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The Evaluation of Marine Sponge–Associated Bacteria Microcapsule to Control Vibriosis in the Shrimp Culture

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Abstract

The most frequent disease on vannamei shrimp is caused by *Vibrio* sp. bacterial infection. *Vibrio* sp. might cause a serious production loss in the shrimp culture, leading to larvae and mature stadium mortality at 50%. To date, vibriosis disease has yet to be controlled properly. Therefore, sponge-associated bacteria are used as an alternative to control vibriosis disease in shrimp. To maintain and protect the potential bacteria from unfavorable environmental influence, combining the ability of sponge-associated bacteria and encapsulation technology is necessary. This research aimed to evaluate the viability, shelf life, anti-vibrio activity, and structure characteristics under Scanning Electron Microscope (SEM) of *Bacillus* spp. microencapsulation using alginate, chitosan, as well as Arabic gum. The method of viability test used Total Plate Count (TPC) and to reconfirm the antibacterial test was conducted using plug agar method. The result indicates that the *B. altitudinis* PH.1 coated with chitosan show the highest viability at $1.8x10^8$ CFU/ml after 14 days preserved in -20°C. The moisture content of this treatment revealed 17% as the highest than the other treatment, therefore the predicted shelf life for chitosan coated reach 9.1 years. Furthermore, the antivibrio of *B. altitudinis* PH.1 show the strong activity with clear zone inhibition at 16.07 mm against *Vibrio alginolyticus*. The structure of *B. altitudinis* PH.1 under SEM observation was irregular shape and size. Therefore, this study found that chitosan could be an alternative source to preserve a potential bacterium using microencapsulation technology.

Keywords: Alginate, bacterial microcapsules, chitosan, Vannamei shrimp, *Vibrio* sp.

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1. Introduction

A demand for vannamei shrimp on local and international scales affects the advancement of vannamei shrimp culture. It leads to a higher potential of diseases for the vannamei shrimp. Bacteria, viruses, and fungal-causing diseases might occur as a result of an imbalance between the host, pathogen agent, and the environment [1]. A frequent disease occurs in shrimp aquaculture caused by *Vibrio* sp. bacteria [2]. This type of bacteria can grow quickly in pond water with abundant organic materials. According to Singh [3] and Hameed [4], if the *Vibrio* sp. population is higher than other bacteria, it might decrease the life level of shrimp during the seeding and breeding period. Bacterial disease caused by the genus Vibrio bacteria has become a "big concern" for industrial shrimp farmers [5]. Vibriosis infection in a shrimp is characterized by the paleness of hepatopancreas; redness or paleness of the carapace of the body; redness of the uropod and telson; and the red antenna[6]. Therefore, Vibrio can cause a serious production loss in shrimp aquaculture [7]. In larvae and mature stadiums, Vibriosis leads to death for around 50% [8].

In recent years, significant research result on the prevention and control of vibriosis disease has yet to be established. Therefore, a study on the alternative control of vibriosis disease using biocontrol agents is urgently needed. A biocontrol agent is a disease control management strategy using micro and macroorganisms as control agents with antagonist's properties to the disease-causing agents. The working system of biocontrol agents in controlling the disease is by producing antimicrobial compounds to inhibit or kill the growth of pathogens [9]. One of the biocontrol agents' sources derived from marine is a sponge [10]. Sponge is a marine invertebrate discovered since 645 million years ago and has existed in recent years. In a marine nutrient cycle, a sponge, which lives as a filter feeder, plays a role as a zoobenthic that allows marine microbes to easily infiltrate and reside in the sponge body. Flagellum cell is attached in the choanosome or middle part of the sponge's porous body, leading to a proper air and nutrient transfer, causing the

marine sponge-associated microbes to be permanent. Numerous bioactive compounds derived from sponge-associated microbes are evident in this case [10-11]. A unique marine environment makes the production of the bioactive compound derived from sponges differ from the ones derived from the land [12]. Several important bioactive compounds are reported to have antibacterial activities, including anti-Methicillin-Resistant *Staphylococcus aureus* (antiMRSA), anti-tuberculosis, antifungal, antiparasitic, antiviral, anticoagulant, anti-biofouling, anti-inflammation, UV protector, and cytotoxic potential as anti-cancer [13]. However, stable preservation with high protection for microbes to survive longer is needed in its implementation as antipathogen bacteria.

A preservation technology where the essential materials are retained in the matrix or encapsulation membrane is called microencapsulation [14] aims to protect, reduce the broken or the loss of cells from the influence of an unfavorable environment [15] extend the lifetime duration/age and increase the survival of the microorganisms [16]. In addition, study on the biocontrol agents of vibriosis disease in vannamei shrimp derived from the sponge in a form of microencapsulation using alginate and chitosan has yet to be reported. This research is urgently needed as an effort to control vibriosis disease in vannamei shrimp.

2. Materials and Methods

2.1. Study area

This research was conducted from January to July 2023 at the Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences (FPIK), Diponegoro University, Semarang as a place to prepare *Vibrio* sp. and bacteria associate-sponge. The process of making bacterial microcapsules is carried out at the Experimental Laboratory, Faculty of Agricultural Technology (FTP), Soegijapranata University, Semarang.

2.2. Procedures

2.2.1. Cell cultivation

The bacterial isolates that showed potential activity to inhibit *Vibrio* spp. growth were cultivated in a liquid medium to stimulate the production of antimicrobial compounds. The fresh colony was inoculated in 25 mL of ZoBell broth medium (peptone and yeast) as the starter of mass culture. The isolate was cultured in a 250 mL flask. The mass cultures were transferred from 250 mL flasks to one liter Erlenmeyer flasks and incubated in a shaking incubator at 110 rpm and 37°C for four days [17]. Harvesting was done by 15 minutes of centrifugation of cultured bacteria at 8,000 rpm and at 4°C. The pellet of bacteria was centrifuged with Phosphate Buffered Saline (PBS).

2.2.2. Freeze drying

The microencapsulation using the freeze-dry method is conducted by suspending the bacteria homogeneously in a suspension medium, such as alginate for *B. paramycoides* PA.4, chitosan for *B. altitudinis* PH.1 and arabic gum for consortium of *Bacillus* spp. (including *B. albus, B. cereus, B. proteolyticus)* Then, around 0.2 ml is put into the glass. The bacteria submerged in a material are soaked in ethanol at 60°C-70°C for 2 to 10 minutes, connected to a freeze dryer with the manifold type for 4 to 20 hours under 1 Pa vacuum, and sealed by heating to maintain the vacuum. The completed microcapsule of bacteria is stored at -20°C in a refrigerator [18].

2.2.3. Cell viability and storage stability

Cell viability was determined using the standard plate count method. A sample of 0.5 g of encapsulated powder (freeze-dry) was reconstituted with 100 mL of 0.85% saline and it was serially diluted and plated onto ZoBell agar. Colony-forming units were enumerated manually after incubation at 37 °C for 24 h [19].

2.2.4. Yield encapsulation

The yield encapsulation test was carried out to determine the efficiency of the microcapsule coating on bacteria. The efficiency of the coating is seen from the number of bacteria that can survive after going through the encapsulation process [20]. The formula to calculate the encapsulation yield is as follows:

$$EY = N/N_0$$

Where:
EY = yield of encapsulation
N = the number of living cells after the process drying
N_0 = the number of living cells added (initial density)

2.2.5. Shelf-life

Shelf life is the time interval of a product as long as it is still of good quality to ensure the quality and safety of the product contents are well protected by packaging materials. The shelf-life test is carried out to determine the shelflife of bacterial microcapsules until the bacteria cannot be used properly and lose their viability [20]. Viability and microencapsulation structure during shelf life 14 days with cell shelf-life test encapsulated using the equation:

$$T = \frac{8 \log S_o}{\log S_o - Sac}$$

Where:
So = viability at zero days or immediately calculate viability after processing encapsulation
Sac = survival rate after being stored for 14 days at optimal temperature cell growth.
T = shelf-life of encapsulated cells

2.2.6. Screening of antipathogenic bacteria

The antipathogenic test will be carried out using the cross-streak method and overlay. The cross-streak method will be conducted by streaking each of the sponge-associated bacteria isolates with the causative bacteria of vibriosis in

vannamei shrimp, which is a collection isolate of the Centre of Fisheries Brackish Water Aquaculture (BBPBAP) Jepara. It is incubated horizontally and vertically on the surface of Marine agar medium for 2x24 hours at room temperature. Meanwhile, the overlay method will be conducted by pouring soft agar of disease bacteria into the surface of ZoBell 2216E medium colonized by healthy coral symbionts [21]. Positive results are obtained by selecting a colony of bacteria producing an inhibition zone to the test strain. The agar diffusion method had been used for rescreening in a ZoBell 2216E medium using agar plug. An observation by measuring the diameter of the inhibition zone of each symbiont bacterium to the test strain has been also conducted.

2.2.7. Moisture content

The average moisture content (wet basis) of the freeze dry powder was measured gravimetrically. A known mass of sample (0.5 g) was placed in an aluminum foil pan and dried in a vacuum oven at 100 °C for a period of 12 h. The initial and final weights were used to calculate the wet basis moisture contents. The experiments were carried out in duplicates and averaged values were taken to calculate the final moisture content [19].

2.2.8. Scanning Electron Microscope (SEM)

Samples tested using SEM are required under circumstances conductive so that the electron beam that is fired can be absorbed by the sample where the electron beam was connected to an amplifier that produces an image on the monitor. During the SEM test, the condition of the room inside must be in a good vacuum so that electrons can move freely and produce a clear image on the monitor. The morphology of freeze-dry microencapsulated samples was examined using a scanning electron microscope. The dried samples were mounted on the specimen holder and sputter-coated with gold (2 min, 2 mbar) and observed at 20 kV and a vacuum of 9.75 105 torr [19].

2.3. Data Analysis

Data analysis is carried out descriptively, to obtain systematic, factual, and accurate results so that it can be interpreted correctly. Therefore, the problem can be analyzed carefully, and appropriate conclusions can be drawn. The data presented in the form of graphics and tables of results which are then strengthened by literature study.

3. Results and discussion

3.1. Cell Viability and Storage Stability

Based on the results (Table 1), the density of *B. paramycoides* bacteria before microencapsulation process was 2.0 x 10^8 CFU/ml, *B. altitudinis* was 2.0 x 10^8 CFU/ml, and consortium of *Bacillus* spp. was 1.3 x 10^7 CFU/ml. The viability test for bacterial microcapsules was carried out by diluting the microcapsules in stages, then at each dilution, spreading them evenly on ZoBell agar media. The calculation results showed that the density of *B. paramycoides* bacteria after the microencapsulation process using alginate coating was 1,8 x 10^8 CFU/ml. Then the density of *B. altitudinis* bacteria using chitosan coating was 1.8 x 10^8 CFU/ml and *Bacillus* spp. using an arabic gum coating was 9.5 x 10^6 CFU/ml. For the last test, bacterial microcapsules were stored at -20°C in a refrigerator for 14 days. The viability showed that for *B. paramycoides* microcapsules was 8.9 x 10^7 CFU/ml, *B. altitudinis* microcapsules was 1.8 x 10^8 CFU/ml, and consortium of *Bacillus* spp. (*B. albus, B. cereus, and B. proteolyticus*) was 1.8 x 10^5 CFU/ml.

3.2. Yield encapsulation

Based on calculations, the yield level for encapsulation of *B. paramycoide*s PA.4 microcapsules coated with alginate was 90%, *B. altitudinis* PH.1 microcapsules coated with chitosan was 90%, and microcapsules of *Bacillus* spp. consortium with arabica gum coating was 70% (Fig. 1). These results support an earlier study which reports a decrease in survival of lactic acid bacteria as dehydration increased [22] and that optimum moisture content is required to maintain the cell survival during storage [23].

3.3. Shelf life

According to the result, the shelf life of *B. paramycoides* PA.4 microcapsules coated with alginate was 8.3 years, microcapsules *B. altitudinis* PH.1 coated with chitosan was 9.1 years, and microcapsules *Bacillus* spp. coated with arabic gum for 6.8 years. These results clearly indicate that the survival of encapsulated cells depends upon the type of coating materials [19]. Sakane and Hiroshima also mention that the difference in stability between the dried cultures of bacteria may result from the difference in the protective media used [24].

3.4. Screening of Antipathogenic Bacteria

After screening with the plug agar method, bacterial microcapsules consistently inhibited *Vibrio* spp. Several studies have reported its antibacterial activity against aquaculture pathogenic bacteria, especially *Vibrio* spp. [25]. The results (Fig. 2) showed that alginate-coated *B. paramycoides* PA.4 bacterial isolate had an inhibition zone of 13.96 mm against *V. anguillarum* bacteria. The chitosan-coated *B. altitudinis* PH.1 bacterial isolate had an inhibition zone of 16.07 mm against *V. alginolyticus* bacteria. Therefore, both *B. paramycoides* PA.4 and *B. altitudinis* PH.1 microcapsules categorized into the strong activity (11 – 20 mm) to inhibit *V. anguillarum* and *V. alginolyticus* bacteria [26]. Then, the arabic gum-coated *Bacillus* spp. (consortium) bacterial isolate had an inhibition zone of 9.88 mm against *V. vulnificus* bacteria. This result revealed that arabic gum-coated consortium of *Bacillus* sp. microcapsules categorized into moderate activity (5 – 10 mm) to inhibit *V. vulnificus* bacteria [27]. The differences in the ability to produce the clear zone were presumably dependent on the secondary metabolites that were produced by test isolates [28]. Nevertheless, the degree of inhibition depends on several other factors and may change in the body.

3.5. Moisture Content

The water content in *B. paramycoides* PA.4 microcapsules coated with alginate was 7%, microcapsules *B.*

altitudinis PH.1 coated with chitosan was 17%, and microcapsules consortium of *Bacillus* sp. coated with arabic gum was 12% (Fig. 3). The lowest moisture content was found in the alginate sample and the highest moisture content was found in the chitosan sample. The higher moisture for freeze-dried powders could be affected by the lower process temperature. The lower freezing temperature results in a smaller pore size in the freeze-dried product due to a higher cooling rate and increased nucleation. Small pores resist mass transfer and act as a barrier against sublimation, retaining moisture in freeze-dried powder [29].

3.6. Scanning Electron Microscope (SEM)

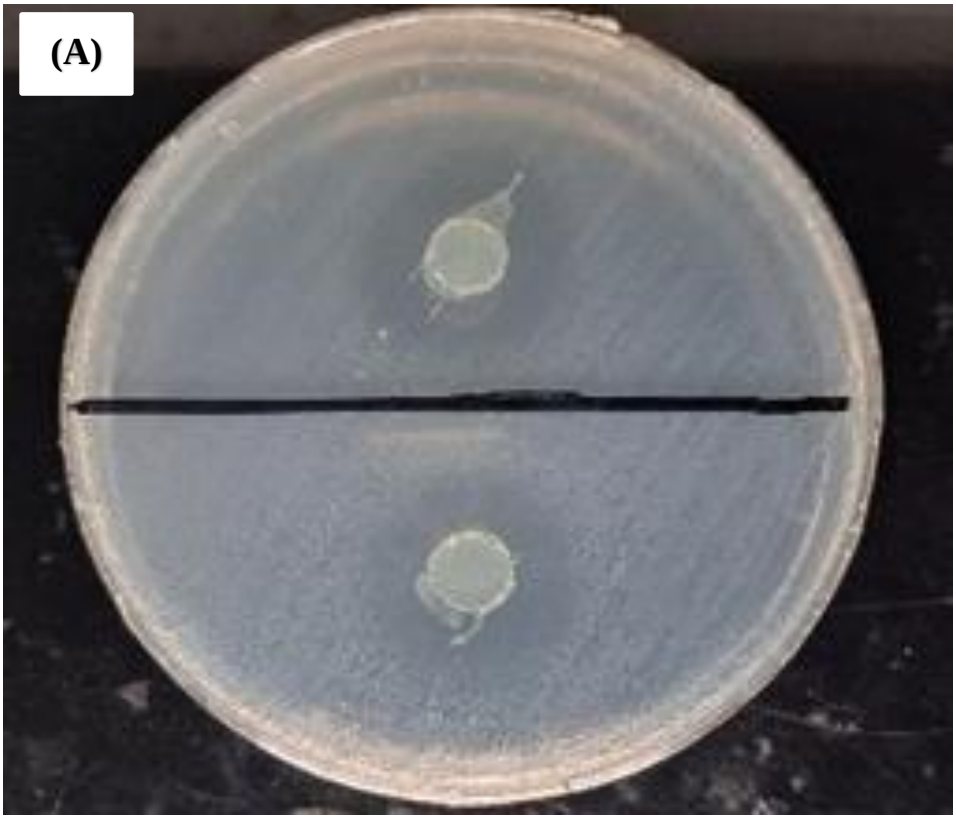
The surface shape of the microcapsules can be seen using a scanning electron microscope (SEM) instrument. The surface of the microcapsules affects the viability of the bacteria contained in the bacterial microcapsules [30]. SEM testing was carried out with magnifications of 500x, 1000x, 6000x, and 15,000x. The morphology of three freeze-dried microcapsule powders (Fig. 4) shows irregular shapes and surfaces, cake-like structures with highly porous surfaces. The freeze-drying involves freezing, primary drying (sublimation), and secondary drying (desorption), the water in the solution sublimates directly from ice to water vapor in the vacuum environment, so the surface of the powder will be microporous due to water loss [31-32]. The pores in the microcapsules are formed as a consequence of ice crystal formation during the freezing step, which sublimes during, freeze-dry [33]. Encapsulation facilitates the bacteria to gain consistent characteristics resulting in higher viability than non-encapsulated bacteria [34]. Based on the results, even the viability of the three strains of marine bacteria in the microcapsules showed decrease after the drying process and storage for 14 days, these three samples indicate that wall material protected the probiotic cells from external environmental conditions such as temperature and moisture [19]. The stability of bacterial microcapsules is related to storage conditions, coating material, and bacterial characteristics. The storage of bacterial microcapsules under conditions with low moisture, temperature [35] and oxygen [36-37] can maintain the structure of the coating material, so that the bacteria are less susceptible to environmental exposure. Furthermore, the Gram-positive bacteria used in this study can form spores, enabling survival under low moisture conditions [38]. The goal of encapsulation is to create a micro-environment in which the bacteria will survive during processing and storage and released at appropriate sites (e.g. small intestine) in the digestive tract [39]. The benefits of encapsulation to protect probiotics against low gastric pH have been shown in numerous reports [40]. Zayed and Roos have shown that *Lactobacillus salivarius* subsp. salivarius with trehalose + sucrose in addition to skim milk yielded a survival rate of 83–85% immediately after freeze dry [23]. The survival rate or viability to which a bacteria will stabilize can be estimated by the accelerated storage test immediately after drying. In another study, it was observed that microencapsulation was found to sustain the viability of probiotic organisms in freeze-dried yoghurt beyond 6 months of storage [41]. In this study, each isolate is capable of inhibiting specific *Vibrio* spp. due to every species having different mechanism to counter non favourable environment through secreting various proteinase and antimicrobial compounds against pathogens [42]. According to El-Kholy and colleagues, competition in nutrient absorption was a common phenomenon in natural habitats [43]. Several bacterial species use the antagonistic activity or inhibition property as a weapon against their competitors. In general, bacteria produce several antimicrobial compounds or bacteriocins to inhibit or kill other competitor bacterial species [44]. The other mechanism of bacteria to survive is producing bacteriocin, from group of *Bacillus* sp. [42, 45]. also producing secondary metabolites such as alkaloids, flavonoids, and saponins that are capable of inhibiting the growth and reproduction of pathogens [46]. Possibly one or several combinations of these mechanisms was responsible for these results. Several studies showed that *B. cereus* is capable of inhibiting *V. alginolyticus* [45-46] by producing secondary metabolites compounds i.e., alkaloid, flavonoid, and saponin. These secondary metabolites compounds are capable of disrupting the peptidoglycan, one of the major constituents of bacterial cell wall, that causes lysis in cells [46]. According to Pudziuvelyte's work, an increased moisture content could negatively affect the freeze-dried powders during storage. Higher moisture content in the freeze-dried powders could reduce the quality of the powders, such as lower flowability, change the color, flavor, reduce amounts of predominant compounds, and their activity [47]. Also, freeze-dried powders with high moisture content could be the perfect environment for microorganisms (bacterial contamination). Using different coating materials impacts moisture content of freeze-dried powders. Ezhilarasi have been reported that using whey protein isolate and maltodextrin the moisture content is higher than using a mixture of these wall materials (15.65%, 12.56%, and 11.53%, respectively) [29]. During the freezing process, the higher protein concentration in the solution may induce aggregation and make interstitial water less available for freezing. The results of *B. paramycoides* PA.4 SEM analysis showed that the microcapsules are irregular, not round and have different sizes. This outcome is possible because when freeze-drying or the freeze-dryer does not dry completely and on time. Stirring using a magnetite stirrer is uneven. Factors influencing shape and morphology microcapsules are stirring speed and viscosity. Quickly stirring the emulsion, its produced becomes smaller. The size of the encapsulation diameter affects the ability. The matrix protects the bacteria inside [48]. It has been confirmed that the nutrient composition, buffer salts, rehydration temperature, and pH of the rehydration medium can affect the recovery of the bacteria [49]. Among them, the addition of relevant components of the growth medium to the rehydration medium is beneficial to the recovery of the viability of the bacterium [50]. Therefore, the rehydration status of the bacterial powder also needs further research in the future.

Figure 1. The results of bacterial microcapsules freeze-dried: a) *B. paramycoides* PA.4; b) *B. altitudinis* PH.1; c) Consortium of *Bacillus* spp.

Table 1. The viability of bacterial microcapsules with different coating materials

Bacteria	Coating	Viability before microencapsulated (CFU/mL)	Viability after microencapsulated (CFU/mL)	Viability 14 days after microencapsulated (CFU/mL)
***B. paramycoides* PA.4**	A	2.0×10^8	1.8×10^8	8.9×10^7
***B. altitudinis* PH.1**	B	2.0×10^8	1.8×10^8	1.6×10^8
Consortium of Bacillus (B. albus, B. cereus, B. proteolyticus)	C	1.3×10^7	9.5×10^6	1.8×10^5

Notes: A = Alginate; B = Chitosan; C = Arabic gum



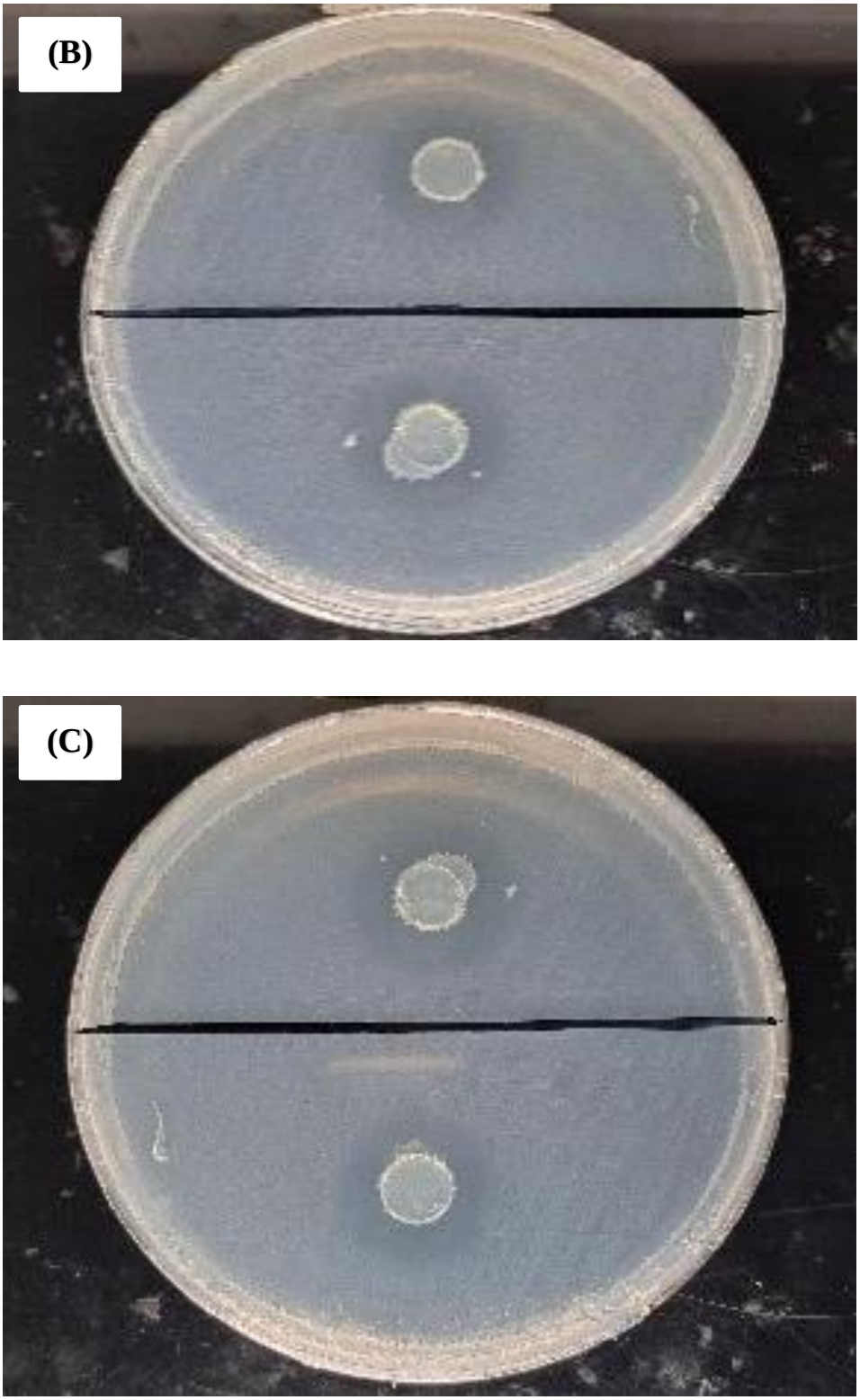


Figure 2. The agar plugs result of bacterial microcapsules: a) *B. paramycoides* PA.4 against *V. anguillarum*; b) *B. altitudinis* PH.1 against *V. alginolyticus*; c) Consortium of *Bacillus* spp. against *V. vulnificus.*

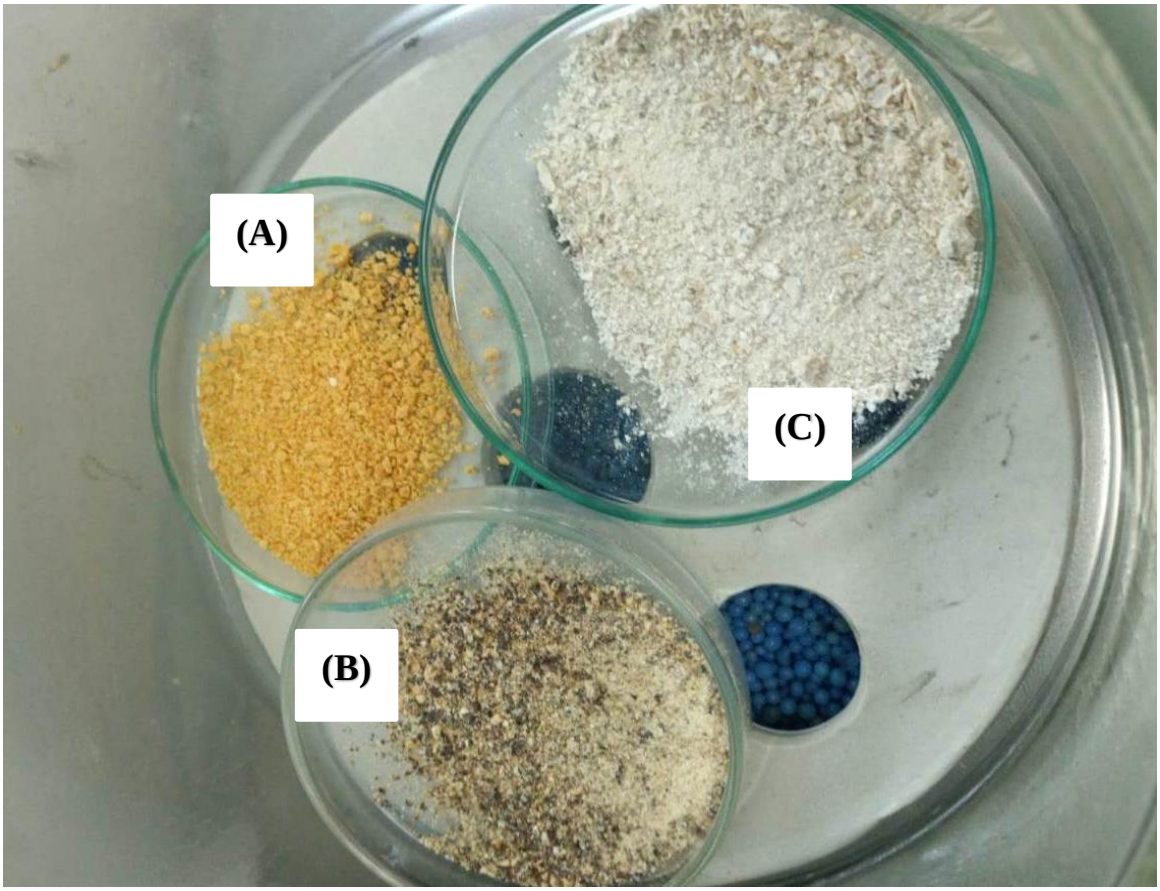
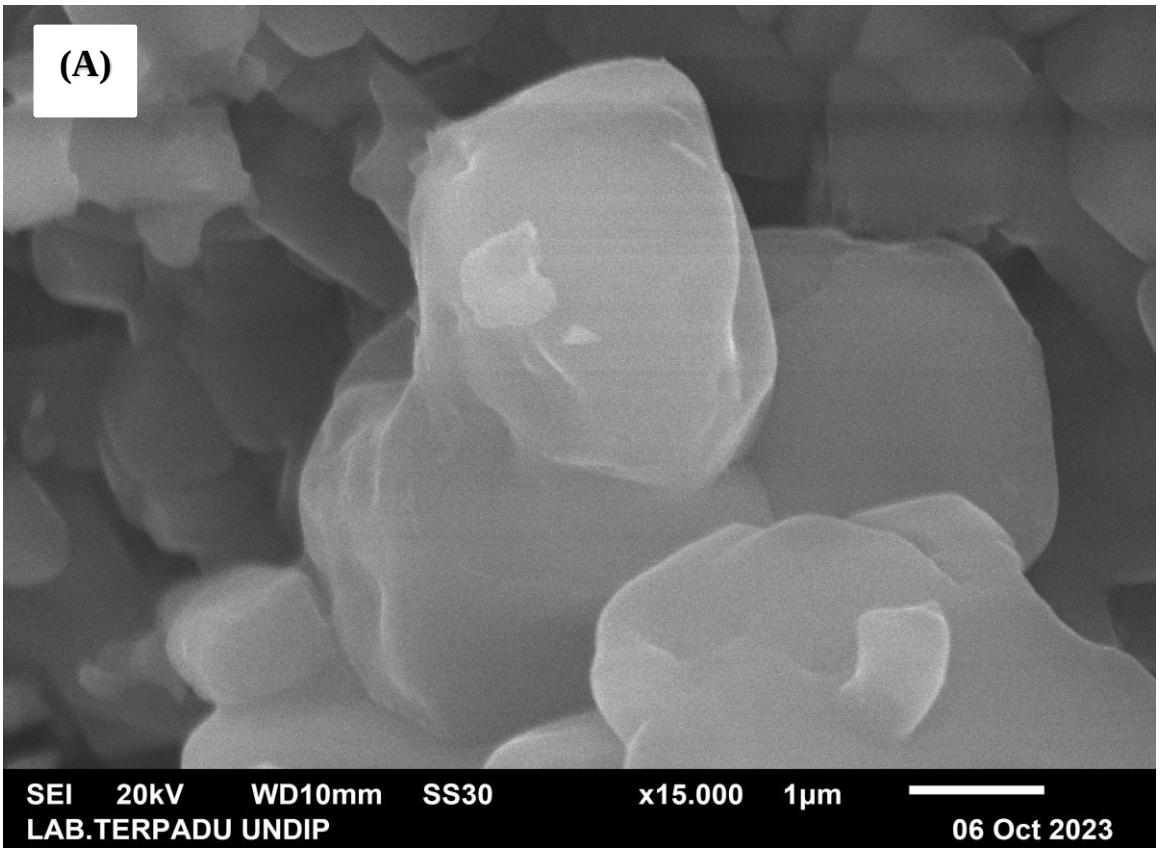


Figure 3. The moisture content result of bacterial microcapsules: a) *B. paramycoides* PA.4 microcapsules; b) *B. altitudinis* PH.1 microcapsules; c) Consortium of *Bacillus* spp. microcapsules inside the desiccator.



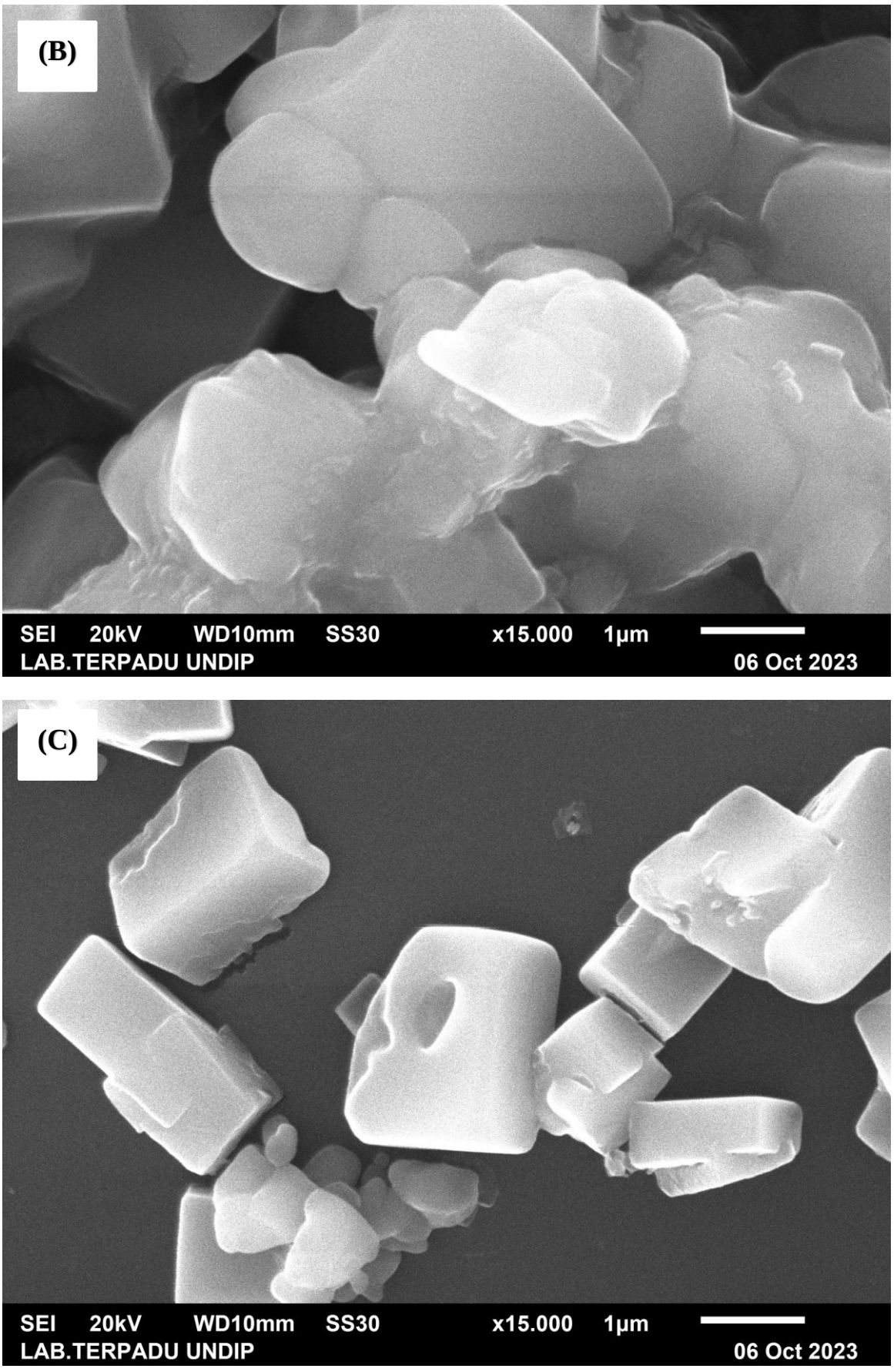


Figure 4. The results of SEM analysis of bacterial microcapsules: a) *B. paramycoides* PA.4 coated with alginate; b) *B. altitudinis* PH.1 coated with chitosan; c) Consortium of *Bacillus* spp. coated with arabic gum

The three bacterial microcapsules samples showed that the wall material used could protect probiotic cells from external environmental conditions such as temperature and humidity. Besides that, the water content contained in bacterial microcapsules requires optimal conditions to maintain cell viability during storage and survive in low pH conditions. Bacterial microcapsules that are tested or inhibited give final results that can produce several antimicrobial compounds or bacteriocins to inhibit or kill other competing bacterial species as proven by bacterial microcapsules capable of inhibiting disease bacteria caused by vibriosis. In addition, bacterial microcapsules with high water content can provide a perfect environment for microorganisms. Based on the observation parameters that have been carried out, the best results were obtained, namely *B. altitudinis* PH.1 bacterial microcapsules, followed by *B. paramycoides* PA.4 bacterial microcapsules, and last *Bacillus* sp. bacterial microcapsules. This is because the best bacterial resistance and long storage period are *B. altitudinis* PH.1 microcapsules. Apart from that, viability plays an important role in bacterial resistance to suppress the spread of vibriosis. This study indicated *Bacillus* spp. bacteria could be developed as antivibriosis and to alleviate the problems of antimicrobial resistance in aquaculture. Several publications showed *B. cereus* could be employed as probiotics in aquaculture, especially in shrimp farming.

4. Conclusions

The result indicates that the *B. altitudinis* PH.1 coated with chitosan show the highest viability at $1.8x10^8$ CFU/ml after 14 days preserved in -20°C. The moisture content of this treatment revealed 17% as the highest than the other treatment, therefore the predicted shelf life for chitosan coated reach 9.1 years. Furthermore, the antivibrio of *B. altitudinis* PH.1 show the strong activity with clear zone inhibition at 16.07 mm against *Vibrio alginolyticus*. The structure of *B. altitudinis* PH.1 under SEM observation was irregular shape and size. Therefore, this study found that chitosan could be an alternative source to preserve a potential bacterium using microencapsulation technology.

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