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Original Article

The efficiency of polysaccharide microencapsulation in improving survival of probiotic bacteria

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Abstract

The objective of this research was to find polysaccharides for encapsulating *B. subtilis* to achieve the highest survival in low pH conditions. In the experiment, *Bacillus subtilis* were encapsulated in gelatin together with alginate and chitosan (GACh), carrageenan together with alginate and chitosan (CACh), or tapioca starch together with alginate and chitosan (SACh). The results showed that SACh encapsulation had the most effective *B. subtilis* release from gel beads. When testing for *B. subtilis* survival at pH 2, the SACh encapsulated *B. subtilis* had the highest percent survival. The survival of *B. subtilis* in SACh when stored at -18, 0, 4, 10, and 25 ºC was evaluated and the results show that 4 ºC is the most suitable storage temperature for the gel beads. In a test of encapsulated *B. subtilis* stored at 4 ºC, *B. subtilis* in SACh had the highest survival rate.

Keywords: bacteria, microencapsulation, polysaccharides, probiotic, survival

1. Introduction

Currently probiotics play important roles and are widely used especially in the animal feed industry, where they are used instead of antibiotics. They help accelerate growth, prevent diseases, and balance microbes in the digestive system (Adeoye *et al.*, 2016; Asaithambi, KumarSingh, & Singha, 2021; Silva, Rossoni, Junqueira, & Jorge, 2016). The probiotic pathway through the GI tract has many physiological barriers. Low pH, bile salts, enzymes, and peristaltic motion can seriously affect their survival (Roobab *et al.*, 2020), and this has reduced the effects of probiotics consumed by animals to insufficient levels.

Bacillus subtilis is a bacterium that has a structure of spores with good heat resistance. It can also produce a large variety of secretory proteins, enzymes, antimicrobial compounds, and vitamins (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017). Also, the use of *Bacillus* spp. As probiotic affects growth, survival rate, and the amount of *Vibrio* in white shrimp (Zokaeifar *et al.*, 2012). To improve the potential of probiotics in the digestive system, they are coated or attached to the inside of capsule material (Martin, Lara-Villoslada, Ruiz, & Morales, 2013). Alginate is widely used in encapsulation and it can increase the probiotic bacteria survival rate to 88-98% (Sathyabama, Ranjith kumar, Bruntha devi, Vijayabharathi, & Brindha priyadharisini, 2014). Gelatin has an excellent membrane-forming ability, biocompatibility, and is non-toxic (Hanani, Roos, & Kerry, 2014). A previous study has indicated that co-encapsulation of alginate together with gelatin can increase the bacterial survival rate. The alginate-gelatin microgels were the most effective at protecting *L. salivarious* Li01 under simulated gastrointestinal conditions when compared to the alginate microgels and non-encapsulated *L. salivarious* Li01 (Yao *et al.*, 2017). Carrageenan is also widely utilized due to its excellent physical functional properties in gelling, thickening, emulsifying, and stabilizing abilities (Li, Ni, Shao, & Mao, 2014). Tapioca starch has a high viscosity and a high content of amylopectin, so it makes the gel resistant to freezing (Seetapan, Limparyoon, Gamonpilas, Methacanon, & Fuongfuchat, 2015). Chitosan is an insoluble derivative of

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chitin but can dissolve in water at a pH below 6. It has antibacterial activity against pathogens (Verlee, Mincke, & Stevens, 2017).

2. Materials and Methods

This study was designed based on prior study results. In a previous study (Khimmakthong, Khumpouk, Saichanaphan, Intarasin, & Tirawanichakul, 2020), *B. subtilis* were encapsulated with alginate (A), alginate + gelatin (AG), alginate + chitosan (AC), and alginate + gelatin + chitosan (AGC). The survival of *B. subtilis* at high temperatures (60 and 90 °C) and low pH (pH 4 and 2) was evaluated. At high temperatures, the highest survival rate of *B. subtilis* was observed in AC. The average survival percentages of AC at 60 and 90 °C were 48.38 and 40.32%, respectively. At low pH, the highest survival rate of *B. subtilis* was observed in AC followed by AGC. The average survival rates in AC at pH 4 and 2 were 98.03% and 45.15%, respectively. Therefore, AC (in this study labeled ACh) was selected for use in this study, where it was combined with various polysaccharides (gelatin, carrageenan, or tapioca starch). The gel beads were tested in critical conditions (pH 2) that have shown low bacterial survival in the previous study. After that, the gel beads were stored at various temperatures, and the survival rates of *B. subtilis* were measured.

2.1 Materials

B. subtilis used in this experiment were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand (TISTR 2057). The other main materials included nutrient agar (NA) (Himedia, India), nutrient broth (NB) (Himedia, India), alginic acid sodium salt from brown algae (Sigma, Norway), gelatine (Merck, Switzerland), carrageenan (KC, Thailand), tapioca starch (RED CAT brand®, Thailand), chitosan (Aldrich, Iceland), $CaCl_2$ (Merck, Germany), NaCl (Merck, Denmark), and glycerol 99.5% (ANaPURE, Hong Kong).

2.2 Culture of *B. subtilis*

B. subtilis were cultured in Nutrient agar (NA) incubated at 37 °C for 24 h. After that, a single colony was added to Nutrient broth (NB) and then incubated in a 180 rpm shake incubator at 37 °C for 24 h. To separate the cells, the bacteria were centrifuged at 5,000 rpm for 15 m and then the culture media were removed. 10 mL of 0.85% NaCl (w/v) was added to wash the cells, and removed by centrifuging at 5,000 rpm for 5 m. After such washing for 3 times 0.85% NaCl (w/v) + 0.1% glycerol was added to adjust the O.D. at 550 nm to 1 (to cell concentration of 10^9 cells/mL).

2.3 Encapsulation of *B. subtilis*

2.3.1 *B. subtilis* encapsulation with gelatin together with sodium alginate (GA), carrageenan together with sodium alginate (CA), or tapioca starch together with sodium alginate (SA)

This experiment was done following the method of Mathews (2017). For GA, 10 mL of *B. subtilis* (cell concentration 10^9 cells/mL) were mixed together with 40 mL of sterile 2% (w/v) sodium alginate + 2% (w/v) gelatin, at pH 6.9. For CA, 10 mL of *B. subtilis* (10^9 cells/mL) were mixed together with 40 mL of sterile 2% sodium alginate (w/v) + 2% carrageenan (w/v), at pH 6.9. For SA, 10 mL of *B. subtilis* (10^9 cells/mL) were mixed together with 40 mL of sterile 2% sodium alginate (w/v) + 2% tapioca starch (w/v), at pH 6.9. Then, each of the polysaccharides-sodium alginate matrices were dropped into sterile 0.1 M $CaCl_2$ using an 18G syringe. The height of syringe tip above the $CaCl_2$ solution was 5 cm. The gel beads were soaked in 0.1 M $CaCl_2$ for 30 min to increase their strength. The gel beads were then separated by filtering through filter paper No. 4, and then washed 2 times with 0.85% NaCl (w/v).

2.3.2 Encapsulation of the gel beads (GA, CA, and SA) with chitosan (GACh, CACh, and SACh)

Chitosan coating starts with the preparation of a chitosan solution (92 percent degree of deacetylation). Weighed 0.4 g of chitosan was dissolved in 90 mL distilled water containing 0.4 mL of acetic acid. The final concentration of the chitosan solution was 0.4 percent (w/v). After that, the chitosan solution was adjusted to pH 5.7 with 1M NaOH solution. Then, the chitosan was sterilized by autoclaving. The gel beads (GA, CA, or SA) were soaked in the prepared chitosan solution, shaking at 100 rpm for 40 min. Then, the beads were filtered from the chitosan solution and washed with 0.1% peptone (w/v) solution containing 0.85% NaCl (w/v) (Chuprom, 2010). Finally, the gel bead size was measured by using a Vernier caliper.

2.4 Characteristics of the gel beads

The sizes of the gel beads (GACh, CACh, and SACh) were obtained by measuring with a Vernier caliper. The average size of the gel beads was estimated as the mean diameter of 20 gel beads. Morphology of the gel beads was observed by an optical microscope (Nikon Eclipse E200, Thailand).

2.5 The efficiency of releasing *B. subtilis* from the gel beads

A 5 gram sample of gel beads was placed into a 150 mL Erlenmeyer flask, 50 mL of 0.1 M KH_2PO_4 at pH 7.4 was added, and the mix was incubated on 100 rpm shaking machine at 37 °C. Supernatant samples were taken at various times and the released bacteria were counted by the drop plate method (modified from Haghshenas *et al.*, 2015). The release of *B. subtilis* at 1 hr was calculated to the encapsulation efficiency (EE) based on the formula below, according to Nami, Haghshenas, and Yari Khosroushahi (2017).

$$EE = (\log_{10} N / \log_{10} N_0) \times 100$$

where N is the number of viable bacteria (CFU) entrapped by biopolymers, and N_0 is the number of free viable bacteria before encapsulation.

2.6 Evaluation of encapsulation effects on the survival rate of *B. subtilis*

2.6.1 The survival of encapsulated *B. subtilis* in low pH

A 1 gram sample of gel beads was placed in a 15 mL test tube, 9 mL of PBS solutions at pH 2 or 7.4 was added, and then the mix was incubated in a water bath at 37 °C for 3 hours. It was then spun at 1,500 rpm for 15 minutes to make the gel beads sediment. PBS solution was poured out, 9 mL of 0.85% NaCl was added to wash the gel, and washing was repeated 2 times. After that, the gel was broken in 9 ml of 0.1 M KH_2PO_4, pH 7.4, by using the vortex machine at maximum speed for 10 minutes. The number of colonies was counted by the drop plate method. The survival rate of *B. subtilis* was calculated as follows.

$$\text{Survival (\%)} = \frac{\text{Number of } \textit{B. subtilis} \text{ after incubation at pH 2 (CFU/ml)}}{\text{Number of } \textit{B. subtilis} \text{ after incubation at pH 7.4 (CFU/ml)}} \times 100$$

2.6.2 The survival of encapsulated *B. subtilis* in various storage conditions

A 1 gram sample of gel beads was placed in a 15 mL test tube. The gel was stored at -18, 4, 10, or 25 °C for 0, 10, 15, and 20 days. Then, 9 mL of 0.1 M KH_2PO_4, at pH 7.4 was added, and the gel was broken by using the vortex machine at maximum speed for 10 minutes. The number of colonies was counted by the drop plate method. The survival rate of *B. subtilis* was calculated as follows.

$$\text{Survival (\%)} = \frac{\text{Number of } \textit{B. subtilis} \text{ at various days after storage (CFU/ml)}}{\text{Number of } \textit{B. subtilis} \text{ at day 0 (CFU/ml)}} \times 100$$

2.7 Statistical data analysis

The efficiency of releasing *B. subtilis* from the gel beads and the encapsulation effects on the survival rate of *B. subtilis* are shown in the form of Mean ± SD and were analyzed by One-way ANOVA using Scheffe Multiple Comparison Test at a 95% confidence level ($P < 0.05$).

3. Results and Discussion

3.1 Characteristics of the gel beads

The size of the gel beads produced by the extrusion method was between 0.25 - 3 mm in diameter. It depends on many factors, including the type of bacteria, needle size, sodium alginate solution concentration, and distance between syringe and calcium chloride solution (Frakolaki, Giannou, Topakas, & Tzia, 2021; Zazzali, Calvo, Ruíz-Henestrosa, Santagapita, & Perullini, 2019). In this study, the results showed that all three types of beads (GACh, CACh, and SACh) had the same external characteristics, being round, smooth, and glossy. They were similar also in size, which was 2.27 ± 0.15, 2.33 ± 0.17, and 2.05 ± 0.12 mm, in the same order. GACh, CACh, and SACh capsules containing *B. subtilis* were observed under an optical microscope. The surface of the gel beads under a microscope was creased as waves passing through the gel beads, as shown in Figure 1A. Under a high magnification, *B. subtilis* cells that were encapsulated within the gel beads could be seen, as shown (at arrow points) in Figure 1B. This is consistent with Chuprom's experiment (2010) of encapsulating 2 species of *Lactobacillus* by extrusion method using 2% sodium alginate and sodium alginate together with chitosan (AC) with needle size 18 G, giving gel capsule size of 2.40 ± 0.050 mm. The large sized gel beads are less sensitive to harmful conditions than smaller size gel beads, and this makes them better able to protect the bacteria inside (Shi *et al.*, 2013). Their disadvantage is breaking or leaking easily (Totosaus, Ariza-Ortega, & Pérez-Chabela, 2013). The beads obtained in this experiment had size of about 2 mm, and could effectively protect the encapsulated bacterial cells in animal feed.

3.2 The efficiency of releasing *B. subtilis* from the gel beads

The release of *B. subtilis* from all gel beads types (GACh, CACh, and SACh) was significantly decreased when compared with the initial bacteria before encapsulation ($P <$

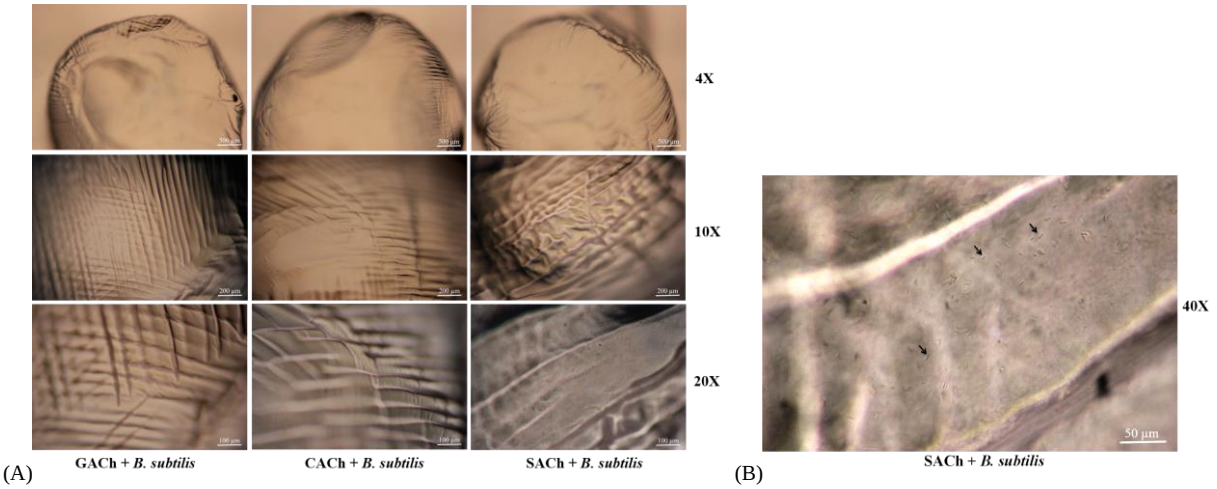

Figure 1. (A) The external surfaces of GACh + *B. subtilis*, CACh + *B. subtilis*, and SACh + *B. subtilis* under an optical microscope at various magnifications. (B) *B. subtilis* cells on the surface of SACh

0.05), as shown in Table 1. When comparing the differences between the three types, SACh was the most efficient in releasing *B. subtilis* from the gel beads ($1.50 \pm 0.57 \times 10^4$ CFU/mL) and significantly different from GACh and CACh ($P < 0.05$). Encapsulation efficiency (EE) of all gel types was very low. The highest EE of 58.70 was for SACh, and the lowest 45.04 was for CACh. This level of EE indicates that the number of bacteria released from the gel beads after encapsulation was low. Therefore, it is necessary to prepare "extra" starting bacteria to have the number of eventually released bacteria on sufficient level.

EE is an important parameter for determining the efficiency of the encapsulation process and of the selected encapsulating agent (Çabuk & Harsa, 2015). Seth, Mishra, and Deka (2017) studied the effects of sodium alginate concentration (1%, 1.5%, 2%, 2.5% and 3% w/v), calcium chloride concentration (0.1, 0.2, 1M), and hardening time (15, 30, 45 and 60 min) on the capsule size and EE. The results showed that sodium alginate concentration increased the microcapsule size and EE, whereas calcium chloride concentration and hardening time had no significant effect. Matulyte, Kasparaviciene, and Bernatoniene (2020) reported that the concentration of sodium alginate had a larger effect on the EE when using 2% $CaCl_2$ compared with the effect on EE when 5% $CaCl_2$ was used. Moreover, Petraitytė and Šipailienė (2019) studied the effects of various cross-linking agents, namely calcium chloride, calcium lactate, and strontium chloride, on EE of *L. plantarum* encapsulation. Extremely low EE of ($52.5 \pm 2.9\%$) was observed when the beads were formed by extrusion technique and calcium chloride was used as the cross-linking agent, while the highest EE (100%) was observed when calcium lactate was used as the cross-linking agent. In this current study, the encapsulation process was done by using 2% (w/v) sodium alginate together with 0.1M calcium chloride as the cross-linking agent. The preparation of gel beads by using various concentrations of sodium alginate and together with another cross-linking agent might be able to increase the EE levels.

The release of *B. subtilis* from gel beads (SACh) at 0, 1, 2, 3, 4, 5, and 6 h was studied and is shown in Figure 2. The results show that *B. subtilis* was completely released from the gel beads at 1 hr. When the *B. subtilis* release in the following hours was determined, the amount remained stable until 6 hr. The average amount of *B. subtilis* released in all 6 time periods was $1.48 \pm 0.29 \times 10^4$ CFU/mL.

3.3 The survival of encapsulated *B. subtilis* in various pH

The percent survival of *B. subtilis* in SACh was significantly greater than in CACh and GACh ($P < 0.05$). The average survival rates were 142.85, 68.88, and 52.50 %, respectively. Surprisingly, the survival of *B. subtilis* in SACh at pH 2 was higher than the survival of *B. subtilis* in SACh at pH 7.4; resulting in the calculated survival rate greater than 100%, as shown in Figure 3. In a previous study, the beads without tapioca starch (only alginate + chitosan, ACh) at pH 2 showed a low survival rate of *B. subtilis* (45.15%) (Khimmakthong, Khumpouk, Saichanaphan, Intarasin, & Tirawanichakul, 2020). Tapioca starch has the ability to absorb water around it to transform into a gel and then bind the ingredients together. Moreover, it makes the gel stabilize in low-temperature storage (Seetapan *et al.*, 2013). In study of Etchepare *et al.* (2016) *L. acidophilus* was encapsulated in alginate and alginate + starch + chitosan. Then, the microbial survival was tested at pH 1.5 for 2 h. They found that *L. acidophilus* encapsulated in alginate + starch + chitosan gave the highest survival with the counts of 4.25 log CFU g^{-1}, follow by *L. acidophilus* encapsulated in alginate with the counts of 4.14 log CFU g^{-1} as compared to uncoated microparticles. These results indicate that tapioca starch is a good polysaccharide material. It is abundant, inexpensive, and protects the encapsulated bacteria well.

Table 1. The efficiency of *B. subtilis* release from the gel beads, and encapsulation efficiency of each gel type

Type of material	Released *B. subtilis* (CFU/mL)	Encapsulation efficiency (EE)
GACh	$5.80 \pm 1.54 \times 10^{3b}$	52.90
CACh	$1.60 \pm 0.70 \times 10^{3b}$	45.04
SACh	$1.50 \pm 0.57 \times 10^{4a}$	58.70

Note: Initial *B. subtilis* concentration (before encapsulated) is $1.30 \pm 0.48 \times 10^7$ CFU/mL. The letters a, b, and c indicate statistically significant differences ($P < 0.05$).

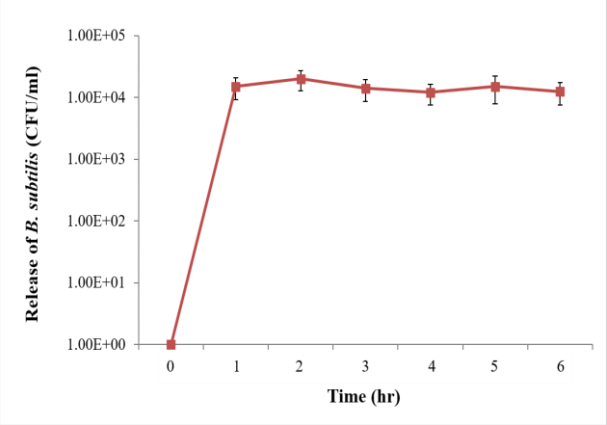

Figure 2. The release of *B. subtilis* from gel beads (SACh) at 0, 1, 2, 3, 4, 5, and 6 hr after shaking the gel in KH_2PO_4 solution

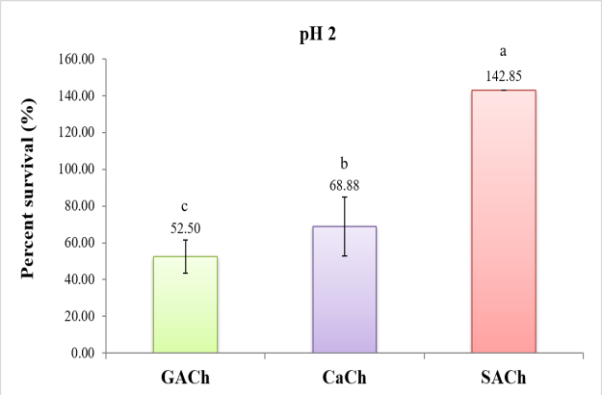

Figure 3. Survival rates of *B. subtilis* after incubation in pH 2 for 1 hr. The letters a, b, and c indicate statistically significant differences ($P < 0.05$).

3.4 The survival of encapsulated *B. subtilis* in various storage conditions

SACh gel beads were stored at temperatures of -18, 4, 10, and 25 ºC. After that, the survival of *B. subtilis* on various days of storage was calculated as percentages compared to the initial number on day 0. *B. subtilis* survival in the gels stored at -18 and 25 ºC decreased rapidly, reaching 0% on day 5. This indicates that the temperatures -18 and 25 ºC are inappropriate for the storage of these gel beads. At 4 and 10 ºC, the survival rate decreased slowly. Especially at 4 ºC, the survival of *B. subtilis* remained at 76.92 until day 10. On day 15, the results at 4 and 10 ºC were similar. That is, the survival rates were less than 50% with nearly no survival on day 20. The results are shown in Figure 4, showing that 4 ºC is the best condition for storing gel beads, matching the results of Zajani *et al.* (2012) and Mirzaei, Pourjafar, and Homayoni (2012). But, for storage shelf life the 10-15 days is still considered a short time. Further studies are needed to extend the storage time and fix the problem of too rapid expiration. For this problem, Zajani *et al.* (2012) suggested that keeping in a glass bottle will give longer shelf life than plastic bottles. Adding some substances to increase the shelf life is a good approach also. Mirzaei, Pourjafar, and Homayoni (2012) reported that sucrose addition as a cryoprotectant could keep the gel beads functional for up to 3 years.

GACh, CACh, and SACh gel beads were stored at a temperature of 4 ºC. After that, the survival of *B. subtilis* on various days after storage was calculated as percentage of the initial number on day 0. The results show that *B. subtilis* in SACh had the highest survival rate throughout the storage period, namely 100, 76.92, 76.92, 38.46, and 4.61% on days 0, 5, 10, 15, and 20, respectively. It was followed by CACh with survival rates 100, 55.56, 55.56, 7.78, and 0.00% on days 0, 5, 10, 15, and 20, respectively. The lowest survival rate was for GACh at 100, 10.60, 5.00, 0.00, and 0.00% on days 0, 5, 10, 15, and 20, respectively. The results show that Tapioca flour, which is the least expensive material choice, has a high efficiency in protecting the bacteria at a low pH, giving the longest storage life. The results are shown in Figure 5.

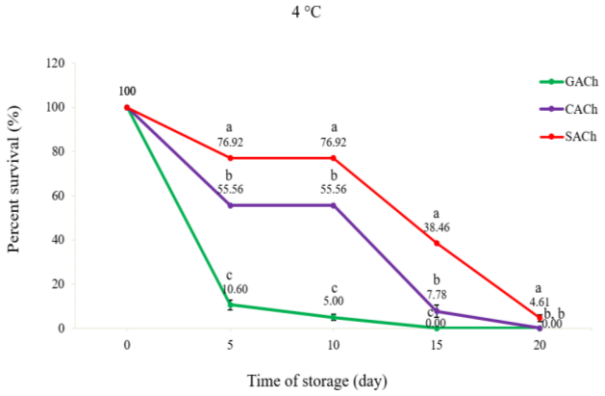

Figure 5. Survival rates of *B. subtilis* in GACh, CACh, and SACh gel beads at 4 ºC on various storage days. The letters a, b, and c indicate statistically significant differences ($P < 0.05$) within each storage day.

4. Conclusions

The use of SACh gave the highest survival rates of *B. subtilis* in both low pH (pH 2) and low temperature (4 °C), while the encapsulation efficiency (EE) and the shelf life in storage of the gel beads are still unsatisfactory. The maximum storage time was only 15 days. In a future study, experiments seeking to increase the EE and increase storage life of the gel beads will be performed. These encapsulation results could also be applied to other probiotic bacteria. Moreover, adding these beads to animal feed can balance the microflora in the digestive tract of the animals.

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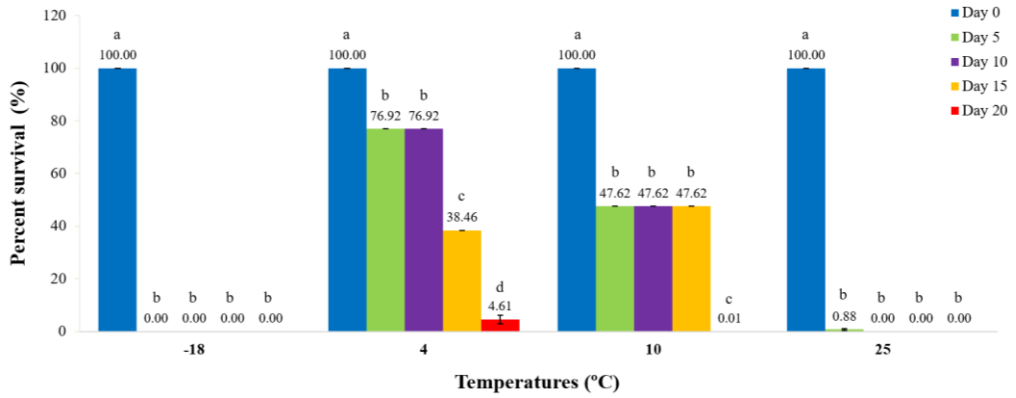

Figure 4. Survival rates of *B. subtilis* in SACh gel beads at the temperatures of -18, 4, 10, and 25 ºC on various storage days. The letters a, b, c, and d indicate statistically significant differences ($P < 0.05$) within each temperature.

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