

The Efficiency of Microencapsulation with Alginate, Gelatin, and Chitosan on the Survival of *Bacillus subtilis*

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ABSTRACT

In this research, the method that helps Bacillus subtilis to survive in high temperatures and low pH was examined. B. subtilis were encapsulated with alginate (A), alginate together with gelatin (AG), alginate together with chitosan (AC), and alginate together with gelatin and chitosan (AGC). Then the gel bead sizes and the release of bacteria from the gel beads were measured. The gel beads using 18G needle has the highest amount of B. subtilis release and was found that AC and AGC encapsulation had the highest efficiency of the release of B. subtilis. The survival of B. subtilis at high temperature (60 and 90 °C) and at low pH (pH 4 and 2) was evaluated. At high temperature, the percentage survival of B. subtilis encapsulated with AC increases when compared to non-encapsulated B. subtilis (BS) and AGC as statistically significant ($P < 0.05$). At low pH, the percentage survival of B. subtilis encapsulated with AC and AGC increased as statistically significant compared to BS ($P < 0.05$). The average percentage survival of AC at pH 4 and 2 are 98.03% and 45.15%, respectively. The average percentage survival of AGC at pH 4 and 2 are 90.90% and 51.02%, respectively.

Keywords: Alginate, *Bacillus subtilis*, Chitosan, Gelatin, Microencapsulation

INTRODUCTION

Probiotics are living bacteria. If the animals receive the right amount, they will benefit the health of the host cell. They will help balance the intestinal microflora appropriately, resulting in good growth of animals and prevention of pathogens replication (Kesarodi-Watson et al., 2008). Good probiotics must not be pathogenic microorganisms, no resistance gene and most importantly, have to withstand in high temperatures that may be encountered in industrial production processes, resistant to high acidity in the stomach and can tolerate in salt bile from the host's small intestine. There are many reports of probiotics used in animals, especially in aquatic animals (Park et al., 2016; Standen et al., 2016; Makled et al., 2017). *Bacillus* spp. is considered as one of the most evaluated bacteria in fish. These bacteria are common in nature, including soil, water, and air, capable of producing endospore for unfavorable environments. And be confident that it is a stable probiotic when used as an animal food additive (Hong et al., 2005). *B. subtilis* is a species that has been commonly reported as a potential probiotic with the ability to produce many types of antimicrobials. In addition, *B. subtilis* is generally considered to be safe in animals and humans (AlGburi et al., 2016).

The problem that occurs when using probiotics in animals is probiotics cell are often destroyed by gastric juices of animals with high acidity. In addition, bile salts that are secreted from the small intestine have the ability to destroy microorganisms, causing the number of probiotics that the animals have received reduced and not enough to function. Microencapsulation is a technology that has been developed and used in nutrition for aquaculture to increase the potential of using probiotics in the gastrointestinal tract. The probiotic particles are coated or trapped inside the capsule material ranging in size from less than one micron to hundreds of microns (Bansode et al., 2010). Alginate, gelatin, and chitosan are the materials that are used to coat probiotics cell and are reported to be effective in preventing bacterial probiotics from various critical conditions (Trabelsi et al., 2014; Gandomi et al., 2016; Wu and Zhang, 2018).

MATERIALS AND METHODS

Materials

B. subtilis were purchased from Thailand Institute of Scientific and Technological Research (TISTR), Thailand (TISTR 2057). The main materials used in the experiment are nutrient agar (NA) (Himedia, India), nutrient broth (NB) (Himedia, India), alginic acid sodium salt from brown algae (Sigma, Norway), gelatine (Merck, Switzerland), chitosan (Aldrich, Iceland), CaCl₂ (Merck, Germany), NaCl (Merck, Denmark), and glycerol 99.5% (ANAPURE, Hong Kong).

***B. subtilis* culture**

B. subtilis were cultured in NA at 37 °C for 16 hours. After that, a single colony bacterium was introduced into NB, incubated in a 180 rpm shaking machine at 37 °C for 16 hours. After that, the bacteria were spun at 5,000 rpm for 15 minutes, removed the media, and washed the cells 3 times by 10 mL of 0.85% NaCl (w/v). Dissolved the cells in 0.85% NaCl (w/v) + 0.1% glycerol and adjusted the cells concentration to 10^9 cells/mL (adjusted O.D. value at 550 nm to 1)

Encapsulation of *B. subtilis* with alginate, gelatin and chitosan

Encapsulation of *B. subtilis* with alginate (A). *B. subtilis* that adjusted to 10^9 cells/mL were encapsulated with sodium alginate in accordance with Pinpimai et al. (2015) method. 10 mL of *B. subtilis* cells are mixed together with 40 mL of aseptic 2% (w/v) sodium alginate, pH 6.9, then sodium alginate matrix were dropped into sterile 0.1 M CaCl_2 using 18G syringes, the height of the syringe tip and CaCl_2 solution equal to 5 cm, incubated the gel beads in 0.1 M CaCl_2 for 30 minutes to increase gel strength. Then separate the gel beads by filtered through filter paper No. 4, washed the gel beads 2 times with 0.85% NaCl (w/v).

Encapsulation of *B. subtilis* with alginate together with gelatin (AG). *B. subtilis* that adjusted to 10^9 cells/mL were encapsulated with sodium-alginate and gelatin in accordance with Mathews (2017) method. 10 mL of *B. subtilis* cells are mixed together with 40 mL of aseptic 2% (w/v) sodium alginate + 2% (w/v) gelatin, pH 6.9, then sodium-alginate matrix were dropped into sterile 0.1 M CaCl_2 using 18G syringes, the height of the syringe tip and CaCl_2 solution equal to 5 cm, incubated the gel beads in 0.1 M CaCl_2 for 30 minutes to increase gel strength. Then, separated the gel beads by filtered through filter paper No. 4, washed the gel beads 2 times with 0.85% NaCl (w/v).

Encapsulation of *B. subtilis* with alginate and chitosan (AC), alginate together with gelatin and chitosan (AGC). Chitosan coating begins with the preparation of chitosan solution according to Chuprom (2010) method. 0.4 g of chitosan (92% degree of deacetylation) was dissolved with 90 mL of distilled water contained 0.4 mL of acetic acid. The final concentration of chitosan is 0.4% (w/v). Then, the chitosan solution was adjusted to pH 5.7 with 1 M NaOH solution. After that, the gel beads (A) and (AG) were soaked into the prepared chitosan solution, shake 100 rpm for 40 minutes, become (AC) and (AGC), respectively. The gel beads were separated from the chitosan solution by filter paper No. 4, washed the gel beads with 0.1% peptone solution (w/v) contained 0.85% NaCl (w/v).

Characteristics of the gel beads. The size of the gel beads (A, AG, AC and AGC) was obtained by vernier caliper. The average size of the gel beads was

calculated from the mean diameter of 20 gel beads. Morphology of the gel bead (AC contained *B. subtilis* and AC not contained *B. subtilis*) was observed by optical microscope (Nikon eclipse E200, Thailand).

Encapsulation of *B. subtilis* in various sizes of the gel beads

Preparation of the gel beads AC by using 16G, 18G and 20G needles were done. Then, the release of *B. subtilis* from the gel beads and the amount of survival of *B. subtilis* from low pH was studied.

Encapsulation of *B. subtilis* with chitosan at various times

Preparation of the gel beads according to method of (AC) was done, but the gel beads were soaked into the prepared chitosan solution, shake 100 rpm for 30, 40, and 50 minutes. Then, the amount of survival of *B. subtilis* from low pH was studied.

The release of *B. subtilis* from the gel beads

The release of *B. subtilis* from various gel bead types was studied by put 1 g of each gel bead type (A, AG, AC, and AGC) into a test tube contained 9 mL of PBS, pH 7.4. Then, broke the gel beads by vortex in the maximum speed for 10 minutes. After that, centrifuged at 5,000 rpm for 15 minutes, removed the PBS solution, and added 2 mL of 0.85% NaCl (w/v). Each tube was made a serial ten-fold dilution from 10^{-1} to 10^{-6} . The numbers of release *B. subtilis* were counted by the drop plate method. The Encapsulation efficiency (EE) was calculated based on the formula below, according to Nami et al., 2017.

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

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

The release of *B. subtilis* from gel bead at various times was studied according to the method of Haghshenas et al., 2015. Briefly, 5 g of gel beads were incubated in 50 mL of 0.1 M KH_2PO_4 , pH 7.4, 100 rpm shaking machine at 37 °C. The samples were taken at different time intervals and the released bacteria were counted by the drop plate method.

Encapsulation efficiency testing for the protection of *B. subtilis*

The protection of *B. subtilis* from high temperature. One gram of gel beads were put into a test tube containing 2 mL of PBS, pH 7.4. Then, incubated in the water bath at 37, 60 and 90 °C for 3 hours. After that, added 7 mL of PBS, pH 7.4 to each test tube. The gel beads were broke by vortex in the maximum speed for 10 minutes. Then, centrifuged at 5,000 rpm for 15 minutes, removed the PBS solution, and added 2 mL of 0.85% NaCl (w/v). Each tube was made a

serial ten-fold dilution from 10^{-1} to 10^{-6} . The numbers of survival *B. subtilis* were counted by the drop plate method. The experiment was done in encapsulated *B. subtilis* compared with free *B. subtilis* (Not encapsulated). The survival rate of *B. subtilis* was calculated based on the formula below:

$$\text{Survival (\%)} = \frac{\text{Number of } B. \text{ subtilis that survived after incubation (CFU/ml)}}{\text{Number of } B. \text{ subtilis that survived after incubated at } 37^\circ\text{C (CFU/ml)}} \times 100$$

The protection of *B. subtilis* from low pH. One gram of gel beads were put into a test tube containing 2 mL of PBS, pH 2, 4 and 7.4. Then, incubated in the water bath at 37°C for 3 hours. After that, centrifuged at 1,500 rpm for 15 minutes to allow the gel to settle, remove the PBS solution. The gel beads were washed 2 times in 0.85% NaCl (w/v). After that, added 7 mL of PBS, pH 7.4 to each test tube. The gel beads were broke by vortex in the maximum speed for 10 minutes. Then, centrifuged at 5,000 rpm for 15 minutes, removed PBS solution, and added 2 mL of 0.85% NaCl (w/v). Each tube was made a serial ten-fold dilution from 10^{-1} to 10^{-6} . The numbers of survival *B. subtilis* were counted by the drop plate method. The experiment was done in encapsulated *B. subtilis* compared with free *B. subtilis* (Not encapsulated). The survival rate of *B. subtilis* was calculated based on the formula below:

$$\text{Survival (\%)} = \frac{\text{Number of } B. \text{ subtilis that survived after incubation (CFU/mL)}}{\text{Number of } B. \text{ subtilis that survived after incubated at pH 7.4 (CFU/mL)}} \times 100$$

Statistical analysis

Every experiment was done in triplicate. The results were shown in the form of Mean $\pm$ SD. Each experiment analyzes the results with one-way analysis of variance (One-way ANOVA) and uses complex comparisons with the Tukey's Multiple Comparison Test at 95% confidence level ($P < 0.05$).

RESULTS

Encapsulation of *B. subtilis* with alginate, gelatin, and chitosan

Characteristics of the gel beads. When encapsulated *B. subtilis* with sodium alginate (A), sodium alginate together with gelatin (AG), sodium alginate together with chitosan (AC), and sodium alginate together with gelatin and chitosan (AGC) using a 18G syringe, found that all forms of gel beads have the same external characteristics that are round and smooth and shiny surface as shown in Figure 1. The gel beads size is 2.31 ± 0.2 mm in diameter. In the figure, the food coloring is added in order to see the difference of each type but does not affect any experimental results.

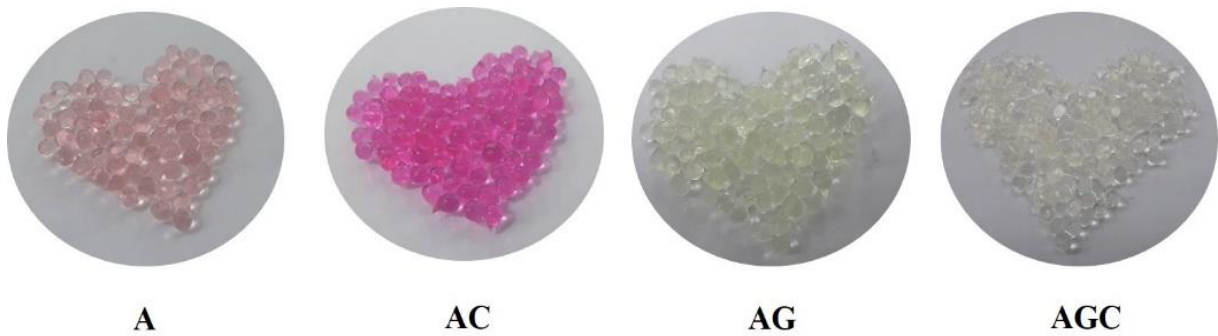
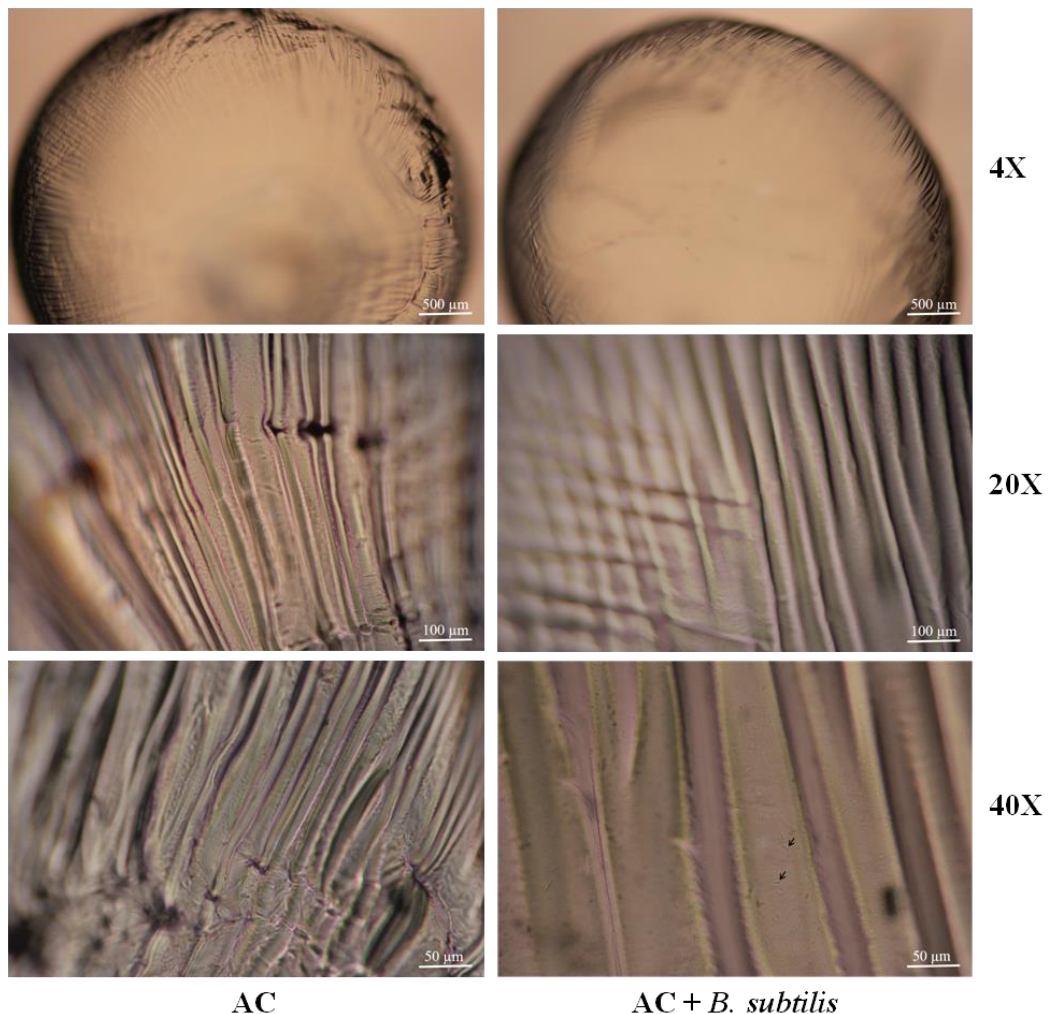


Figure 1. The appearance of various gel bead types. Each type of gel beads was prepared by using 18G needle. The food coloring was added to see the difference of each gel type.

AC contained *B. subtilis* and AC not contained *B. subtilis* were observed under optical microscope. The surface of the gel beads has a wave-like pattern throughout the gel. In gel beads containing *B. subtilis*, when observed at magnification of 20X or above, can see the cells of *B. subtilis* encapsulated in gel beads (the arrow points), as shown in the Figure 2.

(A)



(B)

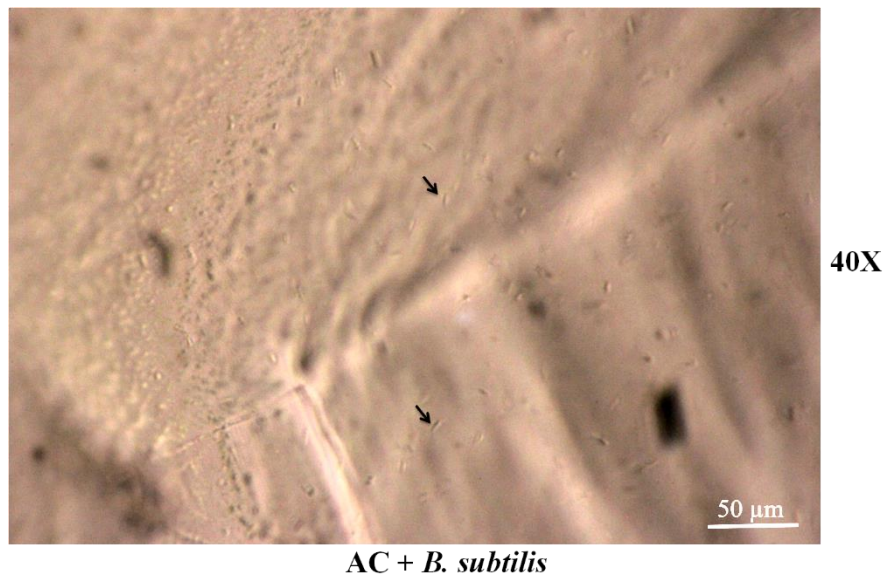


Figure 2. (A) The external surface of the gel beads under a optical microscope at various magnifications. (B) *B. subtilis* cells on the surface of the gel beads.

The release of *B. subtilis* from the gel beads. The amount of release of *B. subtilis* from the gel beads is reduced with statistical significance ($P < 0.05$) when compared with the amount of *B. subtilis* before encapsulation. The group with the highest release of *B. subtilis* is AGC as $1.4 \pm 0.071 \times 10^5$ CFU/mL, followed by AC as $4 \pm 0.053 \times 10^4$ CFU/mL. Encapsulation efficiency of AGC and AC are 70.09 and 62.68, respectively, as shown in Table 1.

Table 1. The efficiency of *B. subtilis* release from various gel bead types. (Each type of gel beads was prepared by using 18G needle).

Type of material	Released <i>B. subtilis</i> (CFU/mL)	Encapsulation efficiency (EE)
A	$9.25 \pm 0.008 \times 10^{3a}$	54.02
AC	$4 \pm 0.053 \times 10^{4b}$	62.68
AG	$7.2 \pm 0.008 \times 10^{3a}$	52.53
AGC	$1.4 \pm 0.071 \times 10^{5c}$	70.09

Note: Initial *B. subtilis* concentration (before encapsulated) is $2.2 \pm 0.45 \times 10^7$ CFU/mL. The letters a, b, and c represents a statistically significant difference ($P < 0.05$).

The release of *B. subtilis* from gel bead (AC) at 0, 1, 2, 3, 4, 5 and 6 h was studied. The results show that *B. subtilis* were completely released from the gel

beads at the 1st h after shaking the gel in KH_2PO_4 solution. And when measuring the amount of *B. subtilis* continuously every 1 h, the amount of *B. subtilis* remained constant until the 6 h, as shown in Figure 3.

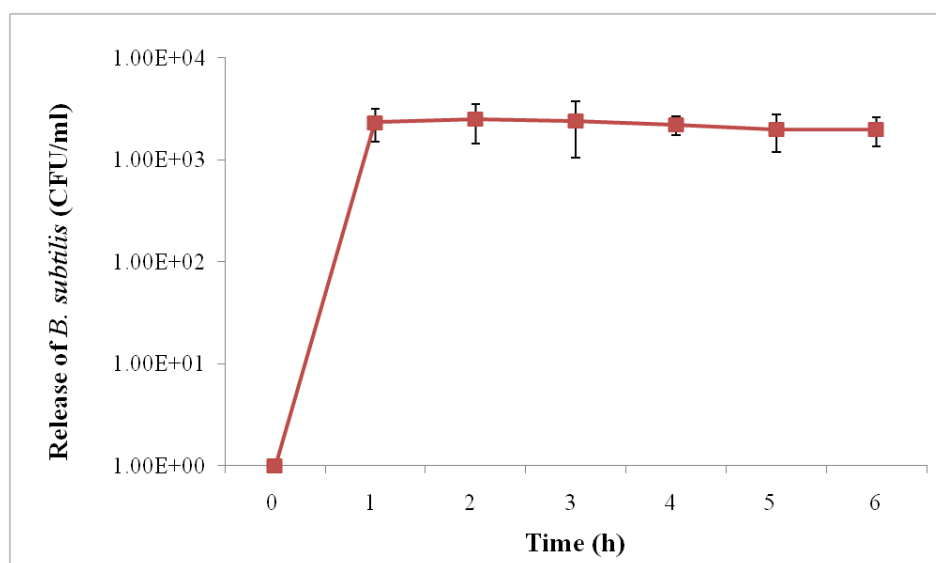


Figure 3. The release of *B. subtilis* from gel bead (AC) at 0, 1, 2, 3, 4, 5 and 6 h after shaking the gel in KH_2PO_4 solution.

Encapsulation of *B. subtilis* in various sizes of the gel beads

Characteristics of the gel beads. *B. subtilis* was encapsulated with AC using various sizes of syringes, including 16G, 18G, and 20G. The results showed the round gel beads and the diameter size of the beads are 3.18 ± 0.27 , 2.31 ± 0.20 and 1.5 ± 0.18 mm, respectively. From Chuprom's (2010) experiment, *L. acidophilus* TISTR 1034 was encapsulated with 2% sodium alginate using needle size 18G, 22G, 24G, and 27G. The results showed that the gel bead sizes are 2.4, 2.1, 2.08, and 1.8 mm, respectively.

The protection of *B. subtilis* from low pH. Base on the survival of *B. subtilis* encapsulated with AC by using the needle sizes at 16G, 18G, and 20G in pH 4, the results were shown in Figure 4. It was found that the gel beads encapsulated using the needle size at 18G has a significantly higher number of surviving *B. subtilis* ($P < 0.05$), that is 6.3×10^4 CFU/mL, compared with the gel beads encapsulated using the needle sizes at 16G and 20G, that are 1.2×10^4 and 1.3×10^4 CFU/mL, respectively. The gel beads encapsulated using the needle sizes at 16G and 20G was not significantly different ($P > 0.05$). Accordingly, in pH 7.4, the gel beads encapsulated using the needle size at 18G has a significantly higher number of surviving *B. subtilis* ($P < 0.05$), that is 2.6×10^4 CFU/mL, compared with the gel beads encapsulated using the needle sizes at 16G and 20G, that are 1.0×10^3 and 4.4×10^3 CFU/mL, respectively. Therefore, the needle size at 18G produced effective gel beads that protect *B. subtilis* from low pH.

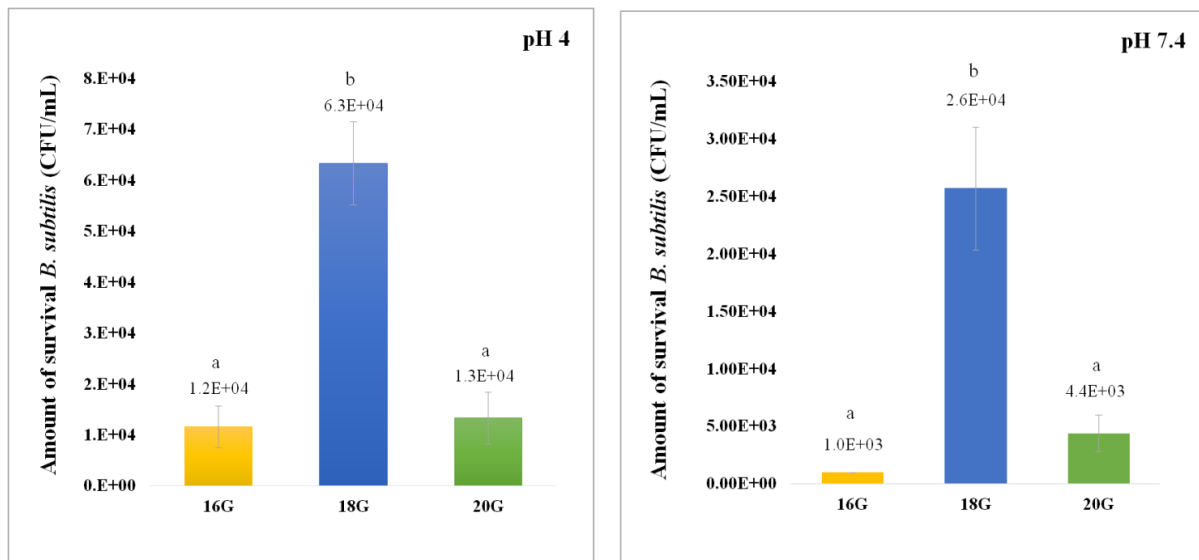


Figure 4. Amount of *B. subtilis* that survived after incubation at pH 4 (left) and pH 7.4 (right) for 3 hours. Gel beads (AC) are prepared using various sizes of needles. The letter (a, b) represents a statistically significant difference ($P < 0.05$).

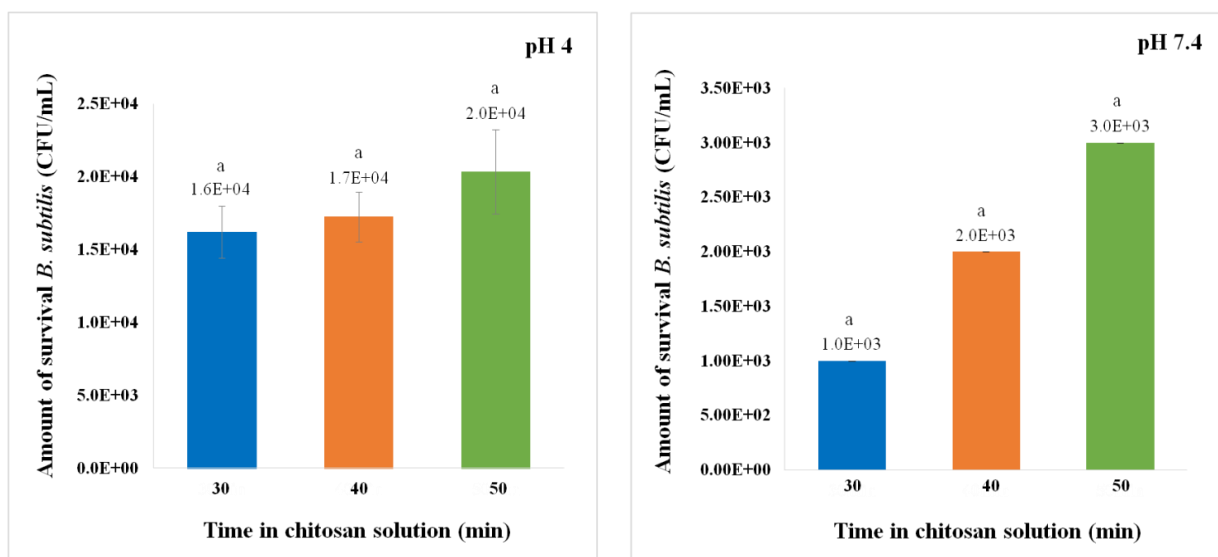


Figure 5. Amount of *B. subtilis* that survived after incubation at pH 4 (left) and pH 7.4 (right) for 3 hours. Gel beads (AC) are prepared using 18G needle and soaked in chitosan solution for 30, 40 and 50 minutes. The letter (a) represents a statistically significant difference ($P < 0.05$).

Encapsulation of *B. subtilis* with chitosan at various times

B. subtilis was encapsulated with AC by using needle size 18G. Then, the gel beads were soaked into the chitosan solution for 30, 40, and 50 minutes and the survival of *B. subtilis* in pH 4 was studied. The amount of the survival of encapsulated *B. subtilis* that soaked in chitosan solution for 30, 40, and 50 min

are 1.6×10^4 , 1.7×10^4 , and 2.0×10^4 CFU/mL, respectively. But when applied to statistical testing, it was found that there was no significant difference ($P > 0.05$) as showed in Figure 5. Correspondingly, in pH 7.4, the amount of the survival of encapsulated *B. subtilis* that soaked in chitosan solution for 30, 40, and 50 min are 1.0×10^3 , 2.0×10^3 , and 3.0×10^3 CFU/mL, respectively.

Encapsulation efficiency testing for the protection of *B. subtilis*

The protection of *B. subtilis* from high temperature. From the experiment of the survival of encapsulated *B. subtilis* at 60 °C, the percentage survival of *B. subtilis* encapsulated with AGC and non-encapsulated *B. subtilis* (BS) is not significantly different ($P > 0.05$), but the percentage survival of *B. subtilis* encapsulated with AC increases when compared to BS and AGC as statistically significant ($P < 0.05$), as shown in Figure 5. The average percentage survival of BS, AC and AGC are 4.83%, 48.38%, and 6.49%, respectively. From the experiment of the survival of encapsulated *B. subtilis* at 90 °C, *B. subtilis* encapsulated with AC has the highest percentage survival and significantly different when compared with BS and AGC ($P < 0.05$). Followed by, AGC which increased percentage survival when compared to BS with statistically significant ($P < 0.05$) as also shown in Figure 6. The average percentage survival of BS, AC and AGC are 9.67%, 40.32% and 27.59%, respectively. According to the study of Praepanitchai et al. (2019), *L. plantarum* was encapsulated with hybrid alginate-soy protein and then the survival of the encapsulated *L. plantarum* was evaluated by treating the beads at 30, 50, 63, and 72 °C. The encapsulated *L. plantarum* was found still alive after incubated at 72 °C for 90 s, while most free cells do not survive at temperatures above 50 °C.

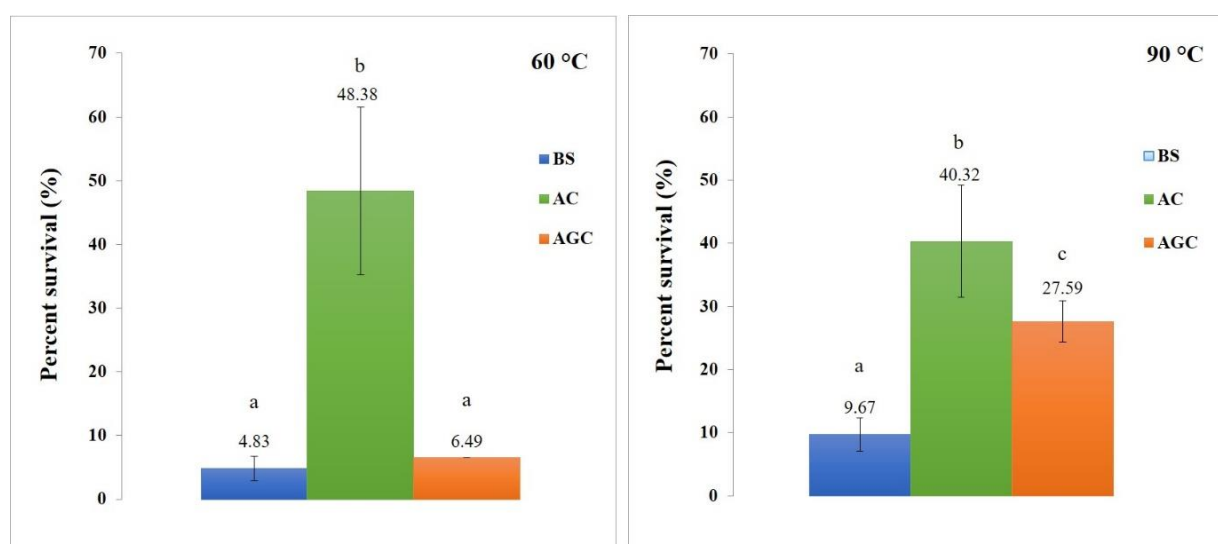


Figure 6. Percent survival of *B. subtilis* encapsulated with AC and AGC after incubation at 60 °C (left) and 90 °C (right) for 3 hours, compared with non-encapsulated *B. subtilis*. The letter (a, b, c) represents a statistically significant difference ($P < 0.05$).

The protection of *B. subtilis* from low pH. From the survival test of encapsulated *B. subtilis* at pH 4, the percentage survival of *B. subtilis* encapsulated with AC and AGC were not significantly different ($P > 0.05$), but increased significantly compared to BS ($P < 0.05$) as showed in Fig. 5. The average percentage survival of BS, AC and AGC are 1.04%, 98.03% and 90.9%, respectively. From the survival test of encapsulated *B. subtilis* at pH 2 corresponding to pH 4, but the survival is less than pH 4. The percentage survival of *B. subtilis* encapsulated with AC and AGC were not significantly different ($P > 0.05$), but increased significantly compared to BS ($P < 0.05$) as also showed in Figure 7. The average percentage survival of BS, AC and AGC are 0.15%, 45.15%, and 51.02%, respectively.

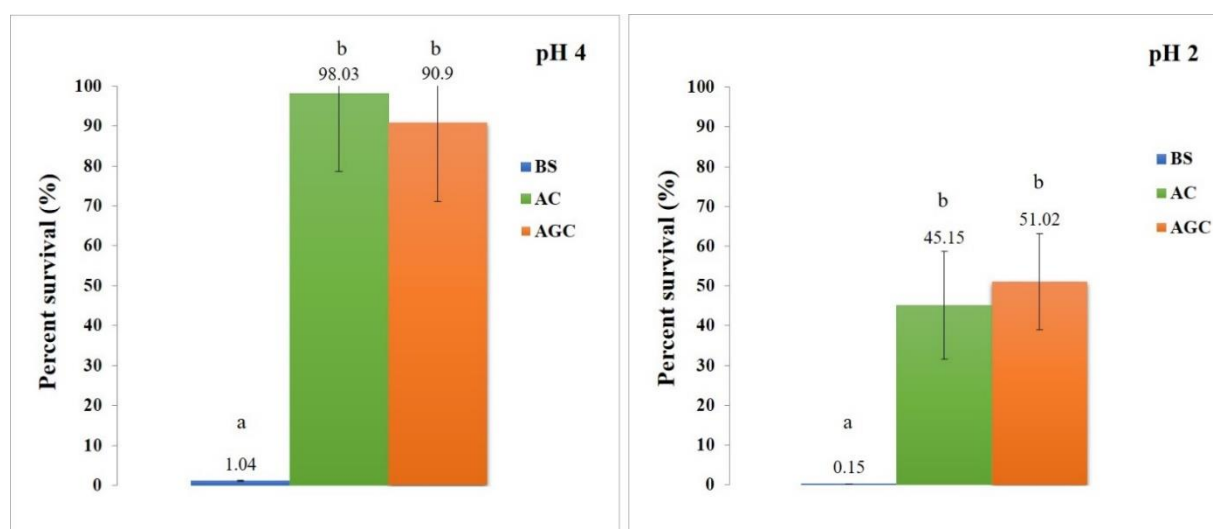


Figure 7. Percent survival of *B. subtilis* encapsulated with AC and AGC after incubation at pH 4 (left) and pH 2 (right) for 3 hours, compared with non-encapsulated *B. subtilis*. The letter (a, b) represents a statistically significant difference ($P < 0.05$).

DISCUSSION

B. subtilis (TISTR 2057) was tested for pH resistance in an intestinal tract. It showed potential to be antimicrobial against broiler-chicken pathogen and to produce digestive enzyme for growth promotion (Sriyam et al., 2011). In addition to *Bacillus* spp., there are other probiotics that are commonly used in aquatic animals, including *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* sp., *Micrococcus*, and etc. (Abd El-Rhman et al., 2009; Safari et al., 2016; Yu et al., 2017; Feng et al., 2019; Yang et al., 2019). However, *Bacillus* spp. have been proven to have better probiotic properties due to their ability to produce antimicrobial agents many types of microorganisms, non-pathogenic and non-toxic. With the ability to create spores, they have two advantages in terms of

survival (resistant to heat and longer shelf life) in various environments compared to other probiotics.

This study reported that the alginate, gelatin, and chitosan can protect the cells to survive in severe conditions. This is new and significant because previously, there are no any reports of *B. subtilis* encapsulation in these materials. Young et al., 2006 encapsulated of a *B. subtilis* CC-pg104 with alginate by enriching the bead microenvironment with humic acid. High viability of the encapsulated bacteria was observed with minimum cell loss upon storage for 5 months. Steady and constant cell release from the bead was observed for 1 week at different pH. Ma et al., 2015 developed a microencapsulation procedure of *B. subtilis* B99-2 using maltodextrin and gum arabic as wall materials. They found that Maltodextrin is an efficient wall material, the mean survival rate of *B. subtilis* was more than 90%, when spray drying was performed at 145 °C and *B. subtilis* microcapsule survival rate was 87.53% after 540 days of storage. Barlow et al., 2017 encapsulated *B. subtilis* in a semi-permeable membrane composed of mPEG-PDLLA. This polymer membrane was sufficiently permeable to permit exponential bacterial growth, metabolite-induced gene expression, and rapid biofilm growth.

Alginate is a natural polymer used in the food and drug industry because of its mild gel formation, biological compatibility, and biodegradability. There is a growing interest in using alginate in a variety of scientific fields such as oral vaccination, development of drug release systems and microencapsulation of cells for continuous control and delivery of treatment products (Amirheidari et al., 2016). Chitosan is an alternative for microcapsule coating. It is also the most positively charged polysaccharide and can be obtained by alkaline removal of chitin. Due to its low toxicity, good biocompatibility and controlled biodegradability, it is used as an active ingredient in encapsulation (Chen et al., 2007, Chen et al., 2009). It is also reported that the substrate is suitable for bio-polymers due to the structure similar to glycosaminoglycans found in tissues. When using alginate and chitosan to encapsulate, alginate-chitosan capsules (AC) occurs due to the reaction between electrostatic charge and both polysaccharides with opposite charge. Live microorganisms, cells, DNA, proteins, and enzymes in AC have been studied extensively (Chen et al., 2009).

The size of the gel beads is determined by many factors including the type of bacteria needle size, sodium alginate solution concentration and distance between syringe and calcium chloride solution. The size of the gel beads produced by the extrusion method will be between 500 µm to 3 mm in diameter (Burey et al., 2008). In this study, the diameter size of the beads prepared by 16G,

18G, and 20G needles are 3.18 ± 0.27 , 2.31 ± 0.20 and 1.5 ± 0.18 mm, respectively. Chandramoulia et al. (2004) studied the survival of the *L. acidophilus* which is encapsulated by 1.5% sodium alginate, by preparing various sizes of gel bead. It was found that the survival of the encapsulated bacteria increased as the size of the gel increased. By reasoning that the large gel beads will have a surface area that will be exposed to various harmful conditions less than small gel beads. While Totosa et al., 2013 indicated that the gel beads that have a less exposed surface area are stronger, more flexible and less viscous. Therefore, the larger gel beads are more likely to break or leak easily than small gel beads. In addition, Lee and Heo, 2000 reported that the large gel beads may cause roughness of the texture in fresh microbial supplements. Therefore, the gel bead size should be in the appropriate range, not too large or too small.

From the experiment found that the amount of release of *B. subtilis* from the gel beads is reduced with statistical significance ($P < 0.05$) when compared with the amount of *B. subtilis* before encapsulation. The amount of *B. subtilis* disappeared, it is possible that some of *B. subtilis* are combined together with the gel and some are still trapped inside the gel. Indicated by, after vortex the gel beads in the maximum speed for 10 minutes, it was found that the gel beads that were encapsulated with chitosan were easier broken than the no chitosan gel beads. In this experiment, AC and AGC are easier broken than A and AG, resulting in a greater amount of bacteria released. In living animals, if the gel beads are sticky and difficult to break when in the digestive tract of animals, the amount of bacteria released is low. This may result in an insufficient amount of probiotics for function. Therefore, the researcher has chosen two groups of gel beads that have the good release of *B. subtilis* that are AC and AGC for further experiments. Haghshenas et al., 2015 reported that *Lactobacillus plantarum* 15HN using alginate-psyllium-fenugreek polymeric blends has release occurred after 2 h in colonic condition and sustained until the 12th h incubation period. While in this study, *B. subtilis* release occurred at 1st h and remained constant until the 6th h incubation period.

When poured the sodium alginate solution containing cell suspension into the calcium solution, the bound ions will interact with other GG blocks to create complexities that lead to gel formation, resulting in protecting cells that are released in the intestine tract (Prakash and Jones, 2005). Encapsulation in 2% alginate containing 2% Hi-maize resistant starch bead improved the survival of *L. plantarum* CM53 in simulated GIT significantly ($P < 0.05$). *L. plantarum* and free cells survival after incubated in stomach gastric juice (SGJ) for 3 h was 89.55% and 69.82%, respectively (Ayama et al., 2014). In this study, the encapsulation efficiency quite low (only 62.68) but the survival of *B. subtilis* is high as the average percentage survivals of AC and AGC at pH 4 are 98.03% and 90.9%, respectively. The previous study indicated that the level of bacteria survival was increased when co-encapsulate alginate with other material. Coating of gelatin microspheres with alginate provided significant protection

for *Bifidobacterium adolescentis* 15703T from the harsh acidic conditions of simulated gastric fluid (SGF) (Annan et al., 2008). Many kinds of research indicated that the alginate-chitosan encapsulation provided significant protection of probiotics bacteria in SGF with statistical significance compared to free cells (Chavarri et al., 2010; Li et al., 2011).

CONCLUSION

The gel beads of A, AG, AC, and AGC have the same external characteristics that are a round and smooth surface. The gel beads using 18G needle has the highest amount of *B. subtilis* release and made effective gel beads that protect *B. subtilis* from low pH. Optimal duration of soaking the gel beads in chitosan is 30 min. *B. subtilis* encapsulated with AC has the highest percentage survival in high temperature. *B. subtilis* encapsulated with AC and AGC provides equally high percentage survival in low pH. This experiment will be the basis for the use of these encapsulated gel beads in animals. To supplement beneficial probiotic bacteria that able to withstand critical conditions in the digestive tract of animals, it may be added to animal feed or allow animals to eat in other methods.

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