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Effect of Calcium Chloride Concentration on Viability and Swelling Power of *Paenibacillus polymyxa* Encapsulated Beads in Vitro

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Abstract

Background: *Paenibacillus polymyxa* is a potent antagonist that can be utilized as a biological agent. The use of biological agents has gained interest among farmers, but their application in liquid formulations has not consistently maintained the optimal stability and viability of microorganisms. One promising approach to overcome this problem is bioencapsulation, which is a method of wrapping biological agents to protect bacteria in the soil and increase their efficiency. This study aims to assess the encapsulation efficiency, measure the viability of microorganisms in the beads, and determine the difference in swelling power of beads made with various concentrations of calcium chloride ($CaCl_2$) as a binding agent. **Method:** Beads were produced using the extrusion method by combining *Paenibacillus polymyxa* suspension, sodium alginate suspension, and adding $CaCl_2$ at concentrations of 1%, 3%, and 5%. **Results:** The results showed that different $CaCl_2$ concentrations can affect the viability of *Paenibacillus polymyxa* in beads. Beads made with $CaCl_2$ at 3% concentration were the best results in the encapsulation efficiency test compared to beads made with 1% and 5% $CaCl_2$ binders. In comparison, beads with 3% and 5% $CaCl_2$ concentrations were able to maintain the viability of microorganisms at a higher level and for a longer time than beads using $CaCl_2$ at 1% concentration. The decrease in viability and swelling power of the beads is thought to be caused by the carrier material used and the storage conditions. **Conclusion:** Bead treatment with 3% calcium chloride concentration was the best treatment for encapsulation efficiency in absorbing Paenibacillus polymyxa, amounting to 98.21%.

Keywords: Bioencapsulation; Swelling power; Calcium chloride; *Paenibacillus polymyxa*; Viability.

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Introduction

Paenibacillus polymyxa is an endophytic bacterium found in rice plants. It is an antagonist bacterium that can be used as a biological control agent for several types of diseases in food crops and horticultural crops. It can also produce various kinds of antibiotics that inhibit the activity of other microorganisms (Jannah et al., 2023). One disease that *P. polymyxa* can control is bacterial leaf blight (BLB) in rice, caused by the pathogenic bacterium *Xanthomonas oryzae*.

The use of *P. polymyxa* as a biopesticide in liquid formulations is common. Still, these formulations are susceptible to inappropriate environmental factors such as extreme temperatures, unbalanced pH, and high osmotic pressure. This is in accordance with the statement by Haspon et al. (2020), that liquid formulations are commonly used, but these formulations are susceptible to environmental stress. Therefore, further research is needed on new formulations that can address the stability and viability of microorganisms. One promising approach is encapsulation technology, including bioencapsulation, which is the wrapping of biological agents to protect bacteria in the soil.

Bioencapsulation is made from natural and synthetic materials with low viscosity

(Rumbiak & Hilal, 2022). Bioencapsulation can protect biological agents from mechanical stress and adverse environmental conditions, as well as provide controlled release of microorganisms, by reducing pollution during transportation and storage (Huq et al., 2013). The use of materials as coatings in bioencapsulation plays an important role. One type of coating material used is a biopolymer, namely sodium alginate, which has the advantages of easily forming a gel, has good solubility and low viscosity, allowing cells to be trapped and reducing the decline in viability (Szczech & Maciorowski, 2016). There are several techniques used in encapsulation, including extrusion, emulsion, and dry spraying (Heidebach et al., 2012). In this study, the technique used was the extrusion technique (droplet method), which is a simple technique because it is relatively easy and inexpensive. The extrusion technique can maintain bacterial viability because it does not use high temperatures (Rokka & Rantamaki, 2010).

The manufacture of beads (bioencapsulation results) requires the addition of polymer chain crosslinking agents; ions that can be used include Ba^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, or Fe^{3+} (Choi et al., 2009). In this study, the binding agent used was calcium chloride ($CaCl_2$) with different concentrations as a determining factor for the results. Using Ca^{2+} because it is the most suitable and safe binding agent, while other binding agents, such as Zn2+ and Fe3+, are heavy metals that have toxicity to microorganisms (Angela & Marzuki, 2021). According to Khazaeli et al. (2008), differences in $CaCl_2$ concentration can affect the swelling power of beads, where the higher the concentration, the lower the swelling power of beads. The swelling power of beads refers to the swelling of beads due to the entry of water into the beads, which can affect the release of core material from the beads (Ibrahim et al., 2014). Variations in $CaCl_2$ concentration affect the swelling power and diameter size. Previous research on differences in $CaCl_2$ concentrations was conducted by Munawwaroh (2019), using variations of 3%, 4%, and 5% $CaCl_2$, resulting in swelling powers of 92.64%, 85.17%, and 84.79%, respectively. This indicates that differences in $CaCl_2$ concentrations affect the swelling power of beads, and different concentrations are required for comparisons to obtain optimum beads in this study.

The survival of biological agents is crucial in bioencapsulation research. This study aims to determine encapsulation efficiency, microbial viability within beads, and differences in swelling power of beads with different binder concentrations.

Methods

The research was conducted from April to June 2024 at the Plant Health Laboratory 1 of the National Development University "Veteran", East Java. The tools used included petri dishes (10 cm diameter), stoves, autoclaves (All American 50x), micropipettes, beakers, loop needles, analytical scales (Kern PCB), incubator cabinets, vortex (Maxi Mix II), media bottles, syringes, stirrers, and Erlenmeyer flasks (150 ml). The materials used included *Paenibacillus polymyxa* bacterial isolates, sodium alginate (Merck), distilled water, CaCl2, NA media, labels, Technical NaCl, and 70% alcohol. The study used a Completely Randomized Design (CRD) with CaCl2 concentration factors: P1 = 1%, P2 = 3%, and P3 = 5%, based on research by Munawwaroh (2019). There were four treatments and three replications.

The study began with the rejuvenation of *Paenibacillus polymyxa* by taking one loop of the isolate, then streaking it on NA media and incubating it for 48 hours at room temperature to obtain *Paenibacillus polymyxa* colonies. After incubation, bacterial colonies were observed to ensure growth. Bacterial suspensions were made using distilled water by rejuvenating the bacteria for 48 hours before use. The isolate was then suspended in distilled water with a population density of 108 CFU/ml and 10 ml as an inoculum source (Weselowski et al., 2016).

To make bioencapsulation formulations, start by weighing 2 g of sodium alginate into an Erlenmeyer flask. Then, add 100 ml of distilled water and heat the mixture. Finally, sterilize it using an autoclave at 120°C for 20 minutes. Making a CaCl2 solution involves weighing 1 g, 3 g, and 5 g of CaCl2, placing each into an Erlenmeyer flask, and then adding 100 ml of distilled water. The tube is shaken until the solution is dissolved, then sterilization is carried out. Making beads by means of biopolymer suspensions, each finished treatment is added

with *Paenibacillus polymyxa* suspension with a ratio of 10:1 (v/v) (Khan et al., 2013). Dropping a mixture of biopolymer suspension and *Paenibacillus polymyxa* suspension with a syringe into 1%, 3%, and 5% CaCl2 solutions, little by little.

Wash the beads with a NaCl solution prepared by weighing 0.85 g of NaCl, then placing them in an Erlenmeyer flask containing 100 ml of distilled water, homogenizing them, and then sterilizing them. The finished beads were filtered and washed three times with 0.85% NaCl, followed by analysis (Ratnasari et al., 2014).

Encapsulation efficiency testing was performed by weighing 1 g of beads, placing them in a sterile Petri dish, crushing them with a scalpel until coarsely ground, and putting them in a test tube containing 9 mL of sterile distilled water. The resulting vortex was then taken with a micropipette and placed onto NA medium for 24-48 hours.

Viability testing using the bioencapsulation formulation involved storing the formulation in a glass jar at room temperature (25–27°C) for 30 days, with periodic observations every six days. Observations were made by weighing 1 g of beads, then placing them in a sterile petri dish and crushing them with a scalpel until they were coarsely crushed. They were then placed in a test tube containing 10 ml of sterile distilled water and vortexed for 1 minute. The vortex was taken with a micropipette as much as 1 ml and grown onto NA media using the spread plate method, incubated for 24-48 hours. The colony population was calculated using the plate count method with the formula according to Hanif (2016).

$$Cfu/grams = \frac{Total\ Number\ of\ Colonies}{Volume\ Spread\ on\ Petri\ Dishes\ x\ Dilution\ Factor}$$

Swelling power was observed to determine how quickly beads absorb water, which would cause swelling, which is thought to affect the release of biological agents. Swelling power was measured by weighing 1 g of beads and soaking them in 10 ml of sterile water for 1 month. The beads were weighed weekly. Swelling power was calculated using a modified formula from Munawwaroh (2019).

$$\text{Swelling Power } (\%) = \frac{Df - Di}{Di} x\ 100\%$$

Information: Df : Final weight of beads
Di : Initial weight of beads

Data Analysis

The observation data were analyzed using ANOVA at a 5% error level. If significant differences were found, further testing using the Duncan Multiple Range Test (DMRT) at a 5% error level was performed. Data analysis was performed using IBM SPSS Statistics 24 software.

Result

Bioencapsulation Formulation

The bioencapsulation formulation shown in Figure 1 is a novel formulation designed to maintain the viability of microorganisms. According to research by Lee et al. (2009), beads made from sodium alginate and $CaCl_2$ have a favorable structure for bioencapsulation applications due to their biocompatibility and biodegradability. These beads can maintain the viability of the encapsulated microorganisms and protect them from harsh environmental conditions. Research by Rojas et al. (2022), also supports the use of beads made from this material in various biotechnological applications, including bioencapsulation, due to their ability to form stable gels with customizable structures. Beads with a 1% $CaCl_2$ concentration, shown in Figure 1A, are less spherical and have a less robust structure than beads made with 3% and 5% $CaCl_2$. This is because the lower calcium concentration results in a weaker and less stable gel network, making the beads more susceptible to breakage. This is in accordance with the statement by Munawwaroh (2019), which states that the

concentration of the binding material affects the viability of bioencapsulation and affects the strength of the beads.

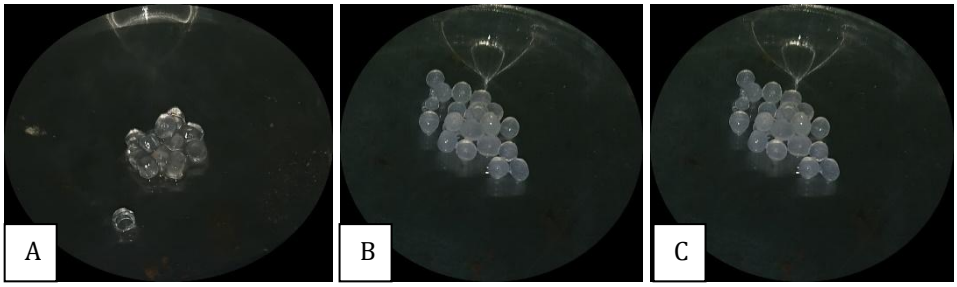

Figure 1. Beads Yield with 1% $CaCl_2$ concentration (a), beads with 3% $CaCl_2$ concentration (B), beads with 5% $CaCl_2$ (C)

Encapsulation efficiency test

The results of encapsulation efficiency are presented in Figure 2. With various $CaCl_2$ concentrations, there were differences in the number of entrapped *Paenibacillus polymyxa* colonies.

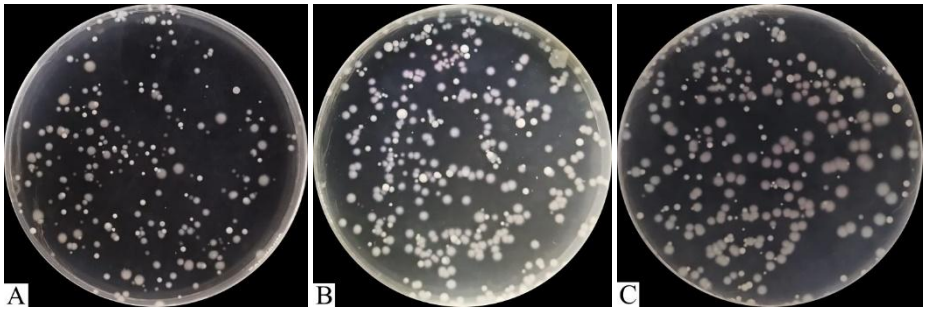

Figure 2. Test results of encapsulation efficiency with 1% $CaCl_2$ concentration (A), 3% $CaCl_2$ concentration (B), 5% $CaCl_2$ concentration(C)

Table 1. Encapsulation Efficiency Test Results

***Beads Treatment* (*$CaCl_2$ Concentration*)**	**EE (%)**
1%	86.86a
3%	98.21b
5%	96.87b

These results were obtained by calculating the bacterial population density and entering it into the formula. The 1% $CaCl_2$ concentration treatment yielded an EE of 86.86%, the 3% $CaCl_2$ concentration treatment yielded an EE of 98.21%, and the 5% $CaCl_2$ concentration treatment yielded an EE of 96.87%. Beads with different concentrations had varying encapsulation efficiencies. The highest encapsulation efficiency was achieved with beads with a 3% $CaCl_2$ concentration.

Based on the results presented in Table 1, it is known that different $CaCl_2$ concentrations affect the encapsulation efficiency of *Paenibacillus polymyxa*. Based on these data, it can be seen that increasing the $CaCl_2$ concentration from 1% to 3% significantly increases encapsulation efficiency. However, further increases to 5% do not provide a significant growth and even slightly decrease efficiency compared to the 3% concentration. Beads with a 5% $CaCl_2$ concentration produce excessively strong crosslinks, which can cause the gel structure to become dense, inhibiting the diffusion of nutrients and oxygen into the beads. In addition, pores in the gel that are too small can hinder the entry of bacteria during the encapsulation process or cause osmotic pressure that reduces bacterial survival and results in decreased encapsulation efficiency. In general, $CaCl_2$ plays an essential role in the alginate gelation process, which forms a gel matrix for bacterial cell encapsulation. At the right concentration, $CaCl_2$ can produce a gel strong enough to protect bacteria, but still allows

diffusion. Too low or too high a $CaCl_2$ concentration can reduce encapsulation efficiency. This is due to suboptimal bead formation, such as the influence of time in bead formation that has not been included in the observation variables. This is in accordance with the research of Zhao et al. (2012), which explains that the optimal $CaCl_2$ concentration is critical to produce high encapsulation efficiency, because it affects the strength and structure of the formed beads. Therefore, it is crucial to determine the right $CaCl_2$ concentration to ensure the viability and efficiency of bacterial encapsulation during storage.

Observations on encapsulation efficiency showed that the optimal concentration for the highest encapsulation efficiency was 3% $CaCl_2$. At this concentration, calcium ions (Ca^{2+}) were sufficient to form crosslinks with alginate, resulting in denser, more stable, and stronger beads. Beads at this concentration could adsorb microorganisms more efficiently than those with lower or higher $CaCl_2$ concentrations.

Viability Test of Paenibacillus polymyxa in Beads

The viability of *Paenibacillus polymyxa* bacterial cells in beads was measured by growth in NA media treated with various $CaCl_2$ concentrations every 6 days. As seen in Table 2, the most significant decrease occurred on day 24. This indicates that the administration of $CaCl_2$ at different concentrations can affect the viability of *Paenibacillus polymyxa* during storage.

The graph presented in Figure 3 shows that the viability of *Paenibacillus polymyxa*, measured in CFU/mL, decreased with increasing storage time for all treatments during the observation period. This indicates that bacterial viability decreases with increasing storage time. Beads treated with 3% and 5% $CaCl_2$ concentrations have more favorable conditions for maintaining bacterial viability longer than beads with a 1% $CaCl_2$ concentration. It is suspected that factors affecting this viability include the type of storage media, environmental conditions, or special treatments given. This is in accordance with the statement of Setiaji et al (2015) that the longer the storage time, the percentage of bacterial viability decreases, related to the bacterial growth capacity, which is influenced by environmental factors, especially temperature and media. This is also supported by the opinion of Capucino & Natalie (2001), who stated that bacterial viability (survival rate) is influenced by bacterial growth capacity, medium, temperature, pH, and nutrients.

Table 2. Colony Density *Paenibacillus polymyxa* (10^8 CFU/mL)

Beads Treatment ($CaCl_2$ Concentration)	**Colony density *Paenibacillus polymyxa* (10^8 CFU/ mL) (Day)**					
	6	**12**	**18**	**24**	**30**	**36**
1%	2.60a	2.28a	2.28a	2.00b	2.02b	1.92b
3%	2.94a	2.92c	2.89b	2.61c	2.62c	2.52b
5%	2.90a	2.75bc	2.72b	2.68c	2.68c	2.50b

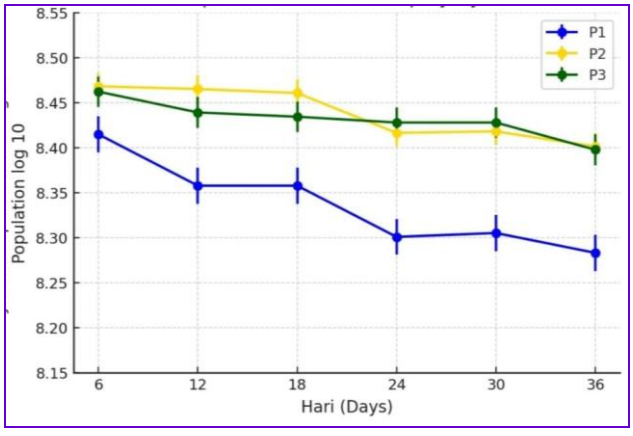

Figure 3. Population of *Paenibacillus polymyxa* in Beads up to 36 days

According to research by Munawwaroh (2019), coating media such as alginate combined with a $CaCl_2$ binder can affect the quality of active ingredients. $CaCl_2$ acts as a binder that forms stable beads with the alginate, thus protecting bacteria from adverse environmental conditions. Furthermore, factors such as storage temperature, media pH, and nutrient availability also significantly influence bacterial viability during storage (Zhao et al., 2020).

Based on data analysis from *Paenibacillus polymyxa* bacterial viability tests, it is understood that carrier materials can affect the viability of active ingredients. Therefore, it is crucial to select the right carrier material and optimal conditions to maintain bacterial viability over an extended period. According to research by John et al. (2011), optimal storage of microbial bioencapsulation is at low temperatures and in a place with a humidity of between 20-30% to maintain stable microbial viability.

Bead Swelling Power Test

The results of the swelling power test, presented in Figure 4, show that the swelling power of the beads increased from week 1 to week 2 and continued to decrease until week 4.

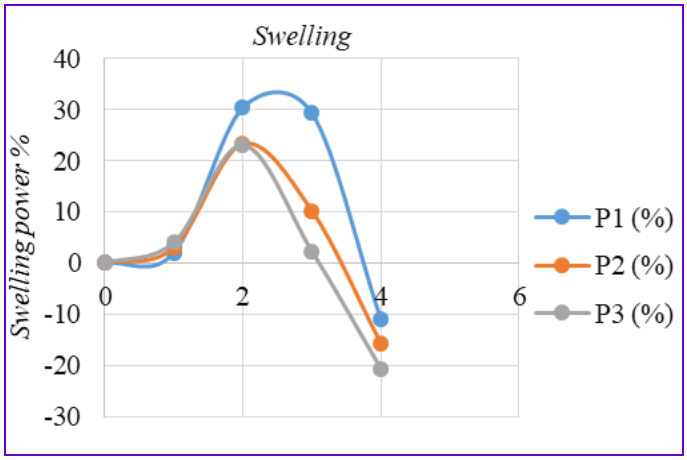

Figure 4. *Swelling Power of Beads*

Beads treated with 1% CaCl2 concentration exhibited the highest swelling power in the 2nd week, followed by a decrease in swelling power value until the 4th week. After the 4th week, the beads treated with this concentration broke. While the treatment with 3% and 5% $CaCl_2$ concentration did not have a significant difference in the observations of the 1st and 2nd weeks, in the 3rd and 4th weeks, the two treatments showed a significant difference in swelling power values. Beads with a 1% $CaCl_2$ concentration were easily broken due to their low stability, whereas beads treated with 3% and 5% $CaCl_2$ concentrations exhibited higher stability, enabling them to survive until the 4th week of observation. The difference in swelling power of beads is thought to be influenced by several factors, one of which is the binder. This is in accordance with the research of Khazaeli et al. (2008), which indicates that differences in $CaCl_2$ concentration can affect the swelling power or swelling of beads.

Conclusions

Bead treatment with 3% calcium chloride concentration was the best treatment for encapsulation efficiency in absorbing *Paenibacillus polymyxa*, amounting to 98.21%. Calcium chloride concentration treatment of 3% and 5% was the most supportive treatment in the viability of *Paenibacillus polymyxa*, amounting to 108 CFU/mL. Different concentration treatments affected the swelling power of beads, with 5% concentration having the highest swelling value.

Declaration statement

The authors report no potential conflict of interest.

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