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Improvement of *Bacillus subtilis* Natto Viability by Alginate and Xanthan Gum as a Wall Material

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ABSTRACT

In this study, *Bacillus subtilis* natto was encapsulated in alginate, either coated with or mixed with xanthan gum as a supplemental component. The encapsulated bacteria were then evaluated for their survival in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The results showed that *B. subtilis* natto biomass had a thrombolytic ability compared to the control sample. The viability of encapsulated *B. subtilis* natto was improved in which alginate 2.5% (w/v) had a high encapsulation efficiency, and there was no difference between the samples with or without the xanthan gum supplement. In the SGF and SIF tests, the viability of *B. subtilis* in samples supplemented with xanthan gum was higher than in samples that contained only alginate. Additionally, there was no significant difference in viability between the samples that mixed xanthan gum with alginate and those that were coated with it. The results indicated that adding xanthan gum is necessary to increase alginate's protective effect on *B. subtilis* natto.

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Introduction

Probiotics are strains of live bacteria introduced directly into the human body through the oral route (food in general and functional foods in particular) in a specific dose to support health (C. Zhang et al., 2021). The probiotic microorganisms include *Bifidobacteria*, *Lactobacillus plantarum*, *Lactobacillus paracasei, Bacillus subtilis* natto, etc. *Bacillus subtilis* natto has received much attention thanks to its outstanding ability to produce Natokinase enzyme which can dissolve blood clots and reduce cardiovascular disease (Wang et al., 2020; Sumi et al., 1987). *B. subtilis* natto is a bacillus, gram-positive, arbitrarily aerobic, has moving ability, is spore-forming, and has been proven to be safe in experimental studies on animals and humans (Hong et al., 2008; J. Zhang et al., 2020). In addition, *B. subtilis* natto is known for its ability to produce nattokinase, a protease containing 275 amino acids, which has been shown to have potent fibrinolytic activity (Lampe & English., 2016). However, although the *B. subtilis* natto viability in intestinal and gastric fluid conditions which have pH 7.4 and 2.0 respectively and temperature at 37°C is strong, the vegetative cells of the strain are susceptible to these environments (Hong et al., 2008). In the spore form, *B. subtilis* natto has the ability to overcome adverse conditions such as simulated gastrointestinal conditions. However, the germination rate of these spores in the gastrointestinal tract is very low only 8% (Hatanaka et al., 2012). Therefore, protecting *B. subtilis* cells in a vegetative form is essential to provide health benefits to the host. Previous studies showed that encapsulation effectively protects probiotic bacteria under adverse conditions such as production process, storage, and simulated gastrointestinal conditions. Many encapsulation techniques and different wall materials have been studied, in which extrusion compression is a simple and low-cost encapsulation method, and the operating conditions are not harsh to improve encapsulation performance (Frakolaki et al., 2021). In addition, alginate is an edible biopolymer derived primarily from brown seaweed and is a widely used material in extrusion encapsulation due to its ability to form gels in the presence of divalent cations. This property enables the encapsulation of a variety of substances (Raus et al., 2021). Alginate is also an inexpensive and non-toxic biomaterial commonly used in

extrusion encapsulation (Afzaal et al., 2019). The extrusion technique with alginate as the primary wall material significantly improved the viability of probiotic bacteria under simulated gastrointestinal conditions (Afzaal et al., 2019). However, due to the porous structure of the alginate gel network, it facilitates the diffusion of H^+ ions, causing cell death and reducing the encapsulation efficiency (Chavarri et al., 2010; Vaziri et al., 2018). To limit this issue, additional coatings such as chitosan, pectin, gelatin, and xanthan gum were included to improve the alginate gel structure network and enhance the viability of probiotic bacteria (Chavarri et al., 2010; Khorshidi et al., 2021; Vaziri et al., 2018). Xanthan gum is a natural material with safe properties and application in a lot of fields such as pharmaceutics, cosmetics, and food. Besides, xanthan gum is unaffected by pH conditions, thermally stable, and entirely biodegradable (Singhvi et al., 2019). These properties could be a potential supplement to limit the porous structure of the alginate gel network. This study assessed the thrombolytic ability of a bacterial strain by comparing the biomass and culture fluid to a control. Additionally, the protective effectiveness of alginate, both with and without the addition of xanthan gum, was evaluated for its impact on the viability of *B. subtilis* in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

Materials and Methods

Materials

B. subtilis natto strain was obtained from strain collection of the Faculty of Food Science and Technology, Ho Chi Minh City University of Industry and Trade. The strain was cultured in nutrient broth for 20 hours. The biomass after culture was collected by centrifugation and continued to wash the biomass with 10 ml of saline water twice. Then, the biomass was collected and suspended in saline water. Alginate (Zhanghai et al.) and xanthan gum (Himedia) Co., Ltd) were sterilized at 121°C for 15 minutes before use.

Test the Ability of Bacteria to Dissolve Thrombosis

The experiments were conducted following a previous study (Prasad et al., 2006) with some modifications. Briefly, 200 µl of pig blood (slaughter pig's blood was stored at 4°C before use) and 400 µl of distilled water were added into the Eppendorf tubes. The tube was then shaken and let for 1 hour to allow the blood to clot. After coagulating blood, the Eppendorf tube was added 100 µl of fluid culture or biomass of *B. subtilis*, or saline water was used as control samples. The Eppendorf tube was incubated at 37°C, and the fibrinolytic ability was every two hours of incubation.

Evaluation of the Effect of Carrier on Encapsulation Performance B. Subtilis Natto

The encapsulated process by extrusion compression technique was carried out according to Lieu et al. (2019) with the following main steps: Alginate (2.5% w/v) with or without xanthan gum 0.5% (w/v) (AX samples) or alginate (2.5% w/v) (A samples) was mixed with *B. subtilis* natto. The mixtures were then put into a piston, dripped into $CaCl_2$ solution through a 0.5 mm needle, and incubated to solidify completely. The inoculants were then collected, and the A sample was further incubated in xanthan gum at 0.5% (w/v) (sample AXc) (by shaking machine at 100 rpm) to form a xanthan gum coating at room temperature. The encapsulation efficiency was calculated according to the following formula (Lieu et al., 2019):

$$\text{Encapsulation yield (\%)} = \frac{\sum \log \text{CFU}_{\text{after}}}{\sum \log \text{CFU}_{\text{before}}} \times 100\%$$

Effect of Simulated Gastrointestinal Conditions on the Viability of B. Subtilis Natto Encapsulated

The viability of *B. subtilis* natto under simulated digestive fluid conditions was carried out according to Lieu et al. (2019). The simulated gastric fluid (SGF) (containing 9 g/l NaCl) was adjusted to pH 2 by 5M HCl, and the simulated intestinal fluid (SIF) (containing 9 g/l NaCl + 3 ml/l cow bile) was adjusted to pH 6.5 by 5M NaOH.

1 gram of the sample was added to 99 ml of NaCl pH 2 (corrected with 5M HCl) and incubated for 2 hours. Then, 1 ml of the SGF solution was transferred to the SIF condition and incubated for 4 hours. The *B. subtilis* natto in free cell form was used as control samples. The viability of *B. subtilis* natto was determined by plating on a nutrient agar medium.

Statistical Analysis

The experimental data were analyzed using one-way ANOVA in IBM SPSS Statistics 25 software. The mean was compared by Tukey's test at the 5% probability level ($p < 0.05$). The experiments were repeated three times, and the results are represented as mean ± standard deviation.

Results and discussion

The Ability of Bacteria to Dissolve Thrombosis

The thrombolytic ability of *B. subtilis* natto was shown in Figure 1. The results obtained show that there was a significant difference in the thrombolytic ability between the three samples. The post-culture liquid and biomass of *B. subtilis* natto showed significantly different hemolysis compared with the distilled water control (Figure 1).

The thrombolytic ability of *B. subtilis* natto is one of the significant properties of this strain (Prasad et al., 2006). Previous studies have shown that *B. subtilis* natto cultures can effectively dissolve thrombosis. Ju et al. (2019) showed *B. subtilis Subsp*. natto WTC016 isolated from the soil had a thrombolytic ability (Ju et al., 2019). A study by Hong et al. (2009) indicated that although neither of the reference strains was haemolytic, all of the isolates exhibited either complete or incomplete haemolysis. Interestingly, lecithinase activity was also produced in some isolates, including Natto. The production of lecithinase activity in some isolates, including *Bacillus subtilis* natto, is reported in the reference for their biochemical profiling. Haemolysis was evaluated by streaking onto tryptose blood agar containing sheep's blood at 5% and incubation for 24 h at 37 °C (Hong et al., 2009). Similarly, Moharam et al. (2019) demonstrated that cultures of the *B. subtilis* Egy strain could dissolve thrombosis (Moharam et al., 2019). Additionally, Pinontoan et al. (2021) confirmed that *Bacillus subtilis* G8 has powerful fibrinolytic activity and this activity has variety thrombolytic enzymes joined (Pinontoan et al., 2021).

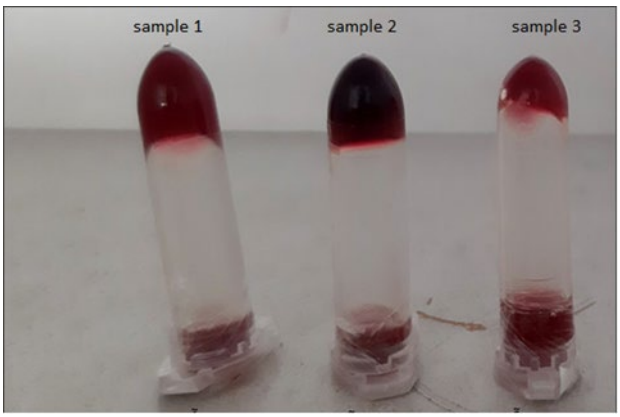

Figure 1. Dissolve thrombosis test.
The order of samples is as follows: Sample 1 is the culture fluid of bacteria, sample 2 is distilled water, and sample 3 is the bacterial biomass solution.

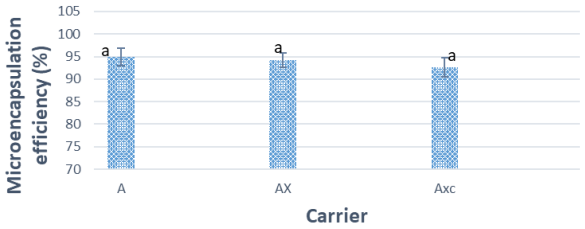

Figure 2. Effect of the carrier on the encapsulation efficiency Bacillus subtilis natto.
(A) alginate 2.5% (w/v); (AX) alginate 2.5% (w/v) with xanthan gum 0.5% (w/v); (Axc) alginate 2.5% (w/v) coated by xanthan gum 0.5% (w/v)

These studies demonstrated that the fluid obtained after the culture of *B. subtilis* natto contains the enzyme nattokinase and has effective thrombolysis. The results obtained from this study showed that, in addition to the culture fluid obtained, the biomass of *B. subtilis* natto also showed thrombolytic potential (Figure 1). This suggests that maintaining the viability of *B. subtilis* natto is necessary to enable them to exert their beneficial effects on the host organism.

The Effect of Carrier on Encapsulation Performance B. Subtilis Natto

The role of wall material in the viability of *B. subtilis* natto was presented in Figure 2. The experiment compared wall materials such as alginate and xanthan gum (mixing or coating to alginate) for encapsulation efficiency. The encapsulation efficiency of A, AX, and Axc was 94.85 ± 1.92%; 94.22 ± 1.55%; and 92.65 ± 2.13% respectively.

During fermentation, *B. subtilis* natto produced beneficial metabolic compounds (Hong et al., 2008). However, *B. subtilis* (natto) can lead to an unique smell, and an obvious stratification can appear in the fermented mixed milk stored for a long time (Nie et al., 2017) which is the cause of consumer pickiness. Therefore, encapsulation of *B. subtilis* natto could reduce the unexpected sensory property. A variety of wall materials are commonly used in the encapsulation process and alginate is used due to its safety, low cost, and high efficiency (Afzaal et al., 2019). Alginate was commonly used as wall material for extrusion and emulsion techniques. The emulsion technique helps to produce particles with a smaller size than the extrusion technique (My Dong et al., 2020). However, particles obtained by the extrusion technique are more straightforward, and the composition does not contain oil, which is a component of the preparation process by emulsification. Previous studies showed that alginate wall material had high encapsulation efficiency. Sohail et al. (2011) demonstrated that the encapsulation efficiency of *L. rhamnosus* GG and *L. acidophilus* NCFM was higher than 90% with alginate 2% (w/v) (Sohail et al., 2011). Similarly, the encapsulation efficiency of *Bacillus subtilis* SL-13 by alginate 2% (w/v) was 90.92% (Tu et al., 2015). Despite high encapsulation efficiency, the alginate structure is easily broken during drying (Kusuktham et al., 2013). Therefore, the combination of alginate with wall material has also received much attention. The combination of alginate with additional wall material has been reported in previous studies. The encapsulation efficiency of *L. plantarum* NCDC201 and *L. casei* NCDC297 in alginate-coated alginate was 72.48% and 63.54%, respectively (Rather et al., 2017). Singh et al. (2019) showed that the encapsulation efficiency of *Lactobacillus gastricus* BTM7 in the mixture of skimmed milk powder and alginate was over 75% and reached the highest in the ratio of 1:1 mixture of skimmed milk powder and alginate with an efficiency of 94% (Singh et al., 2019).

Similarly, the encapsulation efficiency of two bacterial strains, *L. plantarum* and *L. rhamnosu*, by alginate, alginate - Skim milk mixture, and alginate - skim milk coated with chitosan were higher than 94.54 ± 0.03% (Padhmavathi et al., 2021). In addition, the influence of the concentration of wall materials on the ability to protect bacteria has also been studied. The study by Mandal et al. (2006) showed that the higher the alginate concentration, the greater the viability of *L. casei* NCDC298 bacteria (Mandal et al., 2006). The low concentration of alginate wall material affected the shape of the shrink particles, and the convex surface was not smooth after drying (Kusuktham et al., 2013; Ramos et al., 2018). These effects can make the particles easily broken or have high permeability affected probiotic bacteria inside the particles. The results obtained from this study showed that alginate 2.5% (w/v) provided a high encapsulation efficiency, and there was no difference between the samples (Figure 2). This showed that adding xanthan gum, either coated or combined, had no significant effect on the encapsulation efficiency.

Effect of Simulated Gastrointestinal Conditions on The Viability of B. Subtilis Natto Encapsulated

The viability of *B. subtilis* natto after two hours of incubation under SGF and SIF conditions was presented in Figure 3. Under SGF conditions, in samples containing free cells, the density of *B. subtilis* natto decreased rapidly from 9.21 ± 0.19 to 6.78 ± 0.2 Log CFU/ml. In contrast, for encapsulated bacteria, the reduction was significantly less ($p < 0.05$). Specifically, the *B. subtilis* viability in A, AX, and Axc samples was reduced from 9.35 ± 0.11 to 7.41 ± 0.14 Log CFU/ml; 9.33 ± 0.15 to 7.80 ± 0.13 Log CFU/ml, and 9.15 ± 0.16 to 7.96 ± 0.14 Log CFU/ml, respectively.

The viability of *B. subtilis* natto in free cell form was significantly affected by the SIF medium (Figure 3). The survival rate of *B. subtilis* was decreased by 4 Log CFU/ml after incubation in simulated digestive fluid conditions and

remained at 5. 58 Log CFU/ml. In the case of encapsulated samples, the viability of encapsulated *B. subtilis* in A, AX, and Axc samples was 6.25 ± 0.18 Log CFU/ml, 6.91 ± 0.14 Log CFU/ml, and 6.96 ± 0.2 Log CFU/ml respectively.

The fibrinolytic activity, health benefits of *B. subtilis* natto depend on their enzyme production ability (Ju et al., 2019; Nie et al., 2017). *B. subtilis*, in the form of spores, gave the germination rate in the digestive fluid only 8% (Hatanaka et al., 2012). In the spore state, all cellular activity halts, and enzyme production ceases. Therefore, it is essential to exist as vegetative cells. Previous studies showed that *B. subtilis* was sensitive to low pH conditions, leading to spore formation to protect them from the effects of simulated digestive fluid. However, the spore counts after 6 hours of surviving under the above conditions reached about 74 ± 1%, and the re-germination rate of spores was not high, about 8%, leading to not bringing high efficiency to the digestive system (Hatanaka et al., 2012).

The encapsulation technique showed a significant improvement in the viability of the bacteria under SGF conditions Zhang et al. (2021) showed that the survival rate of encapsulated *B. subtilis* 04178 in the alginate-chitosan mixture was 18.19% more effective than that of the unencapsulated control under pH 2.5 conditions (Zhang et al., 2021). Similarly, the protective effect of *Lactobacillus gastricus* BTM7 in the mixture of skimmed milk powder and alginate under the SGF condition was nearly 2 Log CFU/ml higher than that of free-form bacterial cells (Singh et al., 2019). A study by Rather et al. (2017) also demonstrated that the viability of encapsulated *L. plantarum* NCDC201 cells was 47.5% higher than that of free bacteria at pH 2 for 2 hours (Rather et al., 2017). Besides, the survival rate of encapsulated bacteria was 1.5 to 4 Log CFU/ml higher than free cell bacteria, as reported by Xiao et al. (2020) and Shu et al. (2018) (Shu et al., 2018; Xiao et al., 2020). Alginate is a widely used food additive because of its proven safety and is also used to protect probiotics from extreme conditions such as SGF, SIF, and storage at 4°C (Chavarri et al., 2010; Vaziri et al., 2018). However, the porous gel structure of alginate was affected by low pH, high permeability, and low stability, reducing the protective power of this material under low acid conditions (Chavarri et al., 2010; Vaziri et al., 2018). The supplement wall material helps to limit the porous structure of the gel network of alginate, which improves the viability of probiotic bacteria under low pH conditions. Padhmavathi et al. (2021) indicated that a mixture of alginate-skim milk wall material coated with chitosan significantly improved the viability of probiotic bacteria (Padhmavathi et al., 2021). Similarly, My et al. (2020) suggested that the calcium-alginate coated with skim milk showed higher *L. acidophilus* viability than the mixture of calcium-alginate and skim milk or calcium-alginate alone (My Dong et al., 2020). Xanthan gum is a suitable complementary material with low pH stability thanks to its rigid, helical structure and thermal stability (Singhvi et al., 2019). Thanks to the addition of xanthan gum to the alginate gel structure, the viability of the probiotics was significantly enhanced (Khorshidi et al., 2021; Shu et al., 2018). The present study showed that the viability of encapsulated *B. subtilis* in alginate was significantly improved compared with the control sample (Figure 3). The *B. subtilis* viability in samples supplemented with xanthan gum (AX and Axc samples) was higher than that of the samples using only alginate (A sample), and there was no significant difference between containing xanthan gum mixing or coating (Figure 3).

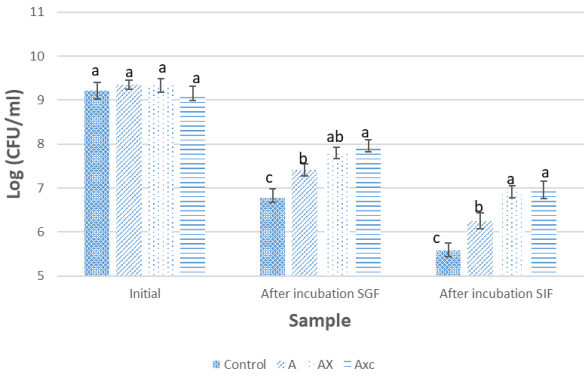

Figure 3. Effect of SGF and SIF on the viability of Bacillus subtilis natto.
The letters a, b, and c represent significant differences between samples ($p < 0.05$)

Besides the SGF condition, the SIF condition also affects probiotic bacteria. Previous studies showed that the viability of encapsulated probiotic cells was significantly higher than that of the free cell samples (Shu et al., 2018; Singh et al., 2019; Xiao et al., 2020). Rather et al. (2017) indicated that the viability of *L. plantarum* NCDC201 and *L. casei* NCDC297 in encapsulated form was higher than in free cell form at about 4 Log CFU/ml and 5 Log CFU/ml, respectively (Rather et al., 2017).

A study by Padhmavathi et al. (2021) showed that the mixed alginate-skim milk coated with chitosan remarkably improved the viability of *L. plantarum* and *L. rhamnosus* in SIF conditions (Padhmavathi et al., 2021). My et al. (2020) suggested that the interference of skim milk with the cross-linking of the calcium ions with alginate degraded the particle structure and decreased the *L. acidophilus* viability in simulated digestive fluid compared to that of the mix of alginate- skim milk (My Dong et al., 2020). Similarly, Padhmavathi et al. (2021) indicated that skim milk was used for encapsulation and chitosan coating, making the microspheres stronger than uncoated ones. In this study, there was no significant difference in the protective effect of AX and Axc, showing that in SIF conditions, the protective effect of the two methods was the same (Figure 3). These results indicated that the xanthan gum concentration (0.5% w/v) was suitable for encapsulation which did not interfere with the alginate structure.

Conclusion

The results obtained from the study showed that *B. subtilis* natto biomass had a thrombolytic ability compared to the control sample. The survival rate of *B. subtilis* natto was significantly affected under SGF and SIF conditions. The wall material by alginate enhanced the *B. subtilis* natto viability, and adding xanthan gum is necessary to increase the protective effect of *B. subtilis* natto of alginate.

The authors declare that there is no conflict of interest regarding the publication of this article.

Declarations

This study was presented at the 7th International Anatolian Agriculture, Food, Environment and Biology Congress, (Kastamonu, TARGID 2024)

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