CHAPTER 4

EFFECT OF CASSAVA STARCH ADDED IN CA-ALGINATE BEADS ON STABILITY ENTRAPPED *vB_salP-p*YM BACTERIOPHAGE.

4.1 Abstract

This study investigated the effect of cassava starch (CS) incorporation on the stability and entrapment efficiency of salmonella bacteriophage *vB_salP-p*YM in Caalginate beads under various conditions. All treatments showed high entrapment efficiency (>97%), indicating that the addition of CS did not significantly affect the entrapment efficiency. The morphological shape of Ca-alginate beads with/without CS was illustrated by SEM. These results showed that the addition of CS in Ca-alginate beads was able to increase the porosity and pore size of the internal structure. The addition of CS in Ca-alginate beads could improve the stability of phages at high temperature, acidic and alkaline pH better than only Ca-alginate beads. In addition, the CS incorporated with Ca-alginate could support the phage stability exposure to SGF and also accelerate phage release in SIF that are targeted for colonization of salmonella phages. The entrapped phages in Ca-alginate with CS prolonged the shelf life of phages during six months of storage. These results suggested that cassava starch could be a promising biopolymer addition for improving the stability and application of alginate entrapped bacteriophages, with potential applications in therapy and food safety.

Keywords: Cassava starch, Ca-alginate beads, Salmonella bacteriophage, Entrapment.

4.2 Introduction

Bacteriophages, or phages, are viruses that infect and lyse bacteria through rapid replication within their hosts. Consequently, phage therapy has been proposed as a method to control *Salmonella* infections in poultry. Numerous studies have demonstrated the efficacy of phages in reducing colonization of *Salmonella enterica* in chickens (Colom, Sarabia, Otero, Cortés, Maspoch and Llagostera, 2015). Bacteriophage have been increased research as an alternative method for pathogen

control for phage therapy in animals and processing aids in the food industry for phage therapy for enteric bacterial infections in animals was interesting. The challenge of phage therapy was oral administration because of survivability when exposed to the gastrointestinal tract, including gastric juice, bile salt and intestinal conditions (Colom et al., 2017). Encapsulation of phages was applied for the prevention of loss of phage viability (Loh et al., 2021). Ca-alginate beads were not stabilized under acidic conditions. Alginate stability can be increased by (1) combining it with other natural polymers like proteins, glycerol, or starch, and (2) coating an extra layer of beads (Moghtader, Eğri and Piskin, 2017). Biswas and Sahoo, (2016) reported that cassava starch–alginate beads were used to deliver metoprolol tartrate (MT), exposure to simulated gastric fluid (pH 1.2), resulting in protection. The entrapment efficiency was increased from 58% to 88% and the release duration was from 45 minutes to 3–4 hours. These findings suggested that blending alginate beads with cassava starch can enhance stability and prolong the release of MT in gastric juice. Additionally, Rohman, Kaewtatip, Kantachote and Tantirungkij, (2021) reported that *Rhodopseudomonas palustris* KTSSR54 bacteria, used as a biofertilizer, were successfully entrapped within alginate/cassava starch hydrogels, leading to reduced cell loss. The entrapment efficiency of cassava starch in these hydrogels was 70.83%, compared to approximately 50.56% without starch. Therefore, the objective of this study to evaluate the effectiveness of cassava starch incorporation with Ca-alginate for protecting phages under various temperatures, pH, bile salt, gastrointestinal environment and storage at 4°C.

4.3 Material and Methods

4.3.1 Bacteria and bacteriophage

The *Salmonella* Typhimurium ATCC 13311 was bought from American Type Collection Culture (Microbiology, Inc, U.S.A.). Bacteriophage *vB_salP-p*YM. (Phage) was isolated from pork meat that was bought from a local market in Nakhon Ratchasima.

4.3.2 *Salmonella* cultivation and enumeration

Salmonella Typhimurium (*S.* Typhimurium) was cultured on xylose lysine deoxycholate (XLD; HIMEDIA®, India) agar and incubated at 37 °C for 24 hours. The *S.* Typhimurium was routinely sub-cultured by using tryptic soy broth (TSB; HIMEDIA®, India). Subsequently, the bacterial cultures were collected by centrifugal at 1,500 x g for 15 min at 4 °C. The pellet cells were washed twice and resuspended with salt magnesium sulphate buffer (SM buffer) composed of 200 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl and 0.01% gelatin per litter pH 7.4. The resuspension was measured the absorbance at OD 600 nm to obtain 0.2 as approximate as $1x10^8$ CFU/ml (Ye et al., 2010). This bacterial concentration was used for lawn preparation.

The *S*. Typhimurium was counted by using the spread plate technique. The ten-time serial dilution was performed using phosphate buffer saline (PBS) that was prepared by 10 mM Na_2HPO_4 and 1.8 mM NaH_2PO_4 per litter pH 7.4 and then cultured on XLD agar. The plates were incubated at 37 °C for 24 h. The colony-forming unit (CFU/ml) was calculated according to the equation below.

CFU/ml = number of colonies / (volume of sample x dilution factor)

4.3.3 Propagation and enumeration of bacteriophage titer

The 500 ul of mixture of phage and bacterial lawn as described as above were added into 5 ml of molten TSB containing 0.6% agar and poured on the basal TSB containing 1.5% agar. The plates were incubated at 37 °C for 24 hours (Batalha et al., 2021). Five milliliters of SM buffers were added to the plate and then placed on a shaker incubator at 100 rpm, 4°C for 24 h. The phage suspension was centrifuged at 4,500 x g for 15 min at 4°C and the supernatant was filtered using a 0.2 µm syringe filter and stored at 4°C.

Phage titers were determined by using the agar overlay assay (Artawinata et al., 2023). Briefly, phage suspension was diluted with ten-time serial dilution by using SM buffer. The 10 µl of diluted phage was dropped on the lawn as described on phage propagation without phage. The titers of phage were determined

as the mean of three independent counts, as plaque-forming units (PFU/ml) (Batalha et al., 2021). The PFU/ml was calculated according to the equation below.

PFU/ml= Average number of plaque x Dilution factor/ Volume of phage

4.3.4 Entrapments efficiency of bacteriophage

The phage suspension was entrapped into sodium alginate (HIMEDIA®, Nashik, India) with or cassava starch (CS; Kriangkrai company limited, Nakornprathom, Thailand) by modified from method of Ma et al., (2012). The the Ca-alginate beads were composed of 3% (w/v), sodium alginate and added with 0.15, 0.3% w/v of SPI or CS, and then mixed with phage suspension (10^8 PFU/ml). The beads were extruded by using a syringe 20 gauge (G) needle and exposed in 4% (w/v) $CaCl_2$ that was stirrings at 100 rpm at room temperature on stirring plate with a magnetic bar. The Ca-alginate beads were left to solidify for 1 hour then washed the beads two times with distilled water and kept modified alginate beads in 50 ml SM buffer pH 7.4 at 4 °C during the experiment.

For enumeration of phage titers, 100 mg of beads were added into 5 ml of sterile PBS pH 7.4 and shaken by an orbital shaker at 300 rpm at room temperature for 1-2 hours, or until dissolved (Ma et al., 2012) and then were determined by using agar overlay assay. Entrapment efficiency (EE) of phage in Ca-alginate beads without/with SPI and CS were determined according to Batalha et al. (2021). The EE was calculated as the following equation below.

EE= (Phage entrapped (PFU)/Initial phage (PFU)) x 100

4.3.5 Scanning electron microscopy (SEM)

The SEM-EDS-JEOL/JSM-6010LV (InTouchScope™, Japan) at 3 kV was used to clarify the morphology of ca-alginate beads. Briefly, the samples were prepared by critical point drying (CPD). Ca-alginate beads were fixed using glutaraldehyde (1%–3%) or a mixture of 2.5% glutaraldehyde and 4% formaldehyde.

Dehydration was carried out using a gradient series of ethanol including concentrations of 10%, 20%, and 100%. After that, the samples were placed in a critical point dryer, which requires connections to a CO_2 cylinder, hot and cold-water supplies, a drain (Bray, 2000), and an exhaust tube and is coated with gold under a vacuum.

4.3.6 Lytic assay of entrapped salmonella phage

The lytic activity assay was used for investigating effiency of entrapment. The hypothesis was if it had free phage released from the entrapped salmonella phage, the amount of *Salmonella* was reduced or if not, the *Salmonella* was still growth. Free phage (100 µl, 10^7 PFU/ml) was added to 5 ml of *S.* Typhimurium suspension at a 10^7 PFU/ml concentration. The 100 mg of Ca-alginate beads were placed into 5 ml of *S.* Typhimurium cells (10^7 PFU/ml) and then incubated at 41 °C with agitation at 120 rpm for 6 hours. Five hundred microliters of sample were collected interval every 2 hours (Zhou et al., 2022). The number of *Salmonella* was counted using the drop plate method and incubated at 37 °C for 24 hours (Reed and Reed, 1948). The colony-forming unit (CFU/ml) was calculated as described above.

4.3.7 Stability of salmonella bacteriophage in Ca-alginate beads under different temperatures and pH

The pH stability of the salmonella bacteriophage-entrapped and free phages was evaluated. The 100 mg of each sample and free phage (10^7 PFU/ml) were placed into SM buffer was adjusted to pH 2.5, 5, 5.5, 6.5, 7.5 and 8 (2.5 is the pH of the gizzard, 5 is the pH of the duodenum, 5.5 is the pH of the crop, 6.5 is the pH of the jejunum and 8 is the pH of the cecum) by using 1 M HCl or 0.5 M NaOH. All samples were incubated at 37 °C for 2 hours. Subsequently, the survival of free phage or entrapped phage in the Ca-alginate bead under each pH was measured by double agar overlay assay according to the method described above (4.3.4) The structure of

the alginate polymer network is destroyed at pH 2.5 and 5, but the structure of calcium alginate is stable between pH 5.5 to 10 (Malektaj et al., 2024).

For thermal stability, the 100 mg of alginate beads were placed into 1 ml SM buffer pH 7.4 and subsequently at different temperatures including 4°C, 25°C, 37°C, 42°C and 50°C (At 4°C is the storage temperature, 25°C is the room temperature, 37°C is the human body temperature, 42°C is the chicken body temperature and 50°C is the representative of high temperature) for 24 hours (Dougan and Baker, 2014). The survival phages in each bead treatment were determined by the same method described above (No. 4.3.4).

4.3.8 Effect of bile salt on the stability of salmonella bacteriophage

The survival of free phages and entrapped phages in each Ca-alginate bead was determined. For free phage, 100 µl of phage suspension (10^7 PFU/ml) was placed into 4.9 ml of 0.01 M bile salt, 0.85% (w/v) NaCl pH to 6.8 as well as that the 100 mg of each Ca-alginate bead was placed into 4.9 ml bile salt solution. Afterward, all treatments were incubated at 41 °C with agitation at 120 rpm for 1 hour (Scanlan et al., 2019: Ma et al., 2008). The titer of survival phage under the bile salt solution was determined immediately by agar overlay assay according to the method described above (No. 4.3.4).

4.3.9 Stability of the phage in Ca-alginate beads under simulated gastric fluid (SGF)

The stability of the entrapped phages under simulated gastric fluid (SGF; pH 2.5) that consposed of 3 mg/ml pepsin (pepsin enzyme, HIMEDIA®, Nashik, India) and 0.85% NaCl; pH to 2.5 that adjusted by using 1 M HCl (Colom et al., 2017) was determined. One hundred milligrams of Ca-alginate beads were placed into SGF and incubated in orbital shaker (S1500, Stuart, Stone, UK) at 120 rpm at 41 °C for 30, 60, 90 and 120 min and then removed SGF from the test tube and replace 2 ml of SM buffer pH 7.4 and left at room temperature for 5 min. The phage titers in each bead treatment were determined as described above (No. 4.3.4). The treatment was

performed in triplicate and the results were presented in percentage of survival phages (Ma et al., 2008).

4.3.10 Phage releasing under simulated intestinal fluid (SIF)

The entrapped phage in Ca-alginate beads treatment were evaluated for the phage releasing under the simulated intestinal fluid (SIF) that consisted of 1 mg/ml of pancreatin enzyme (HIMEDIA®, Nashik, India) and 0.85% NaCl, pH to 6.8 that adjusted with 1 M HCl. The 100 mg of each Ca-alginate bead was incubated at 41 °C for 120 min on orbital shaker incubator at 120 rpm. The 100 ul of sample solutions were sampled at 30, 60, 90 and 120 min. The release of phage titers under SIF was examined by utilizing the double agar overlay assay as described above (No. 4.3.4).

4.3.11 The survival of entrapped phages in Ca-alginate beads during storage

The survival of the entrapped Salmonella phage in Ca-alginate beads during storage at 4 °C in SM buffer was investigated for one month interval until 6 months. The phage titers were counted as described as above (4.3.4).

4.3.12 Statistical analysis.

The experiments were conducted in triplicate, and the results are presented as means ± SD. The resultes was evaluated by using one way analysis of variance (ANOVA) followed by Tukeys multiple comparison statiscal tests by using SPSS 26.0 for Windows (SPSS Inc., Chicago, USA). Significant differences were assessed if $p \leq 0.05$ (95% confidence interval).

4.4 Result and Discussion

4.4.1 Entrapment efficiency (EE)

Salmonella phages were entrapped in Ca-alginate beads containing 0%, 0.15%, and 0.3% (w/v) cassava starch (CS). The entrapment efficiency (EE) of each

treatment was evaluated **(Table 4.1)**. There were no significant differences in EE among treatments, approximately 97.67 to 98.00%, respectively. These results indicated that the addition of CS to Ca-alginate beads did not significantly affect their ability to entrap salmonella phages. This EE was higher than the alginate/carrageenan microcapsules for phage encapsulation, with an efficiency of about 95% (Zhou et al., 2022).

Table 4.1 Entrapment efficiency for bacteriophage *vB_salP-p*YM in Ca-alginate beads.

Sodium alginate (w/v) (%)	Cassava starch (w/v) (%)	Entrapment efficiency
3	0	97.67 ± 0.58^{ns}
3	0.15	98.12 ± 0.19^{ns}
3	0.3	98.00 ± 1.00^{ns}

Data are means ± standard deviation of three replications. ns means each treatment is not significantly different at $p< 0.05$.

4.4.2 Ca-alginate beads size and morphological structure of Ca-alginate beads via scanning electron microscopy (SEM)

The diameters of the extruded beads containing 0% CS, 0.15% (w/v) CS, and 0.3% (w/v) CS were 2.9 ± 0.25 mm, 3.0 ± 0.55 mm, and 2.8 ± 0.8 mm, respectively (**Figure. 4.1**). All beads exhibited nearly perfect roundness, smooth surfaces, and a clear appearance. These results demonstrated that the addition of CS did not influence the size or shape of the beads.

On the other hand, Vazquez et al. (2015) reported that the mixture of alginate and cassava starch significantly increases in viscosity, resulting in a wide diameter and abnormal shape, compared to only alginate. When increasing the biopolymer in gel beads of alginate leads to obtaining the extended size of beads (Biswas and Sahoo, 2016)

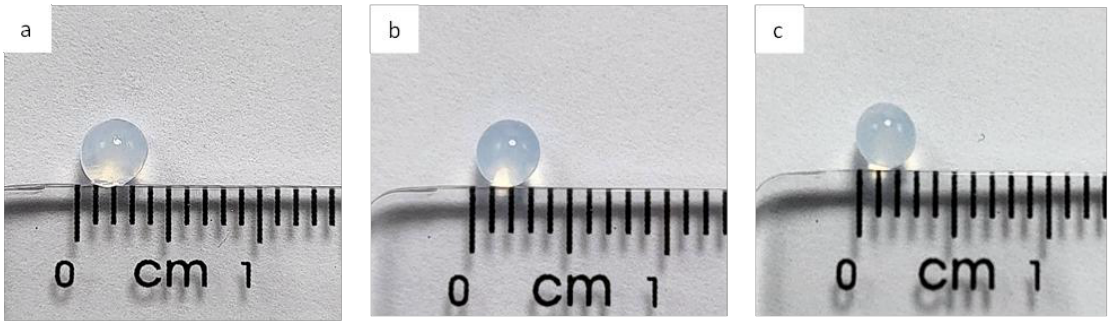

Figure 4.1 Illustration of Ca-alginate beads. A: 0% CS, B: 0.15% w/v CS and C: 0.3% w/v CS.

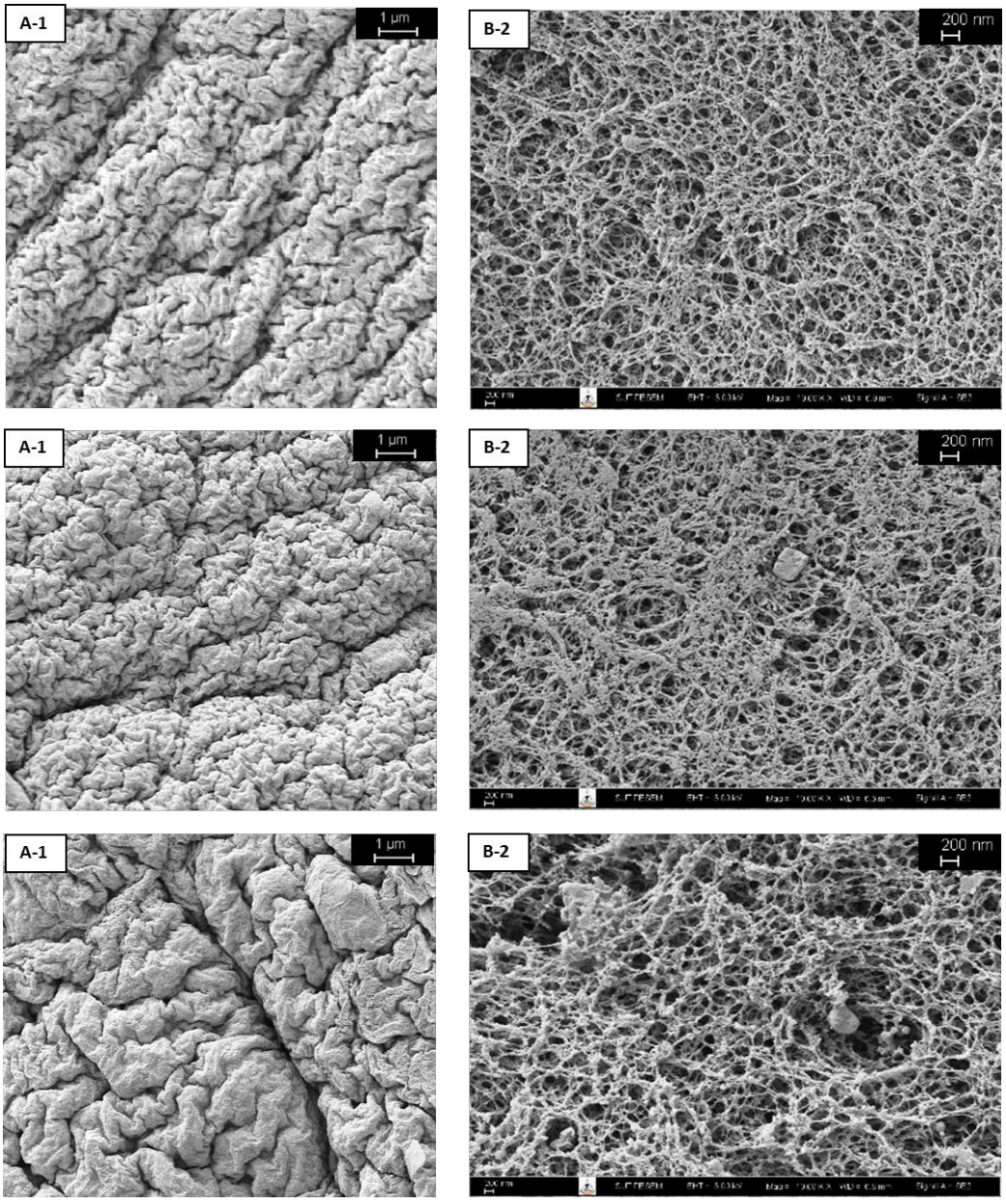

Figure 4.2 The SEM image surface and internal structure of Ca-alginate beads. Images A is surface structure at 5000x magnification and images B is internal structure at 10000x magnification. 1: 0% w/v CS, 2: 0.15% w/v CS and 3: 0.3% w/v CS.

Scanning electron microscopy (SEM) revealed detailed visualizations of both the surface and internal structures of the beads (**Figure 4.2**). The surface of all treatments exhibited a wrinkled, rough, and cracked morphology. The pore size of the internal structure of the Ca-alginate beads was measured by using ImageJ software, with 10 replicates for each treatment (**Table 4.2**). The average pore sizes of Ca-alginate beads added with CS 0.15% and 0.3% were 225.50 and 330.10 μm, respectively, compared to only Ca-alginate beads, about 235.90 μm, which means that the incorporation of a higher concentration of CS led to an increase in pore size. The presence of amylose and amylopectin in cassava starch may contribute to increased viscosity in the alginate solution. When cassava starch is incorporated into alginate, it can fill the 'egg-box' structure and stretch the alginate polymer chains (Belia, Fransiska and Suprianto, 2024). These results reveal that the wide-pore internal structure of CS added Ca-alginate beads was observed (**Figure 21; B2**).

Similarly, Tiamwong, Yukhajon, Noisong, Subsadsana and Sansuk, (2023) found that incorporating cassava starch into Ca-alginate led to increased pore size within the internal structure of the beads. The cassava starch insertion into the molecular arrangement of alginate during cross-linking with calcium ions resulted in the formation of wide spaces, which were observed under SEM.

Table 4.2 Surface morphology and pore size analysis of Ca-alginate beads.

Treatment (T)	Min	Max	Mean diameter(μm)
0% CS	135.24	375.47	235.90 ± 0.85 ^{b}
0.15% CS	141.84	357.14	225.50 ± 0.65 ^{a}
0.3% CS	131.68	726.34	330.10 ± 0.54 ^{c}

CS = cassava starch. Data are means diameter ± standard deviation of ten replications; different letter mean significantly different ($p \leq 0.05$).

4.4.3 Lytic assay of entrapped salmonella phage.

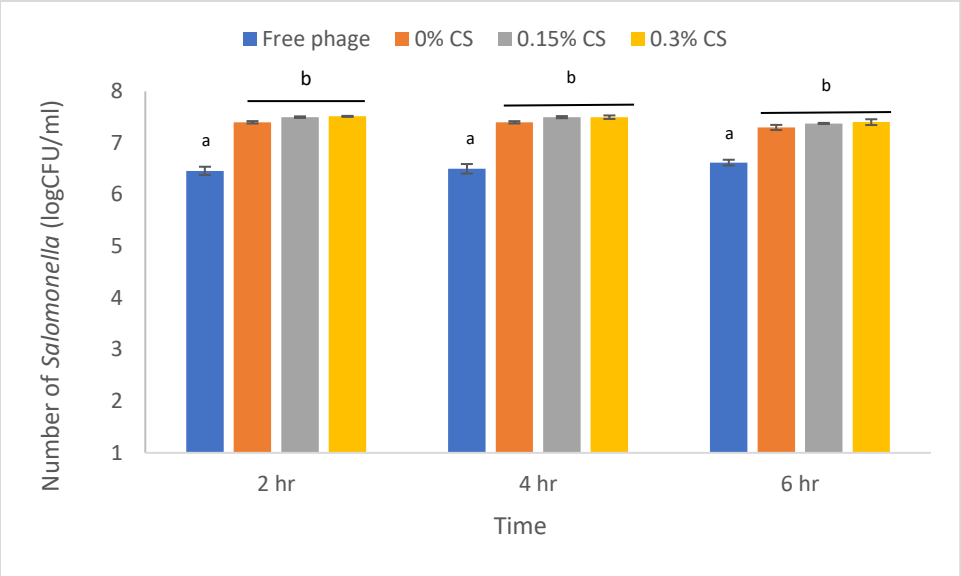

Figure 4.3 Lytic of entrapped salmonella phage on number of *Salmonella* Typhimurium; The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at the same time ($p \leq 0.05$).

All treatments were placed in SM buffer (pH 7.4) and *Salmonella* Typhimurium cells were added at a concentration of 10^7 PFU/ml. The samples were then incubated at 41 °C for 6 hours to evaluate the lytic activity of the free phage and entrapped salmonella phages (**Figure 4.3**). After 2 hours, the number of *Salmonella* incubated with free phages decreased rapidly compared to those incubated with Ca-alginate beads. *Salmonella* incubated with Ca-alginate beads remained close to the initial cell count after 6 hours of incubation, as the phages were not released from the Ca-alginate beads. The results showed that the number of *Salmonella* was significantly different from that of *Salmonella* incubated with free phages at 2, 4, and 6 hours. The number of *Salmonella* did not differ significantly between treatments with Ca-alginate beads for 6 hours. These results indicated that Ca-alginate, with or without CS, can entrap salmonella phages, which is consistent

with our previous evaluations of entrapment efficiency. Because the ionic gelation of sodium alginate with $CaCl_2$ forms a cross-linked network through ionic bonds effective in encapsulating salmonella phages. When CS is added to Ca-alginate beads. The hydroxyl groups (-OH) on starch molecules can form hydrogen bonds with the carboxyl groups (-COOH) on alginate chains. These interactions create a network of cross-linked polymer chains, increasing the overall strength and stability of the material (Merakchi, Bettayeb, Drouiche, Adour and Lounici, 2019). The homogeneous distribution of starch molecules within the gaps of the alginate matrix results in improved encapsulation efficiency (Vazquez et al., 2015). In contrast, Zhou et al. (2022) encapsulated phages in alginate (ALG)/**K**-carrageenan (CG) beads and evaluated their lytic activity under simulated intestinal conditions. In that study, both free and encapsulated phages demonstrated efficacy in reducing *S.* Typhimurium LT2 populations.

4.4.4 Stability of salmonella bacteriophage under different temperatures.

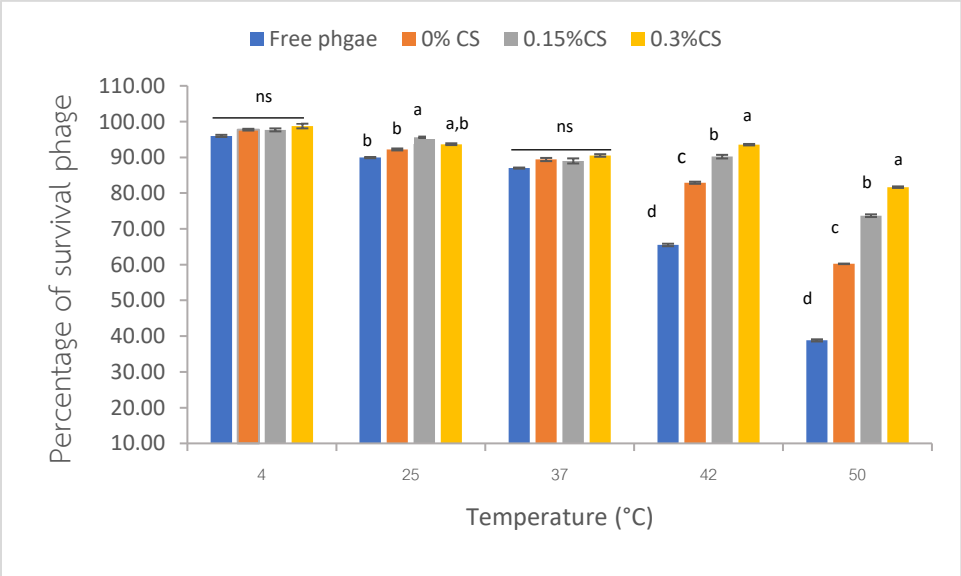

Figure 4.4 The survival of salmonella bacteriophage in the modified bead at various temperatures. The results corresponded to the mean ± standard deviation (n=3). ns means not significantly different, different letter mean significantly different among the same temperature ($p \leq 0.05$).

The Ca-alginate beads, modified with or without chitosan (CS), were evaluated under various temperatures for 24 hours (**Figure 4.4**). The salmonella phages entrapped in Ca-alginate beads were not significantly different at 4 and 37 °C. At 25 °C, the survival numbers of phages in Ca-alginate beads containing 0.15% and 0.3% CS were higher than those without CS and free phages. At 42 and 50 °C, the titers of surviving salmonella phages were significantly different among treatments, particularly the addition of CS in Ca-alginate beads at both concentrations. The number of free phages decreased when the temperature increased due to thermal damage by approximately 65.50% and 38.81%, respectively. These results suggest that incorporating CS into Ca-alginate beads improves the thermal stability of salmonella phages. Because when the starch was autoclaved, the amylopectin from the starch interacted with the carboxyl groups of the alginates and acted as a filler in the egg box. This interaction enhances the crosslinking between starch and alginate, thereby increasing gel stability and reducing syneresis (Belia et al., 2024). Consistent with Abdelsattar et al. (2019), the *E. coli* ZSEC5 bacteriophage encapsulated in chitosan/alginate beads exhibited a 0.8 log reduction in PFU/mL after exposure to 80 °C. These findings suggest that incorporating starch into the matrix enhances phage stability.

4.4.5 Stability of salmonella bacteriophage under various pHs.

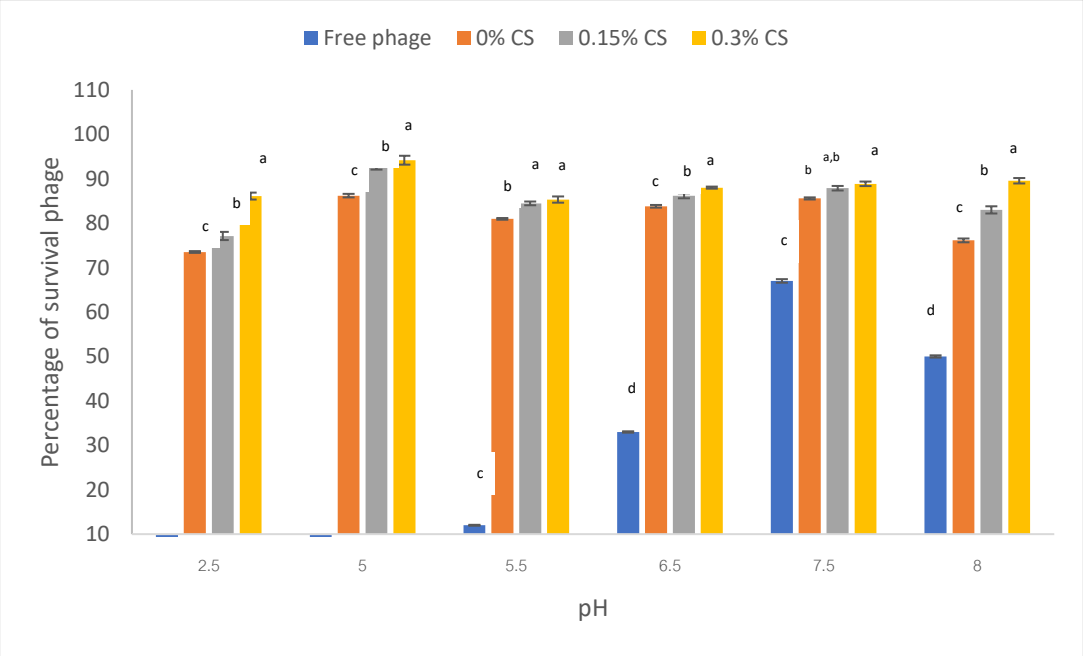

Figure 4.5 The stability of salmonella bacteriophage in a Ca-alginate bead under various pHs. The results corresponded to the mean ± standard deviation (n=3). Different letters mean significantly different at the same pHs ($p \leq 0.05$).

The stability of salmonella bacteriophage entrapped in Ca-alginate beads was evaluated at 37°C for 2 hours under various pH conditions 2.5 (gizzard), 5 (duodenum), 5.5 (crop), 6.5 (jejunum), and 8 (cecum). (**Figure 4.5**). At pH 2.5 and 5.0, free phages were not detected; the entrapped phages in Ca-alginate were examined, resulting in a higher percentage of surviving phages than free phages at all determined pH. It was notable that the addition of CS in Ca-alginate beads could increase the percentage of survival better than only Ca-alginate entrapment. These findings indicate that entrapment in Ca-alginate, particularly with 0.3% CS, enhances phage stability under a wide range of pH conditions. Mixing sodium alginate with cassava starch in hydrogels can improve their performance, especially at low pH, due to reduced shrinkage of the hydrogel matrix. The hydroxyl groups in starch interact with the carboxyl groups in SA through hydrogen bonding, creating a more complex and robust biopolymeric network and cassava starch acts as a structural component, preventing the hydrogel from shrinking excessively. Especially the acidic condition of the chicken's digestive system, where SA can be more susceptible to degradation (Khlibsuwan, Tansena and Pongjanyakul, 2018; Fang et al., 2022). Ma et al. (2008) reported that the survival of bacteriophage in chitosan/alginate microcapsules was enhanced for phage stability under acidic conditions. As cassava starch was integrated into the egg-box structure. Hydrogen bonding and other interactions between hydroxyl groups in starch and carboxyl groups in alginate resulted in a denser and more stable matrix (Rohman et al., 2021; Abdelsattar et al., 2019). This modified matrix improved the protective capability of Ca-alginate beads for salmonella phages.

4.4.6 Effect of bile salt on the stability of entrapped salmonella phage

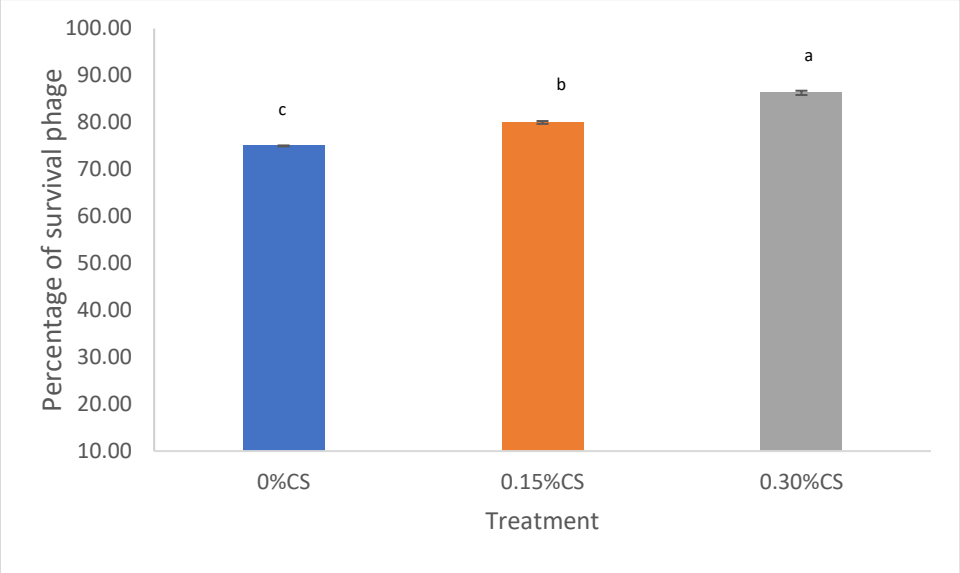

Figure 4.6 The number of phages remaining in Ca-alginate beads were incubated in bile salt solution (pH 6.8) at 41°C for 1 hour. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different at the same condition.

The stability of salmonella phages entrapped in Ca-alginate beads exposed to a 0.01 M bile salt was evaluated (**Figure 4.6**). The results indicated that the survival number of phages in Ca-alginate beads without CS was 75%, while the beads containing 0.15% and 0.3% (w/v) CS were 77.92% and 90.91%, respectively. The percentage of survival phages was increased because of the addition of CS in Ca-alginate beads. The entrapment of materials within a starch–alginate matrix improves mechanical strength and enhances crosslinking between sodium alginate and calcium ions (Ca^{2+}), forming complexes with hydroxyl groups (-OH) and oxygen atoms in the glycosidic bonds of amylose and amylopectin. These interactions contribute to increased gel strength and the formation of a more stable polymer network (Onyido, Ato and Nnamonu, 2012; Bagnolo, Almeida, Silva and Sato, 2023). Batalha et al. (2020) reported that when free phage UFV-AREG1 was exposed to 2% bile salt, survival decreased by 0.4 log, while the phage was encapsulated within alginate/carrageenan beads, the number of survival phages was equal to the initial number (10^9 PFU/g).

4.4.7 Stability of the phage in Ca-alginate beads under simulated gastric fluid (SGF).

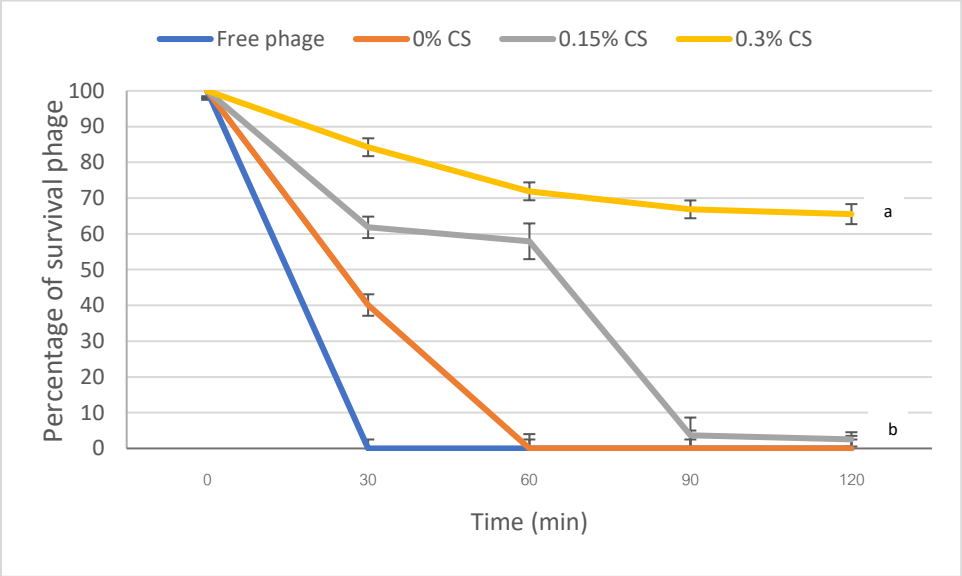

Figure 4.7 The survival of salmonella phage in the modified beads under SGF. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different at the same condition.

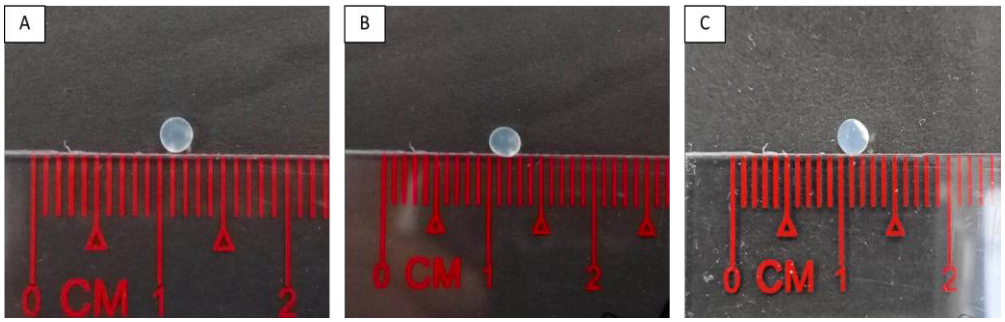

Figure 4.8 Morphology of Ca-alginate beads after in vitro incubation in simulated gastric fluid (SGF) for 120 min. A is Ca-alginate beads without CS, B is Ca-alginate beads wit 0.15% w/v CS and C is Ca-alginate beads wit 0.3% w/v CS.

The stability of entrapped phages was evaluated after incubation in SGF (3 mg/mL pepsin in 0.85% NaCl, pH 2.5) at 41°C for 2 hours **(Figure 4.7)**. The morphology of the Ca-alginate beads after incubation was shown in **Figure 4.8**. The free phages and phages entrapped in Ca-alginate beads with or without cassava starch (CS) were determined. The percentage of survival phages declined rapidly, becoming

undetectable within 30 minutes. Similarly, Ca-alginate beads without CS entrapped phages, which quickly lost viability and were inactivated within 60 minutes. In contrast, phage stability was better maintained in beads containing 0.15% CS than in free phages or 0% CS beads. However, the phages in these beads showed a considerable loss in viability over time, with their numbers decreasing to practically undetectable levels by 90 minutes. The beads with 0.3% CS provided the best level of protection, retaining a significant proportion of live phages even after 120 minutes. These findings indicated that 0.3% w/v of CS addition could enhanced phage stability under acidic conditions. Mixing sodium alginate with cassava starch in beads improves their stability and reduces degradation in simulated gastric fluid because the starch's hydroxyl groups form hydrogen bonds with the carboxyl groups of alginates, creating a denser, more complex network that resists degradation. Additionally, the starch acts as a structural component, preventing over-shrinkage of the alginate, which can improve the bead's ability to maintain its structure and function in a harsh environment (Fransiska, Belia, Nofika, Yohana and Ramayanti, 2025; Fang et al., 2022).

Bacteriophage Felix O1 encapsulated within chitosan-coated alginate microspheres exhibited significant protection when incubated in simulated gastric fluid (SGF, pH 2.4) containing pepsin for 90 minutes (Ma et al., 2008). The encapsulated phages showed only a 2.58 PFU/ml reduction, while no viable free phages were found after 5 minutes of exposure. Ca-alginate beads shrank towards the end of the evaluation period. Under acidic SGF conditions, the carboxylate groups ($-COO^{-}$) in the alginate structure were protonated to carboxylic groups (-COOH), reducing the adhesion between polymer chains linked by calcium ions (Ca^{2+}) and resulting in bead shrinkage (Lee and Mooney, 2012). Cassava starch's hydroxyl groups can create hydrogen bonds with alginate chains, which serve to bridge the gaps in the egg-box structure. As a result, the density and stability of the cross-links are increased (Lin et al., 2021), increasing phage protection under SGF conditions.

4.4.8 Evaluation of phage release from Ca-alginate beads in simulated intestinal fluid (SIF).

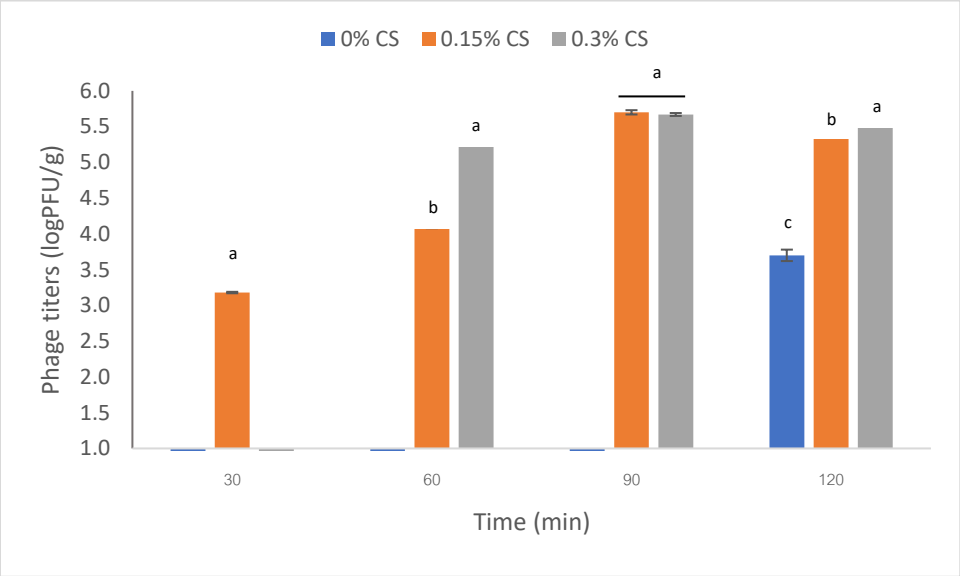

Figure 4.9 Phage stability released in simulated intestinal fluid at pH 6.8. The results corresponded to the mean ± standard deviation (n=3), different letters indicated significant differences at the same time ($p \leq 0.05$).

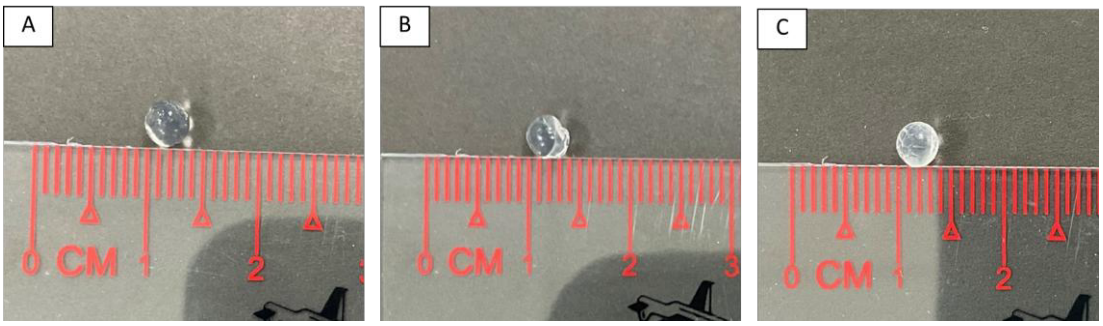

Figure 4.10 Morphology of Ca-alginate beads after in vitro incubation in simulated intestinal fluid (SIF) for 120 min. A is Ca-alginate beads without CS, B is Ca-alginate beads wit 0.15% w/v CS and C is Ca-alginate beads wit 0.3% w/v CS.

Ca-alginate beads, with and without cassava starch (CS), were incubated for 120 minutes in simulated intestinal fluid (SIF) (1 mg/mL pancreatin in 0.85% NaCl, pH 6.8) at 41°C (**Figure 4.9**) and the morphology of Ca-alginate beads was illustrated (**Figure 4.10**). After 30 minutes, only Ca-alginate beads containing 0.15% (w/v) CS released phages, with a concentration of 3.2 log PFU/g. At 60 minutes,

no phage release was observed from Ca-alginate beads without CS, whereas beads containing 0.15% and 0.3% (w/v) CS released 4.1 and 5.7 log PFU/g, respectively. The phage release was not detected in beads without CS, while beads with 0.15% and 0.3% CS could be determined to have approximately 5.7 log PFU/g after 90 minutes. After 120 minutes, phage release from beads without CS reached 3.7 log PFU/g for beads containing 0.15% and 0.3% CS, 5.7 and 6.1 log PFU/g were released, respectively. These results indicated that incorporating cassava starch into Ca-alginate beads could accelerate within 30 minutes in SIF compared to beads without starch. Furthermore, a CS concentration of 0.3% (w/v) led to greater phage release than 0.15% (w/v), suggesting a concentration-dependent effect. Cassava starch contains amylose and amylopectin (Wang et al., 2022), which fill the porous structure of the alginate gel when incorporated. Its highly hydrophilic nature enables cassava starch to promote swelling of the alginate-starch composite in aqueous environments such as SIF. This swelling enlarges the pore size of the hydrogel matrix and facilitates the diffusion of entrapped substances (Das et al., 2024; Poojari, Kulkarni and Wairkar, 2024). In SIF, amylase degrades the starch component enzymatically, disrupting the gel's structural integrity and accelerating the release of entrapped phages (Chen, Song, Huang and Guan, 2021). These combined mechanisms enhance the phage release profile observed in cassava starch–alginate composites under simulated intestinal conditions. Kim et al., (2015) reported that *Escherichia coli* O157:H7 bacteriophages were encapsulated in chitosan/alginate microspheres and evaluated their release in SIF. The results showed that chitosan/alginate microspheres accelerated phage release faster than pure alginate.

4.4.9 Stability of phages in the Ca-alginate bead during storage at 4 °C.

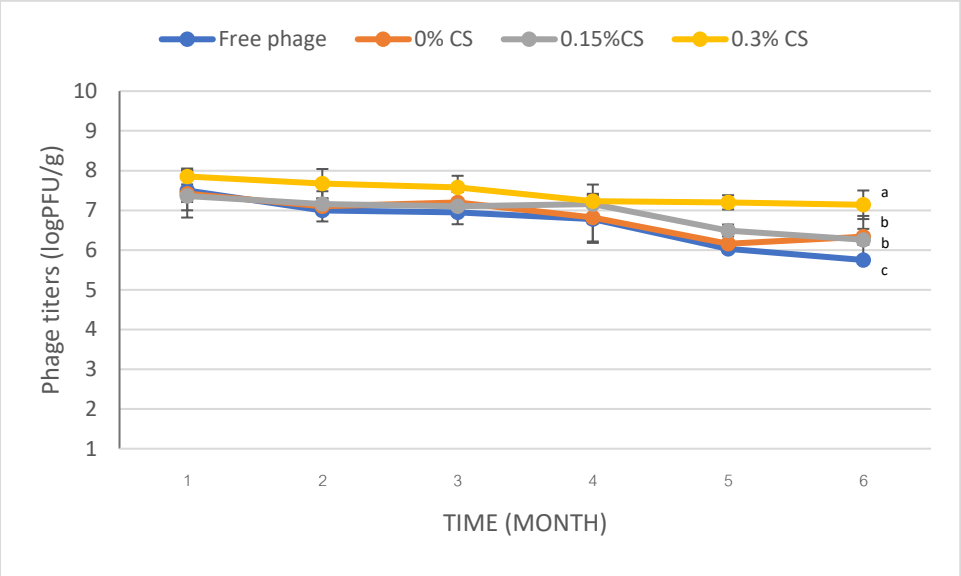

Figure 4.11 The survival of phages *vB_salP-p*YM in the Ca-alginate beads during storage at 4 °C for 6 months. The results corresponded to the mean ± standard deviation (n=3), different letters indicated significant differences at the same time ($p \leq 0.05$).

This study evaluated the survival of phages entrapped in Ca-alginate beads, with and without cassava starch (CS), during six months of storage at 4°C (**Figure 4.11**). After six months, the titer of free phages decreased by 2.25 log PFU/mL from an initial concentration of 8 log PFU/mL. In contrast, phages entrapped in Ca-alginate beads without CS exhibited a smaller reduction of 1.67 log PFU/g from the same initial concentration. The reduction in phage titer for beads supplemented with 0.15% and 0.3% (w/v) CS was 1.74 and 0.86 log PFU/g, respectively. Among all treatments, Ca-alginate beads with 0.3% CS demonstrated the highest phage stability over the six-month period. These results suggest that the incorporation of cassava starch improves phage stability during long-term cold storage.

Cassava starch functions as a filler in the alginate matrix, lowering permeability to tiny molecules while increasing network density. Furthermore, cassava starch has cryoprotective qualities, which serve to reduce phage damage caused by ice crystal formation during low-temperature storage (Anal and Singh, 2007; Ma et al.,

2008; Gogineni, Jain and Arora, 2020). Our findings are similar to those of Colom et al. (2015), who encapsulated bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 in alginate/liposome films and preserved them at 4°C for three months. The encapsulated phages were highly stable, with no appreciable drop in titer from the initial level (10^{11} PFU/mL).

4.5 Conclusions

This study demonstrated that phage *vB_salP-p*YM entrapped in calcium-alginate beads, with and without CS, possessed high entrapment efficiency. CS addition to calcium-alginate beads enhanced survival of the bacteriophages against simulated gastric fluid (SGF), heat, low pH and bile salt. Moreover, CS-loaded beads enhanced phage release efficiency in simulated intestinal fluid (SIF) and enhanced phage viability after prolonged storage at low temperature. These findings showed that CS-loaded calcium-alginate beads represent a good way of protecting phages from degradation when administered orally, thereby moving forward their potential for application in phage therapy.

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