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RESEARCH PAPER

Cryopreservation and Microencapsulation of a Probiotic in Alginate-chitosan Capsules Improves Survival in Simulated Gastrointestinal Conditions

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Abstract The aim of the present study was to focus on the impact of two different methods and the effects of cryoprotectants on the survival of a probiotic bacterium, *Streptococcus phocae* PI80, during storage. For the protection of freeze dried cells, the optimal storage conditions were determined with a high survival rate. After the freeze drying process, all cryoprotectants exhibited a protective effect on cell viability at all storage temperatures. High relative cell viability was observed when cells were incubated at –20°C, which was optimum for the protection of *S. phocae* PI80. Trehalose was the most promising cryoprotectant at all temperatures during the storage period of bacterial cells. The combination of trehalose + skim milk showed more than 85% survivability compared to other combinations at –20°C for 60 days. In addition, encapsulation of probiotic cells into alginate-chitosan gel capsules showed better survival of *S. phocae* cells (5.468 ± 0.15 LogCFU/mL) with high bacteriocin activity at –20°C for six months. The cell-loaded microcapsules remained stable when treated with simulated gastric and intestinal fluids. After 6 h *in vivo* treatment, the capsules were found to be broken, releasing the probiotic cells directly into the intestinal system of rats. Therefore, microencapsulation was found to be the most efficient technique, which not only protected the cells for a longer time but also released the cells into the *in vivo* intestinal system.

Keywords: *Streptococcus phocae* PI80, cryoprotectants, preservation, freeze drying, microencapsulation

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1. Introduction

Probiotics are defined as live microorganisms, which when administered in sufficient amounts confer health benefits to the host [1], including inhibition of pathogenic growth, maintenance of health promoting gut microflora, stimulation of immune system, relieving constipation, absorption of calcium, synthesis of vitamins and antimicrobial agents, and predigestion of proteins [2]. Due to their vast range of industrial applications, it is essential to maintain high cell viabilities of probiotic bacteria. For commercial use, the development of storable formulated lactic acid bacteria, which have stable initial cell population with high cell viability is one of the major obstacles. Lactic acid bacteria are majorly preserved by freeze drying method with suitable cryoprotective media. The composition of the cryopreservation media, nature of the croprotectants, the freezing and thawing procedure, and intrinsic susceptibility of the cells to freeze damage are some of the essential factors for successful preservation [3]. Moreover, the cryoprotectants can reduce the bacterial cell damage while freeze drying and promote the formation of an amorphous state in cells rather than ice crystals during cooling-cryostorage-warming cycle [3]. Smirnoff and Cumbes [4] reported that sugars and sugar derivatives were used as effective cryoprotectants for lactic acid bacteria, as they have carbohydrate hydroxyl groups that replace water, thereby stabilizing the cell membrane lipids and proteins, and the presence of polyols, which inhibit the oxidative cell damage caused by scavenging of free radicals. Many studies revealed that the protective effect toward lactic acid

bacteria was excellent during freeze drying and long-term storage with influenced cryoprotective sugar [5,6]. Further, high survival rates of lactic acid bacteria were achieved in freeze dried form by combination of sugar and sugar derivatives with salt ions or complex media, including skim milk [6]. Probiotics are highly stable in dairy products when compared with non-dairy products, which depends on the selection of the durable strains in harsh environmental conditions and the prebiotics used [7]. In addition, the probiotic bacteria were found to be more stable in host natural barriers against ingested bacteria. Some of the probiotic bacteria did not have the capacity to survive in the adverse conditions such as high acidity and bile salts presented in the gastrointestinal tract [8].

Microencapsulation technique was investigated to overcome these problems. Microencapsulation is a safe, reliable, and simple method for the stable encapsulation of live cells resulting in a controlled and continued release of *de novo* produced metabolites. Microcapsules contain a liquid core surrounded by a semipermeable membrane, which facilitates the passage of nutrients and oxygen supply to the entrapped live bacterial cells and excretes the valuable products, including secondary metabolites. In addition, ultrathin semipermeable polymer membrane protects the probiotic bacteria from mechanical stress and adverse environmental acidic conditions, and retains the high cell viability of probiotic bacteria inside the microcapsules as well [9]. Productive nature, stimulation of production and secretion of metabolites and prevention of interfacial inactivation are the main advantages of the microcapsules. Recently, microencapsulation has been used as an efficient and successful method for long-term storage of bacterial cells and biological materials with suitable cryoprotectants and prebiotics [10,11]. Chitosan, sodium alginate, gelatin, CMC, sodium cellulose sulphate, starch, poly-L-lysine, *etc*., were used as natural and synthetic polymers for encapsulation of bacterial cells and other materials [10-12]. Alginate and chitosan are effective and most commonly used polymers for entrapping live bacterial cells because of their nontoxicity, biocompatibility, and inexpensive nature. Most research studies used alginate-chitosan microcapsules for entrapping yeast cells in ethanol production and encapsulation of *Escherichia coli* DH5 cells for oral delivery for disease therapy [12]. Nazzaro *et al*. [13] used alginate polymers for entrapping probiotic bacteria and analyzed their resistance to gastric conditions and vegetable juice fermentation. The use of alginate as polymers and the addition of prebiotic showed good protection, high viability, and activity of probiotic bacteria against gastric acidic conditions [13].

In the present study, maintenance of high viability, productivity, and resistance of probiotic bacterium *Streptococcus phocae* PI80 was investigated during long-term preservation using freeze drying and microencapsulation techniques with different cryoprotectants and prebiotics.

2. Materials and Methods

2.1. Materials

Carbohydrates such as glucose, sucrose, trehalose, galactose, and glycerol were used as protective agents for preservation of the probiotic bacterium *Streptococcus phocae* PI80. All these carbohydrates, skim milk, calcium chloride, sodium acetate, tween 80, and lactobacillus MRS broth were procured from HiMedia (Mumbai, INDIA). Composition of the MRS medium are as follows: Peptone (10.0 g/L), Beef extract (10.0 g/L), Yeast extract (5.0 g/L), Dextrose (20.0 g/L), Tween 80 (1.0 g/L), Ammonium citrate (2.0 g/L), Sodium citrate (5.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $MnSO_4 \cdot 7H_2O$ (0.5 g/L), and Dipotassium PO_4 (2.0 g/L). Sodium alginate, inulin, pepsin, pancreatin, bile salts, and chitosan were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Bacterial strains and culture conditions

The probiotic bacterium *S. phocae* PI80 was isolated from the gut of shrimp *Penaeus indicus* and was grown in lactobacillus MRS broth and maintained in glycerol stocks at –20°C [14]. For experimental purposes, the culture was precultured and grown in a 250 mL Erlenmeyer conical flask containing 50 mL of MRS broth and was subsequently incubated at 37°C for 12 h. This culture was transferred to 2 L of MRS broth in a 5 L hopkin flask and incubated at 37°C. After 24 h of incubation, the bacterial cell biomass was harvested by centrifugation at 6,000 rpm for 15 min and re-suspended with 100 mL of physiological saline (8.5 g/L) for preservation.

2.3. Freeze drying and storage

The protective agents and their levels were selected based on previously reported data for freeze drying lactic acid bacteria [15]. The afore-separated probiotic bacterial cell biomass (10 mL) was mixed separately with different protectants such as glucose, sucrose, trehalose, galactose, glycerol (35%), and skim milk (10%). Along with each experiment, the probiotic cells without any protectants were used as control for comparison. For equilibration, cells with protectants were incubated at room temperature for all experiments. All experiments were carried out in triplicates. After 1 h of incubation, the samples were kept at –20°C for freezing the cells and the frozen samples were desiccated under vacuum in a freeze dryer (VirTis Advantage Plus, SP industries, New York) for 20 h. The dried

samples were tightly packed and stored at –20, 4, 25, and 35°C for six months. For survival of microbial cells, temperature is an important factor during the storage of cells. At monthly intervals, the survival of bacterial cells was calculated as LogCFU/mL by plate count method in lactobacillus MRS agar medium. Before and after freeze drying, the bacterial cells were determined and expressed as percentage of relative viability by the equation: (%) = Viable cells after freeze drying (LogCFU/mL)/Viable cells before freeze drying (LogCFU/mL) × 100

2.4. Preparation and encapsulation of probiotic bacterium

Lyophilized powder (1 g/mL) of probiotic cells were mixed with 3% sodium alginate solution containing sterile MRS broth (55 g/L), trehalose (50 g/L), prebiotic inulin (10 g/L), and tween 80 (2 g/L). The mixture was extruded drop-wise through a 0.35 mm needle of 10 mL sterile syringe into a sterile calcium chloride (0.2 M) solution. The resulting granular shaped alginate capsules were agitated for 30 min in the same $CaCl_2$ solution to complete ionic gelation and hardening. Subsequently, they were transferred to chitosan solution (0.5%) containing 0.02 M sodium acetate buffer for coating the capsules. After 30 min agitation, the alginate-chitosan capsules were washed with water and immersed in 0.05% alginate solution for 30 min. The microcapsules were recovered and washed with water and stored at 4°C in minimal medium containing 10% MRS broth and 90% saline.

2.5. Long-term storage of microcapsules

For long-term preservation, half of the microcapsules were transferred to a sterile petri-dish and dried at 37°C for 48 ~ 72 h in an incubator. The dried capsules were transferred to airtight cryovials or containers and stored at –20, 4, and 35°C for six months. The viable cells and bacteriocin activity were analyzed at monthly intervals during the six month period. To determine the viability of cells, 100 mg of dried capsules were weighed and transferred to 10 mL of sterile water and kept at room temperature for 1 h for complete homogenization. The homogenized solution was serially diluted and plated on MRS agar plate. After 24 h incubation period, viable cells were calculated and expressed as LogCFU/mL. For bacteriocin activity, preserved capsules (50 mg) were transferred to 50 mL of MRS broth and incubated at 37°C for 24 h. The culture supernatant was collected by centrifugation and the bacteriocin activity (AU/mL) was determined using agar well-diffusion method.

2.6. Assay of bacteriocin activity

The bacteriocin activity of the cell-free supernatant from *S. phocae* PI80 was measured by the method of Todorov and Dicks [16]. Bacteriocin activity was estimated by agar well-diffusion method and expressed as an arbitrary unit (AU/mL), which was calculated as $a^b \times 100$, where "a" represents the dilution factors and "b," the last dilution that produces an inhibition zone of at 2 mm in diameter. Activity is expressed per mL multiplied by 100. One arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest two-fold dilution showing a clear zone of growth inhibition of indicator strain *Listeria monocytogenes*.

2.7. Mechanical stability study

To determine the strength of microcapsules, 2 mL of encapsulated capsule suspension (50 ~ 100 nos) were applied to 20 mL of simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) taken in 250 mL conical flask and incubated at 37°C for 144 h in a rotary shaker. At every 24 h interval, samples were withdrawn and analyzed for their morphological shape under the microscope. Moreover, the viable cells and capsule swelling or size were also examined. The simulated gastric fluid (SGF, pH 2.0) was formulated by MRS broth and pepsin (3 g/L). MRS broth, pancreatin (1 g/L), and bile salts (4.5 g/L) were used for the production of simulated intestinal fluid (SIF, pH 7.5). Microcapsules (2 mL) and free cells (5.615 LogCFU/mL) were incubated separately in SGF or SIF and incubated at 37°C for aforementioned time intervals. The viable cells were calculated by plate count method. In all experiments, the results were given as mean ± SD. One-way ANOVA (Tukey's method) was used for comparing experimental data and p-value of 0.05 or less was considered significant. The statistical package software (SPSS) was used to perform one-way ANOVA.

2.8. *In vivo* stability test

The stability of the microcapsules was also investigated under *in-vivo* conditions. For this experiment, six albino *wistar* male rats were procured from King's Institute, Chennai, India. (Ethical committee Registration no: 1159/C/07/CPCSEA, Pondicherry University, INDIA). The rats were fasted for 24 h prior to experiment and each rat (160 g) was orally administered with microcapsules (20 ~ 30) containing probiotic strains. After oral administration, each rat was fasted up to experimental finishing point. At every 2 h interval, one rat was sacrificed and their stomach pouch and intestines were dissected, their inner portions rinsed with sterile water to recover the microcapsules, and were examined under the microscope.

3. Results and Discussions

S. phocae PI80 is a potent probiotic strain, which was

isolated from the gut of shrimp *Penaeus indicus* [14]. During the fermentation period, it produced some metabolites including exopolysaccharide and bacteriocin [17,18]. Moreover, *S. phocae* PI80 was found to exhibit potent probiotic role against vibriosis in shrimp *Penaeus indicus* and *Aeromonas* infection in fishes *Cyprinus carpio* and *Tilapia mossambicus* [19,20]. Recently, Paari *et al*. [21] reported that probiotic *S. phocae* PI80 could be used as a potent biopreservative agent in the preservation of Tiger shrimp *Penaeus monodon*. Maintenance of high viability of *S. phocae* PI80 is important due to the aforementioned application. Hence, we have attempted a trial to preserve the potent probiotic culture for a long term using cryopreservation and microencapsulation techniques.

3.1. Effect of cryoprotectants on storage of probiotic bacterium *S. phocae* PI80

One of the most considerable prerequisites for the use of lactic acid bacteria is that they survive throughout the production process and storage time till the end of their shelf life. Using this aspect, we planned and studied the survival of the probiotic bacterium *S. phocae* PI80 by freeze drying and microencapsulation methods and subsequent storage at different temperatures. In this study, protective agents such as glycerol, skim milk, monosaccharide glucose, disaccharide sucrose, trehalose, and galactose were used in this experiment in order to ensure the viability of probiotic bacterium *S. phocae* PI80 during freeze drying. All cryoprotectants were selected based on their protective ability and previous literatures. The addition of disaccharides was compensating the water loss by replacing the hydrogen bonds through interactions with the polar head groups of the cell membrane and cellular proteins [22]. Storage conditions such as temperature and atmosphere were also important factors for the survival of bacterial cells [23]. The concentrated bacterial cells (14.489 ± 0.16 LogCFU/mL) were freeze dried with different cryoprotectants and subsequently stored at −20, 4, 25, and 35°C for six months. The bacterial cells without any cryoprotectants were used as control group for comparison.

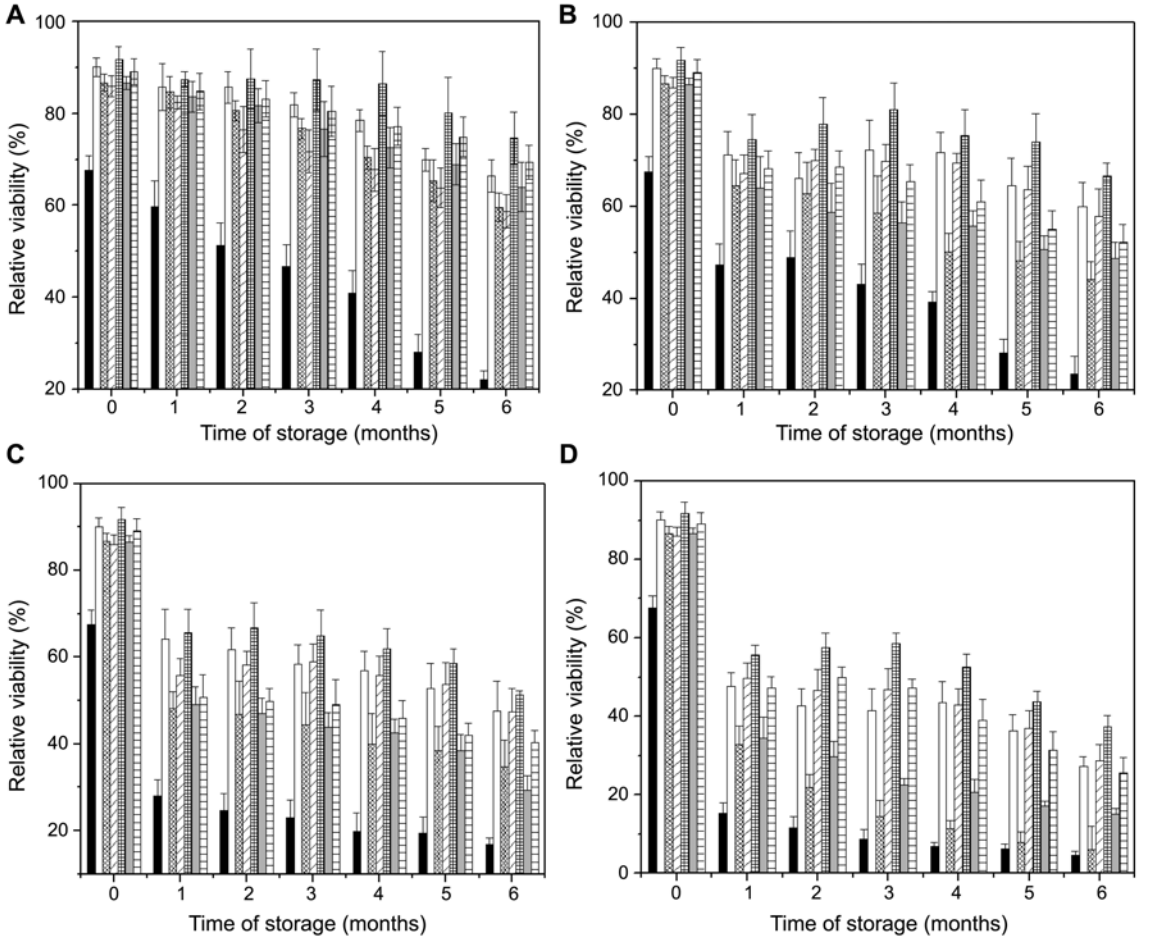

Fig. 1. Relative cell viabilities of freeze dried *Streptococcus phocae* PI80 cells in the absence ( ) and presence of cryoprotectants glycerol ( ), glucose ( ), sucrose ( ), trehalose ( ), galactose ( ), and skim milk ( ) after six months of storage at temperatures −20°C (A), 4°C (B), 25°C (C), and 35°C (D).

There was no significant loss of initial viability of freeze dried bacterial cells in all protective agents. After freeze drying, the highest initial viability of bacterial cells were observed in trehalose (91.8 ± 2.8%), followed by glycerol (90.1 ± 2.1%) and skim milk (89.1 ± 2.8%) as cryoprotective agents. Moreover, a low initial viability of bacterial cells (86.7 ± 1.8 and 85.9 ± 2.3%) was obtained when cells were incubated with glucose and sucrose. The viability of bacterial cells without any cryoprotectants was reduced by more than 30% of bacterial cell survival. Potts [24] reported that the exact mode of action of protective agents is indeed complex and not fully understood to date and water replacement may be the crucial mechanism underlying the protection mediated by some protective agents.

Fig. 1 depicts the relative cell viability of *S. phocae* PI80 after freeze drying in the presence or absence of cryoprotectants over a six-month period at different temperatures. At −20°C storage temperature, trehalose and skim milk significantly retained relative cell viabilities (74.6 ± 5.9 and 69.4 ± 3.7%, respectively) compared to other protectants after six months, which showed that only 25.4 and 30.6%, respectively, of the cell viabilities were lost (Fig. 1A). Formulating cells with glycerol, galactose, glucose, and sucrose decreased the relative cell viability to 66.4 ± 3.6, 64.0 ± 5.4, 59.6 ± 3.1, and 58.7 ± 3.6%, respectively, after six months of storage. Freeze dried cells without any protective agents showed very low cell viability (22.0 ± 1.9%) compared to cells dried with protectants, after six month storage. Moreover, trehalose and glycerol proved to be excellent protectants at 4°C storage temperature, which showed stabilized cell viabilities of 66.6 ± 2.8 and 60.0 ± 5.2%, respectively, after six months storage period (Fig. 1B). At 4°C storage temperature, skim milk failed to protect the cell viability (52.2 ± 3.9%) compared to cells incubated with sucrose (57.9 ± 5.9%) after six months. Using glucose and galactose as cryoprotectants caused significant loss of relative cell viabilities of 44.2 ± 3.8 and 48.7 ± 3.6%, respectively at 4°C over a period of six months. At 25 and 35°C storage temperatures, trehalose also significantly retained relative cell viabilities of 51.3 ± 1.0 and 37.3 ± 3.0% than cells stored without any cryoprotectants (16.8 ± 1.6 and 4.5 ± 1.1%) after six month storage (Figs. 1C and 1D). After six months, glycerol and sucrose showed higher relative cell viability (47.6 ± 6.8 and 47.3 ± 5.4%) than glucose and galactose at 25°C. In general, the relative cell viability of *S. phocae* PI80 was dramatically reduced from initial storage period to final period in all cryoprotectants at all temperatures. Overall, trehalose was the most significant protective agent, which showed higher relative cell viabilities among other protective agents at all storage temperatures followed by glycerol and sucrose (Figs. 1A, 1B, 1C, and 1D). Trehalose was not like the other sugars that did not produce moisture during freeze drying [5]. During the thawing process, the presence of residual water crystals could cause damage to the cell structure. These damages were eradicated by the presence of trehalose that replaces water molecules. Trehalose forms a covering layer surrounding the biostructures, which stabilizes their mobility thereby maintaining their functional conformations [22]. Moreover, trehalose has a superior capacity as a water structure breaker molecule [25]. De Giulio *et al*. [5] reported that trehalose was the most promising cryoprotective agent, which ensures high cell viability of *Lactobacillus acidophilus* and *Streptococcus salivarius* sub sp. *thermophilus*. At all storage temperatures, glucose failed to protect the high cell viability of *S. phocae* PI80, among all the carbohydrates used in this experiment.

3.2. Effect of combination of cryoprotectants on storage of probiotic *S. phocae* PI80

Fig. 2 shows the relative viability of *S. phocae* PI80 after freeze drying in the presence of mixed cryoprotectants over a 60-day storage period at −20°C. After freeze drying (0 day), the relative percentage of initial viabilities were observed in the combination of cryoprotectants such as sucrose + trehalose, glucose + trehalose, glycerol + trehalose, galactose + trehalose, skim milk + trehalose, and glycerol + skim milk trehalose. Among them, highest initial cell viabilities were observed in the combination of

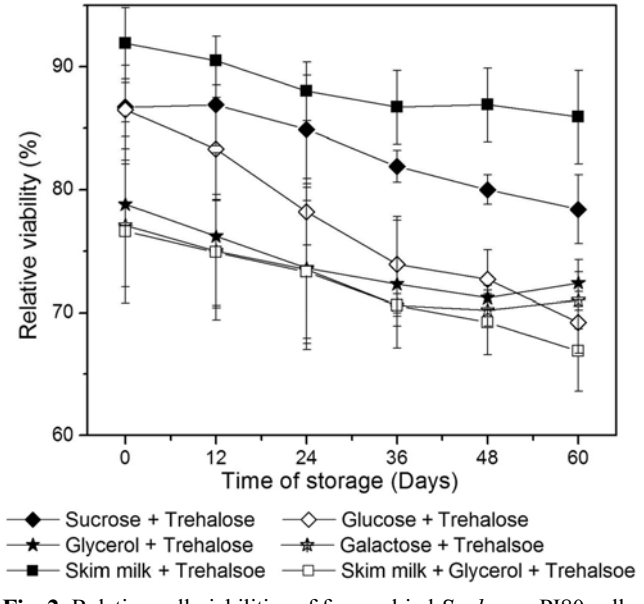

Fig. 2. Relative cell viabilities of freeze dried *S. phocae* PI80 cells in the presence of combined protectants sucrose + trehalose (-◆-), glucose + trehalose (-◇-), glycerol + trehalose (-★-), galactose + trehalose (-✱-), skim milk + trehalose (-■-), and skim milk + glycerol + trehalose (-□-) after two months of storage at temperature −20°C.

Table 1. Effect of various temperatures (–20, 4, and 35°C) on bacteriocin activity and viable cells of microencapsulated *S. phocae* PI80

Month	Different temperatures					
	–20°C	4°C	35°C	–20°C	4°C	35°C
	Bacteriocin activity (AU/mL)			Total viable cells (LogCFU/mL)		
1	16,966 ± 1,021	15,233 ± 1,245	16,966 ± 865	5.701 ± 0.20	5.672 ± 0.27	5.387 ± 0.11
2	16,066 ± 1,443	16,966 ± 865	16,133 ± 1,021	5.671 ± 0.15	5.639 ± 0.12	5.133 ± 0.16
3	16,200 ± 856	16,066 ± 1,245	15,233 ± 1,245	5.633 ± 0.11	5.394 ± 0.20	4.290 ± 0.10
4	15,233 ± 1,443	14,466 ± 1,443	15,300 ± 1,021	5.170 ± 0.15	5.071 ± 0.21	4.383 ± 0.12
5	17,800 ± 1,021	16,066 ± 1,021	15,366 ± 865	4.943 ± 0.13	4.829 ± 0.16	3.793 ± 0.28
6	16,200 ± 1,245	16,133 ± 1,021	14,466 ± 2,400	4.668 ± 0.15	4.505 ± 0.11	3.333 ± 0.31

Data represented as mean ± SD.

skim milk + trehalose (91.9 ± 2.9%) followed by sucrose + trehalose (86.7 ± 3.4%) and glucose + trehalose (86.5 ± 2.2%). However, trehalose + skim milk combination has the capacity to maintain higher relative cell viability (85.9 ± 3.8%) up to end of the 60 days storage period. In contrast, the trehalose + lactose mixture was found to be the most promising agent, which maintained more than 75% of *Lactobacillus paracasi* NFBC 338 and *L. rhamnosus* GG survival rate [26]. Five percent of sucrose was able to maintain a more concentrated starter culture of *Lactobacillus lactis* subsp. *lactis* than glycerol at storage temperature 20°C [27]. Similarly, sucrose was effective in retaining more than 86% of cell viability when combined with trehalose whereas, glycerol + skim milk + trehalose showed lower cell viability (66.9 ± 3.3%) than other combinations.

3.3. Long-term preservation of probiotic bacteria loaded in microcapsules

Sodium alginate-chitosan coated dried microcapsules containing *S. phocae* PI80 were stored at –20, 4, and 35°C to check the survival rate and bacteriocin activity of probiotic bacteria over a period of six months. To increase the cell viability after drying, 5% of trehalose and 1% of inulin were added to the sodium alginate solution to ensure the encapsulated probiotic strains. Li and Chen [28] reported that the microcapsules with trehalose as a carbon source actively protect the probiotic bacterial cells during drying process. Inulin is a type of fructans and fructo-oligosaccharide, which improves the survival of probiotic bacteria and is approved for use in food industry as prebiotic substances as well [29]. The addition of probiotic fructo-oligosaccharide to the alginate capsules ensured high cell viability [30]. The maximum bacteriocin activity (16,200.0 ± 1,245.0 AU/mL) and total viable cells (4.668 ± 0.15 LogCFU/mL) were obtained when encapsulated capsules were stored at –20°C for six months (Table 1). In contrast, low number of cells (3.333 ± 0.31 LogCFU/mL) and bacteriocin activity (14,466 ± 2,400 AU/mL) were obtained when probiotic containing capsules were stored at 35°C. To summarize, significant loss of initial viability and bacteriocin activity were not observed till the end of the six month storage period at all storage temperatures. Similarly, Brinquez and Ayub [31] reported that the chitosan-alginate capsules significantly improved the high cell viability of *L. plantarum* BL011 under refrigerated storage conditions (4°C). Moreover, results of microencapsulation indicated that alginate-chitosan-carboxymethyl chitosan capsules successfully increased the cell numbers of *L. casei* ATCC 393 after storage at 4°C for four weeks [32]. In contrast, the yield of microencapsulated chitosan-alginate capsules showed low number viable cells for probiotics *L. gasseri* and *B. bifidum* when the capsules were stored at 4°C [33]. Heidebach *et al*. [34] attempted the encapsulation of probiotics *Lactobacillus* F19 and *Bifidobacterium* Bb12 in casein based microcapsules. Under optimized conditions (4°C), after three months of storage, about one log cycle of encapsulated *Bifidobacterium* Bb12 was lost and the capsules averaged to about 3.8×10^9 CFU/g powder. For *Lactobacillus* F19 the reduction is almost two log cycles with about 1.7×10^8 CFU/g powder at the end of storage.

3.4. *In vitro* stability of microcapsules in SGF and SIF

The probiotic cell loaded microcapsules were introduced to simulated gastric fluid (SGF) and intestinal fluid (SIF) for 144 h. Alginate-chitosan coated capsules remained in a uniform spherical shape with smooth surface when incubated with SGF, whereas microcapsules became wrinkled and broken in SIF at 37°C for 144 h (Fig. 3). The cell release was not observed from microcapsules in SGF, whereas capsules from SIF released very high number of cells (4.222 ± 0.41 LogCFU/mL) in 144 h incubation period (Table 2). In SIF, the capsules remained stable till 24 h, but were broken after 48 h of incubation. In case of free cells, the cell viabilities were decreased dramatically from initial to end period when incubated in both SGF and SIF. Similarly, Chavarri *et al*. [33] reported that the chitosan coated alginate capsules significantly enhanced the survival

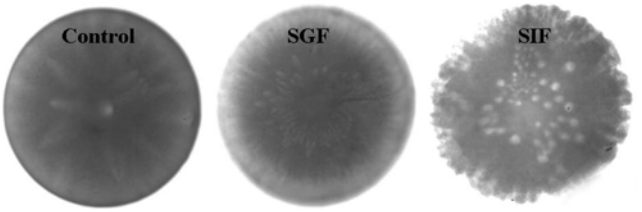

Fig. 3. Morphology of microencapsulated capsules with *S. phocae* PI80 after 144 h incubation in simulated gastric (SGF) and intestinal fluid (SIF).

of probiotic bacteria *L. gasseri* and *B. bifidum* when compared with free bacteria in simulated gastric conditions and bile salts. Moreover, sodium alginate capsules coated with chitosan greatly improved the survival of *L. plantarum* when compared with uncoated capsules in SGF and SIF [31]. In contrast, Gbassi *et al.* [35] and Sultana *et al*. [36] reported that probiotic encapsulation in alginate capsules without chitosan coating was ineffective in protecting probiotic cells in highly acidic environments. Encapsulation of bifidobacteria without chitosan coating calcium alginate capsules failed to protect probiotic cells in SGF [37]. Mokarram *et al*. [38] reported that encapsulation of *L. acidophilus* and *L. rhamnosus* in calcium alginate capsules without coating materials did not significantly improve the probiotics cell survival in SGF. In addition, Krasaekoopt *et al*. [39] found that for *L. acidophilus* with initial cell count in the range of 2.171 ~ 1.970 × 10^9, the survival of cells in coated capsules was significantly higher than that of uncoated capsules.

Fig. 4 showed that the size of the swollen capsules in SGF and SIF. The swelling of capsules increased day by day when capsules were incubated in SIF, whereas capsules were not found to be swollen in SGF. More than 30% of swelling was observed in SIF for 144 h. In contrast, the capsule size remained stable and shrank in SGF with by about 10%. Wenrong and Griffiths [40] reported that the alginate capsules were not swollen when incubated at low

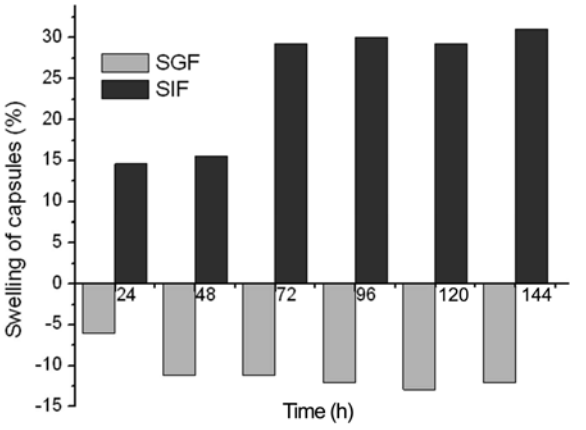

Fig. 4. Size of swollen microencapsulated capsules after 144 h *in vitro* treatment at 37°C.

pH. But the carboxyl group of alginate was not cross-linked with Na^+ and found that the capsules absorb water at higher pH. Dainty *et al*. [41] reported that the disruption without chitosan coating calcium-alginate matrix occurred in phosphate buffer solution by chelating action of phosphate ions in pH above 5.5. Further, the cell containing capsules were first introduced into SGF and the same capsules were introduced to SIF. At 144 h, higher amounts of cells (5.638 ± 0.24 LogCFU/mL) were released from capsules when they were transferred from SGF to SIF. The cells were continuously released from microcapsules in SIF containing pH (7.5). This may happen by the partially broken sodium alginate bonds during the preparation of capsules, which makes capsules unstable at a higher pH. These results explain that the alginate-chitosan microcapsules are much more stable in gastric conditions and broke when applied to intestinal fluid, which facilitated the release of cells from capsules directly into the intestinal system. Similar results were observed for lactic acid bacteria using alginate as gel matrix [13,28].

3.5. *In vivo* delivery of microcapsules

Table 2. Mechanical stability of capsules in SGF and SIF

Time (h)	Simulated gastric fluid		Simulated intestinal fluid	
	Encapsulation (LogCFU/mL)	Free cells (LogCFU/mL)	Encapsulation (LogCFU/mL)	Free cells (LogCFU/mL)
0	Nil	5.610 ± 0.23	Nil	5.610 ± 0.23
24	Nil	2.433 ± 0.18	Nil	6.973 ± 0.30
48	Nil	2.008 ± 0.15	1.234 ± 0.21	4.560 ± 0.18
72	Nil	1.156 ± 0.12	1.261 ± 0.13	4.089 ± 0.21
96	Nil	1.132 ± 0.15	2.605 ± 0.12	4.027 ± 0.36
120	Nil	Nil	3.917 ± 0.43	2.194 ± 0.13
144	Nil	Nil	4.255 ± 0.41	Nil

Data represented as mean ± SD.

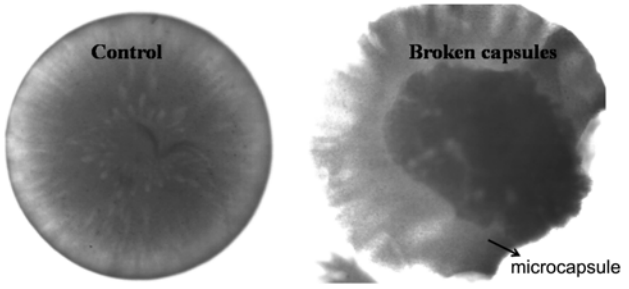

Fig. 5. Morphological shape of microencapsulated capsules with *S. phocae* PI80 after 6 h of oral administration period in rats.

The stability of cell loading microcapsules was analyzed *in vivo* using rats. The capsules were orally delivered to the rats and observed for 12 h. Stable and uniform sized microcapsules were retrieved from the gut of rat from up to 4 h, whereas the capsules broke within 6 h of incubation (Fig. 5). This result was a good method for easy and direct delivery of probiotic cells into intestinal regions using alginate as a support material. Similarly, Murua *et al*. [11] used alginate capsules as a delivery system for genetically engineered myoblasts (Epo) releasing in to mice.

4. Conclusion

The probiotic bacteria *S. phocae* PI80 was preserved for a long time by both freeze drying and microencapsulation methods. All cryoprotectants were able to retain cell viability during the storage time. The high cell viabilities were obtained when freeze dried cells were incubated at –20°C. Among the cryoprotectants, trehalose was the most promising cryoprotective agent at all temperatures during storage period. Moreover, the combination of trehalose + skim milk showed high survival rate (85%) than other combinations at –20ºC for 60 days. *S. phocae* PI80 were also microencapsulated with alginate and chitosan coat and prebiotic additives by the extrusion method and dried at 35°C. The dried capsules retained good survival and bacteriocin activity at –20°C for six months. Additionally, the immobilization of cells had been shown to improve the survival of cells when exposed to acidic conditions. After 6 h *in vivo* treatment, the capsules were broken and probiotic cells were directly released into the intestinal system of rats. These results explained that both freeze drying and microencapsulation methods have the potential to be applied in food and pharmaceutical industries.

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