulating microaerophilic; loosely, simulating aerophilic & slight tightly simulating facultative anaerobic incubated at 37^{0}C for nearly 3 hours& then plated out on MRS agar medium for checking viability in the form of cfu/mL after 48hours at 37^{0}C. Similarly the orange containing probiotic strains with different oxygen conditions were also checked for the LDH assay test to understand the extent of cell damage imposed due to variable oxygen.

Effect of oxygen on encapsulated symbiotic

For encapsulation,1.5g of sodium alginate was dissolved in 25ml of orange juice, and homogenized. 1ml of cultured cells are mixed with the solution properly 2% of CaCl2 solution was prepared and kept at 4'C for 10minutes. Sodium alginate solution containing microbial cells were added drop wise to the chilled CaCl2 solution with the help of a pipette. Beads are formed. Filter the beads with the help of normal filter paper. After filtration wash the beads 2-3 times with sterile distilled water. Then beads are subjected to test the effect of oxygen in a similar manner. Viability is determined through CFU/mL& survivability is assessed through LDH test.

Table 1: Effect of ph on survivalance of probiotic symbiotic and encapsulated symbiotic

DILUTION	PH	PROBIOTIC		SYMBIOTIC		ENCAPSULATEDSYMBIOTIC	
		NO. OF COLONIES	CFU/ML	NO. OF COLONIES	CFU/ML	NO.OF COLONIES	CFU/ML
10^{-1}	Ph1	45	45	45	45	49	49
	Ph3	53	53	59	59	92	92
	Ph5	107	107	110	110	121	121
	Ph6	96	96	99	99	109	109
10^{-3}	Ph1	16	0.16	43	0.43	54	0.54
	Ph3	47	0.47	51	0.51	62	0.64
	Ph5	78	0.78	78	0.78	82	0.82
	Ph6	57	0.57	58	0.58	69	0.69
10^{-5}	Ph1	30	0.030	30	0.030	43	0.043
	Ph3	85	0.085	89	0.089	92	0.092
	Ph5	95	0.095	96	0.096	99	0.099
	Ph6	50	0.050	53	0.053	59	0.059

Table 2: Effect of temperature on survivalance of probiotic symbiotic and encapsulatedsymbiotic

DILUTION	TEMPERATURE	PROBIOTIC		SYMBIOTIC		ENCAPSULATED SYMBIOTICS	
10^{-1}	4^0C	47	47	53	53	69	69
	45^0C	53	53	59	59	70	70
	55^0C	58	58	63	63	81	81
	65^0C	21	21	34	34	41	41
10^{-3}	4^0C	27	0.27	36	0.36	38	0.38
	45^0C	22	0.22	36	0.36	49	0.49
	55^0C	18	0.18	25	0.25	42	0.42
	65^0C	10	0.10	18	0.18	20	0.20
10^{-5}	4^0C	45	0.045	61	0.061	63	0.063
	45^0C	48	0.048	54	0.054	57	0.057
	55^0C	42	0.042	49	0.049	51	0.051
	65^0C	38	0.038	46	0.046	59	0.051

Table 3: Effect of dissolved oxygen on survivalance of probiotic symbiotic and encapsulated symbiotic

DILUTION	D/O	PROBIOTIC		SYMBIOTIC		ENCAPSULTED SYMBIOTIC	
		No. of colonies	CFU/mL	No. of colonies	CFU/mL	No. ofcolonies	CFU/mL
10^{-1}	MICROAEROPHILIC	27	27	29	29	31	31
	AEROBIC	90	90	99	99	105	105
	FACULTATIVE ANAEROBIC	26	26	23	23	29	29
10^{-3}	MICROAEROPHILIC	6	0.6	9	0.9	15	0.15
	AEROBIC	23	0.23	28	0.28	32	0.32
	FACULTATIVE ANAEROBIC	10	0.10	15	0.15	18	0.18
10^{-5}	MICROAEROPHILIC	11	0.011	19	0.019	20	0.019
	AEROBIC	42	0.042	53	0.042	58	0.058
	FACULTATIVE ANAEROBIC	8	0.08	10	0.010	11	0.011

LDH pH

pH	Pro	Syn	Imm	P.C	N.C
1	0.8259	0.6459	0.3942	0.9038	0.00
2	0.7227	0.5233	0.2406	0.8708	0.00
5	0.7017	0.3583	0.2344	0.7297	0.00
6	0.5029	0.2972	0.1284	0.7209	0.00

LDH Temperature

Temperature	Pro	Syn	Imm	P.C	N.C
4	0.92202	1.1181	0.6055	0.9843	0.00
45	0.93261	1.1813	0.4977	0.7784	0.00
55	1.0399	1.2937	0.5244	0.9583	0.00
65	0.95613	1.1471	0.5359	0.9698	0.00

LDH oxygen

Oxygen preferences	Pro	Syn	Imm	P.C	N.C
Micro aerophilic	0.8537	0.8033	0.8116	0.7316	0.00
Aerophilic	0.8913	0.82213	0.7864	0.6820	0.00
Facultative anaerobe	0.9086	0.8085	0.8388	0.5839	0.00

Result & Discussion

In this study,*Lactobacillus casei and Lactobacillus rhamnosus*. These microorganisms are usually evaluated for their probiotic characteristics and all these strains were made to undergo many stress conditions to check their viability. All these probiotic strains were expected to tolerate the stress conditions in order to be able to provide its beneficial effect on the host. The ability to tolerate these stress conditions are considered as good indicator of the survival of probiotic bacteria.

In this study pH1, pH3, pH5 and pH6 were used to investigate the acid tolerance of the selected strains as these pH ranges are a challenge for these strains to survive.

The results were shown Table 1 that which was a tolerance of probiotics at pH5, in the sense they showed good results at pH5 (107CFU/mL). At pH1 the survivability was very poor(45CFU/mL), when compared to others studies we found that the lowest pH recorded was pH 1.59[10],[11]. As demonstrated by[6], aciduric members such as *L.acidophilus*, generally could not survive in low pH environment as these cells were proven to be vulnerable at pH 2.0 and below. Low pH environments are thought to inhibit the metabolic activity and growth of *L.cidophilus*, thus reducing the viability. Another study conducted by[7] also confirmed that the viability count of the bacteria declined tremendously when exposed to simulated gastric juice of pH 1.5 after an incubation period of 3 hours.

In our study we also observed that the pH3 and pH5 showed the quite good survivability (53CFU/mL and 107CFU/mL, respectively), according to one study good probiotic should withstand at least pH 3.0[9]. In one study there were strains of *L.acidophilus* that survived perhaps because the pH was not too high so as to cause complete destruction of all the cells, according to this study good tolerance properties of exhibited by the bacteria are closely related to their strains specification as they are always strain dependent[12,13].

After this we made use of orange juice as the prebiotic that is capable of enhancing the growth of probiotics. As the prebiotics are added to probiotics, this combination was called as synbiotics and were exposed to the same stress condition at pH1, pH3,pH5 and pH6; the results shown increased

survivability compared to the previous because of the prebiotic(orange juice).

The other stress condition that was taken into consideration in this study was different temperatures. The isolated strains were incubated for 2 hours at different temperatures such as at 4^0C, 45^0C, 55^0C and 65^0C. After the growth of 2 days the results shown in table 2 that the strains at 55^0C showed the highest survivability 58CFU/mL. At a temperature of 4^0C there was a decrease in viability 47CFU/mL, at 45^0C also there was not much difference with the survivability 53CFU/ml. But at 65^0C there was less viability 21CFU/ml, as the viability was affected by very high temperature. According to a study[5] the survival of *Lactobacillus casei* at different temperatures was investigated; their results showed that the strains remained cultivable up to 65^0C. The viability was studied for an initial population of about 1008CFU/g. It was shown that they were able to survive at 75^0c for 10 minutes. Remarkably, at 45^0C ,they observed an increased viability of the culture. Likewise, we also made use of prebiotics to check if they can increase the viability of the strains. We made use of orange juice as the prebiotic and proceeded with our work. The probiotic strains along with the prebiotic were made to undergo different temperature such as 40^0C, 45^0C,55^0C and 65^0C. As a result there was a marked increase in viability of the strains 53CFU/ml,59CFU/ml,63CFU/ml,34CFU/ml, respectively.

The stress condition that the probiotics were exposed to was exposing them to different concentrations of dissolved oxygen. Here in this study the probiotics were made grow under aerobic, facultative anaerobic and microaerophilic conditions. The results in Table 3 show that the growth of probiotics under aerobic conditions(90CFU/ml) was better compared to the facultative anaerobic(26CFU/ml) and micro aerophilic (27CFU/ml). Only a few studies have been conducted on the oxygen tolerance of probiotic bacteria. Majority of probiotics are gut bacteria thriving as micro aerophillic or aertolerant oxygen concentrations are considered to be sensitive to the survival of probiotics. In one study [15] found that bifidobacteria survived well over a 30 days of period in yogurt, regardless of oxygen content keeping negative redox potential through addition of oxygen scavengersto the yogurt. This contradicts the popular belief that*Bifidobacteria* being most susceptible to oxygen toxicity in yogurt. Similarly, Millerand others (2002) found counts of *Bifidobacteria* to remain well above the recommended 10^6 CFU/g, even as the dissolved oxygen of the yogurt increased steadily over the shelf life, provided if the probiotic are stored in gas barrier packaging mateial. In contrast, counts of *L.acidophilus* were observed to decrease below 10^3CFU/g by just the 3^{rd} week of storage. Oxygen sensitivity in probiotic bacteria, particularly bifidobacteria, has been reported to be strain dependent [14],[17] as dependends on inducible enzymes found in some species.

Once the viability of synbiotics are checked under physiological stress such as pH, temperature and dissolved oxygen. The cultured probiotics along with the prebiotic that is, synbiotics are subjected to encapsulation. The microencapsulation was done by using sodium alginate as the matrix. These encapsulated probiotics were then exposed to the same physiological stress conditions to which the free probiotics and synbiotics were exposed. First of all the encapsulated synbiotics were grown in different pH levels, interestingly the microorganisms showed the increased viability in all the ph levels that is at ph1(49CFU/ml), pH3(92CFU/ml), pH5(121CFU/ml) and pH6(109CFU/ml).

In one study, Eight strains of probiotic bacteria, including *Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis type Bl-04 and B. lactis type Bi-07*, were studied for their acid and bile tolerance[5]. Microencapsulation in an alginate matrix was used to enhance the survival of the bacteria in acid and bile. Free probiotic organisms were used as a control. The acid tolerance of probiotic organisms was tested using HCl in MRS broth over a 2-hour incubation period. Bile tolerance was tested using 2 types of bile salts, oxygall and taurocholic acid, over an 8hour incubation period. Results indicated microencapsulated probiotic bacteria survived better ($P < 0.05$) than free probiotic bacteria in MRS containing HCl. When free probiotic bacteria were exposed to oxygall, viability was reduced by 6.51-log CFU/mL, whereas only 3.36-log CFU/mL was lost in microencapsulated strains. Overall microencapsulation improved the survival of probiotic bacteria when exposed to acidic conditions, bile salts. After the survivability at different pH is increased through microencapsulation, we again subjected encapsulated probiotics to different temperatures, here we also observed the improved viability of the probiotics. The viability count at 4^0C was 69CFU/ml, at 45^0C it was 70CFU/ml, at 55^0C count was 81CFU/ml, and at 65^0C 41CFU/ml. In one study, Heat tolerance was tested by exposing the probiotic organisms to 65^0C for up to 1 hour (Ding and Shah 2007). At 30 min of heat treatment, microencapsulated probiotic bacteria survived with an average loss of only4.17-log CFU/mL, compared to 6.74-log CFU/mL loss with free probiotic bacteria. However, after 1 hour of heating both free and microencapsulated probiotic strains showed similar losses in viability. Some studies are already analyzing the behavior of probiotic microencpsules in various foods. Khalil and Mansour(1998) incorporated *Bifidobacteria* encapsulated with alginate in mayonnaise and evaluated the survival of cells during refrigerated storage at 5^0C for 12weeks and also obtained results above 10^6CFU/ml. In another study, Ozer et al. (2009) added *Lactobacillus acidophilus* microencapsulated in 2% of alginate gel by extrusion technique in white cheese and analyzed it throughout 90 days of storage, at 4^0C and obtained a count of above 10^6CFU/ml.

Then the encapsulated cells were again exposed to the other stress condition of oxygen. They were checked for the oxygen tolerance by growing them under aerobic, facultative anaerobic and microaerophilic. The results showed that the viability was improved compared to the probiotics,which were non-encapsulated. In a study[19], suggested the use of alginate beads for micro encapsulation. Few studies, however, have investigated the protective effect of microencapsulation specifically against physiological stresses during gastro intestinal transact. It is known that alginate restricts the diffusion of oxygen through the gel, creating anoxic regions in the center of the beads. Research into immobilized systems[18] has suggested that increased survival ability of *Bifidobacterium spp* in yogurt over six days of storage than unencapsulated, suggesting microbial aggregates could develop anaerobic parts in the center of the beads. Encapsulated cells [20]of *B bifidum*increased the viabilty for 14 days at a temerature of 4^0C. Cell counts of encapsulated cells were found significantly higher compared with their free cell counterparts, when grown in optimum conditions of temperature (37°C) and culture broth . Interestingly, when tested in yogurt incubated at the storage temperature of 6°C, mixed results were obtained.

Microencapsulation was found to offer significant protection to only a few strain. The large and variable bead size in that study, however, could have resulted in poor cell distribution within the beads and, therefore, exposed more cells to oxygen in the yogurt. Nevertheless, preliminary evidence suggests that microencapsulation, if optimized, could become a useful technique to protect probiotic bacteria from oxygen toxicity in yogurts.

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