VOLUME 10 NOMOR 2 DESEMBER 2023

ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

EVALUATING THE LONG-TERM STORAGE TIME VIABILITY AND SIZE DYNAMICS OF *BACILLUS* SP. BIOENCAPSULATION IN SODIUM ALGINATE MATRIX

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ABSTRACT

The use of biological agents such as Bacillus sp. bacteria has begun to be widely used by farmers as a new form of control. Bacillus sp. needs to require special methods in its application because it has certain living conditions, and Bacillus sp. is vulnerable to environmental pressures. Bioencapsulated formulation in the form of granules is considered effective as a form of bacterial application in soil because it is able to protect Bacillus sp. and maintain survival. This study aims to determine the best concentration of sodium alginate in maintaining the viability of Bacillus sp. at the in vitro stage and to see changes in the size of the beads. The beads were made using extrusion method by combining Bacillus sp. suspension and sodium alginate suspension at 1%, 1.5%, and 2% concentration. The concentration of sodium alginate was not a major factor in changes in bead size and viability test results experienced significant changes in each observation. 1.5% and 2% concentrations can reach the highest viability value of $3x10^6$ CFU/mL and decreased during 1 month of observation. Changes in bead size and viability were caused by the alginase enzyme produced by Bacillus sp.

Keywords: *Bacillus* sp., Bioencapsulation, Sodium alginate, Viability, Beads size

ABSTRAK

Penggunaan agen hayati seperti Bacillus sp. bakteri sudah mulai banyak digunakan oleh petani sebagai bentuk pengendalian baru. Bacillus sp. perlu memerlukan metode khusus dalam penerapannya karena mempunyai kondisi kehidupan tertentu, dan Bacillus sp. rentan terhadap tekanan lingkungan. Formulasi bioenkapsulasi berbentuk butiran dinilai efektif sebagai bentuk aplikasi bakteri pada tanah karena mampu melindungi Bacillus sp. dan menjaga kelangsungan hidup. Penelitian ini bertujuan untuk mengetahui konsentrasi sodium alginat terbaik dalam menjaga viabilitas Bacillus sp. pada tahap in vitro serta melihat perubahan ukuran manik-manik. Manik-manik dibuat menggunakan metode ekstrusi dengan menggabungkan suspensi Bacillus sp. dan suspensi sodium alginat konsentrasi 1%, 1,5%, dan 2%. Konsentrasi sodium alginat tidak menjadi faktor utama dalam perubahan ukuran manik-manik dan hasil uji viabilitas mengalami perubahan yang signifikan pada setiap pengamatan. konsentrasi 1,5% dan 2% dapat mencapai nilai viabilitas tertinggi yaitu $3x10^6$ CFU/mL dan menurun secara selama 1 bulan pengamatan. Perubahan ukuran manik-manik dan viabilitas disebabkan oleh enzim alginase yang dihasilkan oleh Bacillus sp.

Kata Kunci: *Bacillus* sp., Bioenkapsulasi, Sodium alginat, Viabilitas, Ukuran manik-manik

***Received**: 16 November 2023* ***Accepted**: 19 December 2023* ***Published**: 20 December 2023*

INTRODUCTION

Bacillus sp. is a known biological control agent that can inhibit various pathogens, including *Colletotrichum capsici* (Sutarti and Wahab 2010), *Sclerotium rolfsii* (Abidin *et al*. 2015), *Rhizoctonia* sp., *Pythium* sp., *Fusarium* sp., *Phytophthora* sp., *Xanthomonas* sp., and *Erwinia* sp. (Fakhruddin 2020). The application of *Bacillus sp*. to plants often requires formulation for ease of use, with liquid formulation being a commonly used option (Rojas-Padilla *et al*. 2022). However, liquid formulations have several drawbacks, particularly in terms of storage stability and susceptibility to contamination (Tu *et al*. 2015), and they have a limited shelf life (Stephens and Rask 2000). This contradicts the opinion of Soesanto (2017) that a good formulation should exhibit product stability during storage.

Bioencapsulation is a formulation that is widely recognized for its effectiveness as a means of delivering biological control agents. According to Kim *et al*. (2012), bioencapsulation is an effective approach for safeguarding microorganisms in the soil and controlling their sustained release. One of the key advantages of bioencapsulation as a formulation is the enhancement of product stability and the safety of active ingredients (Mishra 2016).

Bioencapsulation is created using sodium alginate, which is the most common material for the encapsulation process because this material has non-toxic, biocompatible, and cost-effective characteristics. It can be employed with simple methods (Uyen *et al*. 2020). According to Szczech and Maciorowski (2016), sodium alginate offers advantages such as easy gel formation, good solubility, low viscosity, and suitability for mild conditions, which enables the trapping of cells with minimal loss of viability.

The bioencapsulation process also affects product stability and viability. Making bioencapsulation using extrusion or the dropping method (Solanki *et al*. 2013), has advantages such as not causing damage to bacterial cells and maintaining high viability. This method utilizes the force of gravity to form beads by dripping a mixture of sodium alginate and *Bacillus* bacteria under strong pressure.

Previous research related to the viability of bioencapsulated formulations with bacteria as the core material in carbofuran media, conducted by Priyani *et al*. (2018) showed that bacterial viability increased to 96.4%. Research by Bevilacqua *et al*. (2020) demonstrated that encapsulated Saccharomyces cerevisiae fungi stored at 4 °C for 30 days did not exhibit a significant decrease in viability, while storage at 25 °C for 7 days showed a notable decrease in viability. Bevilacqua *et al*. (2020) in their research, also mentioned that one of the factors affecting the viability of bioencapsulation is the concentration of the polymer material used. Chi *et al*. (2020) In this research, used the *Bacillus megaterium* microencapsulation method for saline soil remediation. The utilization of this bioencapsulation formulation has also been widely adopted as a Plant Growth-Promoting Bacteria (PGPB) formulation, as demonstrated by Trivedi and Pandey (2008); Farhat *et al*. (2014); Saberi-Rise and Moradi-Pour (2020)

The viability and size of beads are crucial in bioencapsulation, influencing both the stability during storage and the effectiveness of the results. This study aims to assess changes in the viability and size of beads during storage and determine the most efficient sodium alginate concentration that can uphold the viability and size of beads throughout the storage period.

MATERIALS AND METHODS

Location and Time

The production of beads, along with testing their viability and monitoring changes in size, was conducted at the Laboratorium Kesehatan Tanaman, Faculty of Agriculture, UPN Veteran Jawa Timur. The research spanned from May 2023 to June 2023.

Tools and Materials

The tools used in the study were petri dishes, stoves, autoclaves (all american 50x), micropipettes (DLAB Single Channel Fixed 200 µl), beakers, ose needles, analytical scales (Kern PCB), incubator cabinets, vortex (Maxi Mix II), media bottles, 10 ml syringe Ø 0.21 cm, glass stirrers, hockey sticks, 500 ml beakers, 100 ml vials,

Compond microscope (Olympus CX33), mitutoyo vernierre.

The materials used in the study were *Bacillus sp*. isolate cz 30 collection of Dr. Ir. Yenny Wuryandari MP., Sodium alginate (Merck) viscosity 256 mPas medium category, distilled water, $CaCl_2$ (calcium chloride), Nutrient Agar media (Himedia M001-500G), labels, technical NaCl, 70% alcohol.

METHODS

Rejuvenation and Suspension Preparation of *Bacillus sp*.

Preparation of bacterial suspensions using distilled water by rejuvenating the bacteria first by taking 1 ose isolate and then scratched on NA media and then incubated for 48 hours at room temperature to obtain *Bacillus sp*. The isolate was then suspended in sterile water in a separate form with a population density of 10^8 CFU/ml as much as 10 ml as a source of inoculum (Saputra *et al*. 2015).

Preparation of Bioencapsulation Formulation

The preparation of bioencapsulation formulation consists of making biopolymer suspension and making $CaCl_2$ solution. Biopolymer suspension was made by dissolving sodium alginate into distilled water using three different concentration treatments of 1%, 1.5%, 2%. The biopolymer suspension was sterilised using an autoclave. $CaCl_2$ solution was made by dissolving $CaCl_2$ into distilled water with a concentration of 3% and sterilised using an autoclave at 120 ^{O}C for 20 minutes.

Making of Beads

The biopolymer suspension for each treatment was combined with a *Bacillus sp* suspension at a ratio of 10:1 (v/v) (Khan *et al*., 2013). The resulting mixture was then slowly dripped into a 3% CaCl2 solution using a syringe (Li *et al*. 2013).

Beads Washing

The beads were washed three times with sterile 0.85% NaCl solution to eliminate surface contamination, as described in the analysis by (Ratnasari *et al*. 2014).

Beads Observation

The surface of the beads was examined under a microscope to identify the presence of *Bacillus sp*. trapped inside the beads (Figure 1). Khimmakthong *et al*. (2020) conducted research, noting colonies of *Bacillus sp*. bacteria on the surface of the beads. This observation suggests the successful entry of *Bacillus sp*. into the beads.

Beads Diameter Measurement

The bead diameter was measured using a caliper at the start of the observation (week 0) and at the conclusion of the observation (week 4) to assess any alterations in bead diameter throughout the storage period. Changes in bead diameter were determined using the formula for the rate of change as follows:

Rate of Diameter Change (dD/dt) =
(Final Diameter - Initial Diameter) / Time

Viability test

The beads were stored in a jar at room temperature (20-25°C) for 30 days, with periodic observations conducted once a week. One gram of beads was crushed with a scalpel until coarsely ground. The crushed material was then placed in a test tube containing 10 ml of sterile distilled water and vortexed for 5 minutes. Subsequently, 1 ml of the vortexed solution was extracted using a micropipette and inoculated onto NA media, followed by incubation for 24-48 hours (Pringginies 2020).

Viability of *Bacillus sp*. in the bioencapsulated formulation was obtained by counting the population of colonies that grew using the total plate count (TPC) method using the formula according to (Hanif 2016).

$$FU/gram = \frac{Total\ number\ of\ colonies}{Volume\ spread\ to\ the\ petri\ dish\ x\ dilution\ factor}$$

Screening of Enzym Alginase

The alginase enzyme screening method was conducted following the procedure outlined in (Tavafi *et al*. 2017). The alginase enzyme screening medium comprised 0.5% sodium, 1.0% peptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar

(pH 7.0). The bacteria were incubated at 37°C for 48 hours. Confirmation of the presence of the alginase enzyme was achieved by the formation of a clear zone around the bacteria.

Data Analysis

The data were processed using Microsoft Excel and SPSS Statistics 25, visualized with GraphPad Prism 10, and the images in the article were edited using Photoscape X.

RESULT

Beads Observation

The surface of the beads was examined under a microscope to identify the presence of *Bacillus sp*. trapped inside the beads (Figure 1). Khimmakthong *et al*. (2020) conducted research, noting colonies of *Bacillus sp*. bacteria on the surface of the beads. This observation suggests the successful entry of *Bacillus sp*. into the beads.

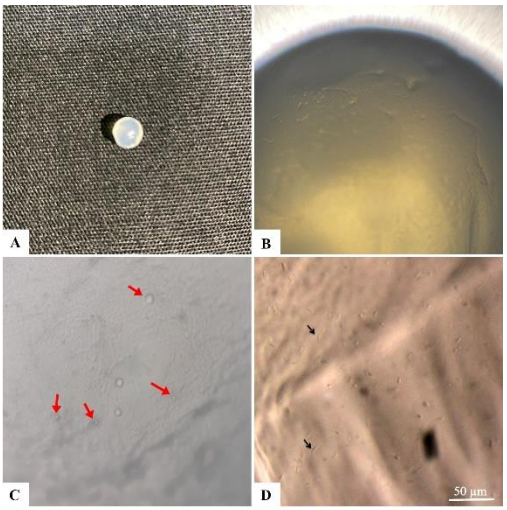

Figure 1. Observations of the beads included: (A) visual shape, (B) surface shape at 20x magnification, (C) *Bacillus Bcz* 30 found on the surface at 100x magnification, and (D) *Bacillus*subtilis cells on the surface at 40x magnification (Khimmakthong *et al*., 2020).

The presence of bacteria on the surface of the beads aligns with research conducted by Fanucci and Seese (1991), demonstrating that alginate is capable of absorbing bacteria through the absorption process and retaining bacteria during the gel matrix formation process. As shown in Figure 1, it can be inferred that the bacteria *Bacillus sp*. are not concentrated at one point but are spread throughout the beads.

Diameter change

According to table 1., the results of the ANOVA test, it indicates that there is no significant difference in the relationship between sodium alginate concentration and storage time. This lack of significance may be attributed to the presence of other factors not considered in the statistical analysis that could influence the association between sodium alginate concentration and storage time. Such factors include humidity and temperature during the storage period. During the observation of diameter changes, temperature and humidity became potential anomalies because in this study, they were not measured and controlled. Nevertheless, temperature and humidity are among the factors contributing to changes in the size of the beads.

Table 1. Statistical Test Results of Beads Diameter Change Rate

Treatment	Observation initial bead diameter (mm)	Diameter of beads at end of observation (mm)	Beads Diameter Change Rate
K1	3,75	3,04	0,33b
K2	3,5	2,9	0,15a
K3	3,4	2,1	0,17ab

*Numbers followed by the same letter are not significantly different in the 5% DMRT table.

Figure 2. indicates a decrease in bead diameter over the 4 weeks of observation. At the beginning of the observation, beads with a sodium alginate concentration of 2% had a diameter of 3.75 mm, which decreased to 3.03 mm at the end of the observation, resulting in a rate of change in diameter of -0.18/week. Similarly, beads with a sodium alginate concentration of 1.5% started with a diameter of 3.5 mm and ended with 2.89 mm, reflecting a rate of change in diameter of -0.15/week. Beads with a sodium alginate concentration of 1% began with a diameter of 3.43 mm, and by the end of the observation, the diameter had reduced to 2.08 mm, with a rate of change of -0.33/week.

These results suggest that sodium alginate concentration may have a mitigating effect on the decrease in bead diameter. Additionally, beads with a 1% concentration exhibited a greater rate of diameter reduction compared to beads with concentrations of 2% and 1.5%. This difference in diameter reduction is attributed to variations in viscosity and concentration—higher concentrations result in increased viscosity and denser bead formation, consequently reducing the rate of size reduction.

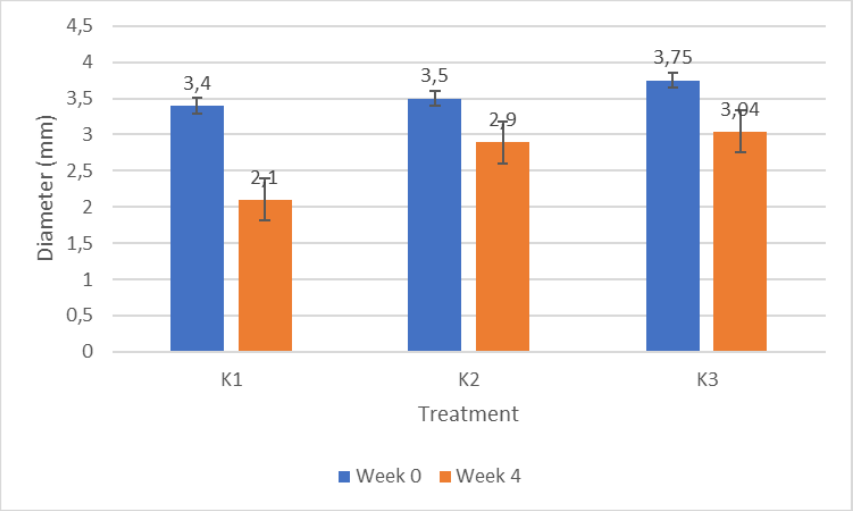

FIgure 2. Changes in Beads Diameter

Viability

The graph (Figure 3) illustrates changes in cell viability at various concentrations over a four-week period. At week 0, the 2% concentration exhibited a cell viability of 10^6, while the 1.5% and 1% concentrations had initial cell viabilities of approximately $4x10^5$ and 10^6, respectively. However, during the first week, the 2% concentration experienced a drastic decrease to $3x10^2$, whereas the 1.5% concentration increased to 10^6. In week 2, the 2% concentration rebounded to $3x10^6$, while the 1.5% concentration remained high at $3x10^6$. Additionally, in week 3, the 2% concentration returned to 10^6, while the 1.5% concentration decreased to $8x10^5$. By week 4, the 2% and 1.5% concentrations reached 10^4 and 10^5, respectively.

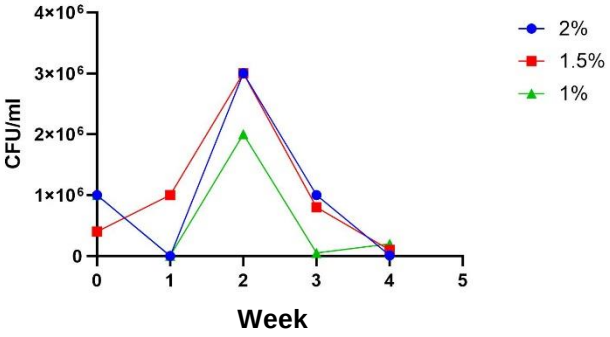

Figure 3. *Bacillus sp*. Viability

The graph (Figure 3) illustrates noteworthy changes in cell viability at various concentrations over a four-week period. The 2% concentration initially decreased before rebounding, whereas the 1.5% concentration experienced an initial increase followed by a subsequent decrease. These observations suggest that cell concentration can significantly influence cell viability over time.

Screening of Enzym Alginase

Based on (Figure 4), *Bacillus* Bcz 30 bacteria, grown on growth media containing sodium alginate as the sole carbon source, exhibited alginase enzyme activity after a 48-hour incubation period. The presence of a clear zone demonstrated *Bacillus* Bcz 30 capability to produce the alginase enzyme, highlighting its enzymatic activity (Subaryono *et al*. 2015). The successful formation of clear zones suggests that these bacteria possess the potential to enzymatically degrade sodium alginate through their alginase activity.

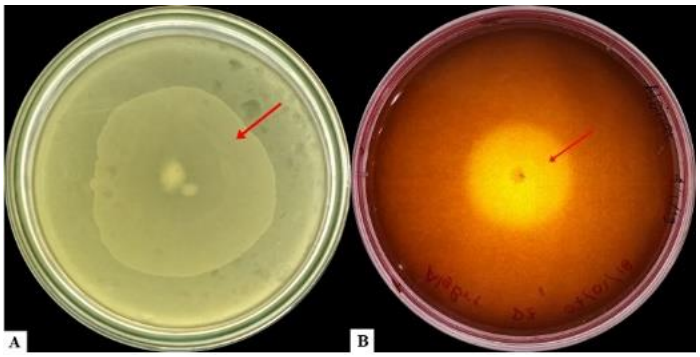

Figure 4. Alginase Clear Zone. (A) *Bacillus Bcz* 30. (B) Isolat T513 (Zilda *et al*. 2019)

DISCUSSION

The changes in bead diameter and the viability of *Bacillus sp*. within the beads are attributed to a common factor—the presence of the alginase enzyme produced by *Bacillus sp*. The decrease in bead diameter during the storage period results from the degradation of bead walls by *Bacillus sp*. This degradation is possible because *Bacillus sp*. isolate Bcz 30 possesses a specific enzyme capable of breaking down beads. According to Subaryono *et al*. (2016), *Bacillus sp*. secretes a special enzyme called alginate lyase or alginase.

The presence of the alginase enzyme (Figure 4) is believed to be a key factor controlling the release of active ingredients from the beads. This is corroborated by Barzkar *et al*. (2022), who state that the endolytic alginase action mode of the alginase enzyme can break down alginate glycosidic bonds into smaller fragments. Breaking these bonds in the sodium alginate beads allows the release of *Bacillus sp*. within the beads. Thus, the activity of the alginase enzyme in breaking alginate glycosidic bonds serves as a crucial factor in controlling the release of microorganisms from the beads.

The graph (Figure 2) illustrates differences in the initial size of observations; higher concentrations yield larger bead sizes. This aligns with the findings of Kalalo *et al*. (2022), who note that increasing sodium alginate concentration leads to higher viscosity, resulting in larger droplet bead sizes. Diameter measurements also reveal that denser beads experience a slower diameter decrease, while looser beads exhibit a faster decrease. This suggests that the movement of the alginase enzyme and the bond-breaking process can be inhibited by high viscosity.

Tavafi *et al*. (2017) mention that the alginase enzyme is found in organisms synthesizing both non-alginate and alginate. In organisms producing non-alginate, alginase plays a vital role in absorbing alginate as a carbon source (Kim *et al*. 2011). The increase in viability value is thought to be due to the metabolic activity of *Bacillus sp*. in the beads. Sodium alginate, as a coating material, comprises carbon with the molecular formula (C6H7O6Na)n (Yunizal 2004). Ac-

cording to Potdar *et al*. (2019), bacteria utilize carbon from sources such as glucose (C6H12O6) for metabolism. Thus, it can be assumed that *Bacillus sp*. utilizes the carbon in sodium alginate for metabolic processes, influencing the viability value changes.

The viability value results (Figure 3) indicate an influence between storage time and viability. Longer storage periods correlate with decreasing viability values. The decrease is influenced by various factors, including insufficient carbon sources from sodium alginate, which occurs due to bead decomposition caused by degradation from the alginase enzyme. Changes in viability values are also influenced by the number of *Bacillus sp*. within the beads, leading to nutrient competition. Cell aging, associated with the decrease in viability, occurs 12–24 hours after incubation, according to Steiner (2021). The aging process in bacteria is linked to reduced viability as the lineage, strain, or population ages.

Earlier studies on viability have indicated that the beads' viability decreases over time during storage. Berninger *et al*. (2016) conducting research on the encapsulation of plant growth-promoting bacteria for seed coating revealed a decrease in viability during storage, dropping from an average of 3.5×10^7 to 1.3×10^6 CFU/100 mg of beads. Souza-Alonso *et al*. (2021) this study stated that Pseudomonas libanensis, encapsulated in alginate beads, experienced a decrease in viability from 10^8 to 10^7 CFU/gram after 90 days of storage occurred away from direct light, with a constant temperature (22-24 °C), and under controlled humidity conditions (30-40%).

Based on the results obtained, this study has implications for the action of beads, particularly as a formulation application of antagonistic agents for the biological control of agricultural plant diseases. Understanding the changes in the diameter of beads during the storage period can help identify factors influencing the quality and applicative potential of *Bacillus* sp beads in various contexts, including biomedical, agricultural, or food industry applications. Furthermore, comprehending the interaction between storage duration and changes in bead diameter can provide insight into the potential use of *Bacillus* sp beads in developing new products and technologies that are more efficient and sustainable. However, this study still offers a preliminary understanding of the viability and size of the beads during storage, so further tests need to be conducted using other microorganisms and different storage locations.

CONCLUSION

The concentration of sodium alginate employed in bead formation influences the alteration in diameter and viability of the beads during the storage period. Concentrations of 2% and 1.5% were determined to be optimal for bead preparation, exhibiting minimal changes in diameter and the highest viability during the observation period. However, the 1.5% concentration was deemed the most efficient option in bead formation.

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