Study of Morphology and Protein Crystal Character of *Bacillus thuringiensis* Isolates

Kusuma Handayani[1], Rachmat Nugraha Indra[1], Rochmah Agustrina[1], and Sumardi[1]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Lampung University, Jl. Prof. Sumantri Brojonegoro No.1, Bandar Lampung 35145, Indonesia
kusuma.handayani@fmipa.unila.ac.id

Abstract. *Bacillus thuringiensis* (Bt) has characteristics that it can produce protein crystals that are toxic to insects. The crystals consist of crystalline (Cry) and cytolytic (Cyt) proteins, known as δ-endotoxins. The specificity and activity of Bt on the substance are associated with the type and form of the δ-endotoxin. This research was conducted to determine the initial formation of protein crystals and to measure the number of bacterial cells during their growth and to characterize the shape of protein crystals in Bt isolates from the soil of the Liwa Botanical Garden Lampung. Forming Bt bacteria through protein crystal morphology can be done by Scanning Electron Microscope. The results showed that each isolate had more than one protein crystal shape with seven different variations, namely ovoid, spherical, triangular bipyramidal, rectangle, elongate, cuboid, and geometrical. By observing the time of formation, protein crystals were seen at the 3rd to 18th hour of growth.

Keywords: *Bacillus thuringiensis*, Crystal Protein, Morphology.

1 Introduction

Bacillus is a Gram-positive rod-shaped bacterium often used in biotechnology because it has a variety of physiological abilities. In addition, *Bacillus* has the unique ability to replicate rapidly, is allowed to tolerate adverse environmental conditions, and acts as a biological control. *Bacillus* is capable of producing endospores intracellularly with various shapes, such as round, oval, elliptical, or cylindrical (Siallagan et al., 2020). The endospores are formed in response to unfavorable environmental conditions, so *Bacillus* can tolerate changing environmental conditions. *Bacillus thuringiensis* (Bt) has the characteristic that it can produce insecticidal crystal proteins during the stationary and sporulation phases of its growth cycle. During sporulation, Bt can create one or more parasporal insecticidal crystals consisting of one or more crystalline (Cry) and cytolytic (Cyt) proteins, which are known as endotoxins (Palma et al., 2014) and are specific to certain insect orders (Akhtar et al., 2021). When ingested by insects, the endotoxin crystals will dissolve and turn into protoxins due to proteases in the insect's midgut. This protoxin acts as an active poison that causes the formation of pores in the intestinal wall resulting in severe damage and death to target insects (Jacups et al., 2013) All reported Bt insecticidal proteins do not contain negative effects on animals, humans,

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plants, and other beneficial bacteria (Liu et al., 2021), so their use in the field of biotechnology, such as biological control is very potential. The specificity of *Bacillus thuringiensis* to its host is associated with the type and form of the δ-endotoxin produced during the sporulation phase (Mukhija & Khanna, 2018).

Protein crystals in *Bacillus thuringiensis* can be classified based on their shape which determines their specificity to insects. Protein crystals with a spherical shape are found in the Israelensis subspecies which has toxic activity against Diptera, flat rectangular protein crystals are found in the tenebrious subspecies which are toxic to Coleoptera, protein crystal cubes are toxic to certain Diptera and Lepidoptera, while protein crystals with a triangular bipyramidal shape in the kurstaki subspecies toxic to Lepidoptera (Addiniyah, 2019). It was strengthened by Mafazah & Zulaika (2017), who stated that protein crystals have a variety of shapes, and these shapes correlate with their toxicity. Protein crystals with a triangular bipyramidal shape are toxic to the Order Lepidoptera, while protein crystals are toxic to Diptera and are cubic, oval, and amorphous. The occurrence of differences in the morphological distribution of crystalline proteins can be caused by genetic variations due to environmental conditions or the effects of different habitats and their protoxin composition.

Based on the research by Janah et al. (2022), seven *Bacillus* isolates were obtained from the soil of the Liwa Botanical Gardens (KRL) Lampung. The isolate has no known protein crystal character, so characterization is needed to detect *Bacillus thuringiensis*. Each crystal morphology can be formed by different strains of *Bacillus thuringiensis* so that it can be ascertained whether there are differences in the crystal structure of the samples that might explain certain differences in terms of toxicity. As mentioned by Mukhija & Khanna (2018) that observing the parasporal crystal protein during the sporulation phase can be done with a Scanning Electron Microscope (SEM), which is the most direct and effective method for identifying *Bacillus thuringiensis* bacteria so that the shape of the protein crystals in the target isolate can be observed by comprehensive and representative.

2 Materials and Methods

2.1 Place and Time of Research

This research was conducted from January to May 2023 at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences and the Integrated Laboratory and Center for Technology Innovation, University of Lampung.

2.2 Material

The tools used in this study were various types of glassware, such as beaker glass, measuring cups, Erlenmeyer, Petri dishes, volumetric pipettes, and test tubes. There are also a variety of instruments such as analytical balance, hotplate magnetic stirrer, autoclave, water bath, refrigerator, biological safety cabinet, microscope, incubator, shaker incubator, centrifuge, sonicator, oven, vortex mixer, and Scanning Electron Microscope (SEM). The materials used are various types of growth media such as Luria Bertani,

Luria Bertani Acetate Buffer, HCl, NaOH, and T3 media. Disposable materials such as distilled water, methanol, acetic acid, 70% alcohol, spirits, cotton, tissue, methylene blue, Whatman filter paper no. 1, Gram stain, as well as personal protective equipment. Bacillus sp. isolates The collection used is the collection of the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung. The isolate had previously been isolated from the soil of the Liwa Botanical Garden, Lampung.

2.3 Method

This study used research objects in the form of three bacterial isolates of *Bacillus* sp. which had previously been isolated from the soil of the Liwa Botanical Gardens, Lampung which were then coded as isolates Bt 4, Bt 5, and Bt 6. The data obtained were then analyzed descriptively qualitatively. Descriptive analysis used is to use analysis in the form of pictures, tables and diagrams which aims to provide a general description of the morphology of protein crystals from the results of three-dimensional scanning electron microscopy (SEM) images and the time of formation of protein crystals by determining the growth curve.

2.4 Subculture of *Bacillus* sp. Isolates

This research used a growing medium named Luria Bertani (LB). On Luria Bertani medium, one loop of isolates was taken aseptically, inoculated onto LB medium for slant using the scratch method, and incubated at room temperature ± 24 hours. The growing colonies were observed until pure colonies were obtained. Validation was carried out by looking at the shape of the cells using simple methylene blue staining. Then, observation was done under a 1000x magnification microscope. If the cell shape is the same, it can be conclude that the bacterial isolate is pure (Mafazah & Zulaika, 2017).

2.5 Validation of *Bacillus thuringiensis* Isolate Purity

To further ensure that the colonies obtained on Luria Bertani media were *Bacillus thuringensis*, the procedure was achieved according to (Bahri et al., 2021) with modifications. The treatment was started by taking one ose of colonies that were in the media and then putting them into acetate buffer LB solution with a pH of 6.8. Acetate buffer LB media with a pH of 6.8 used is a medium with an optimum pH. This solution is a selective medium for *Bacillus thuringiensis*. Once the single colonies were transferred to the selective media, they were incubated by shaking at 150 rpm for a day. After one day, the colony results were seen at the bottom of the tube and then heated at 800C for 15 minutes (Nair et al., 2018). Then, homogenization was done using a vortex mixer. The purpose of heating is other bacteria except *Bacillus thuringiensis* cannot live or die. Then a 10^{-1} to 10^{-5} dilution was carried out to get good and not too-dense colony results. Furthermore, colonies were grown on Luria Bertani Agar media which was added to an acetate buffer solution. Purity validation was again carried out by observing the shape of the cells on a 1000x magnification microscope through Gram staining.

2.6 Protein Crystal Morphology Detection and Staining

Coomassie Brilliant Blue (CBB) solution was prepared by weighing 0.1 gram of Coomassie dissolved in 30 ml of methanol, then adding 5 ml of acetic acid and 65 ml of distilled water. After that, the solution was stirred using a magnetic stirrer for 2 hours and then filtered using Whatman paper no. 1 and stored at 20°C. After that, staining with one loop of *Bacillus thuringiensis* was inoculated on a sterile glass object and then fixed. After that, coomassie brilliant blue dye was added and left for 3 minutes. CBB dye can be replaced by crystal violet as an alternative dye for protein crystals. After staining, the glass objects were rinsed with running water, then dried and observed under a microscope with a magnification of 1000x (Siallagan et al., 2020). Colonies that were confirmed to form protein crystals were harvested and collected as new isolates and stored in slanted acetate buffer LB and coded as isolate.

2.7 Determination of the Time of Protein Crystal Formation

One dose of isolate was inoculated in T3 sporulation media (per liter containing 3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.005 g MnCl2, 6 g NaH2PO4, and 7.1 g Na2HPO4) (Hassan et al., 2021) and incubated at shaker incubator. Every three hour interval, the formation of protein crystals was observed by taking one dose of isolate that had been prepared previously, then spreading it flat on a glass object. After that, staining is done using Coomassie brilliant blue or crystal violet for three minutes and then rinsed with running water. After drying, observe using a microscope at 1000x magnification. Observations were carried out for 48 hours to ensure the formation time of protein crystals and the growth characteristics of each isolate.

2.8 Acquisition of Scanning Electron Microscope (SEM) Images

For the scanning electron microscopy procedure described by (Loutfi et al., 2021), ZEISS Scanning Electron Microscopy was used to capture images of crystals and spores. With a voltage of 15 KV, the distance between the sample and the target is set at 4 mm. A simple preparation protocol is followed so that crystals and spores always have the same concentration for different strains when taking SEM images. Bt potential isolate cells were grown on T3 media. Then incubated by shaking at 120 rpm at 37°C for 7 days to induce sporulation. The Bt spore suspension was then centrifuged at 15,000 rpm and 4°C for 10 minutes to harvest the spore-crystal mixture (Yilmaz et al., 2017). After that, 100 µL of stock solution (a mixture of crystals and spores) was added to 1900 µL of distilled water to get a total of 2 mL. The tube was sonicated for 5 minutes at a frequency of 37 kHz to break up the aggregation and then the tube was vortexed. After that, 50 µL was taken from the tube and placed in the sample holder which was covered with mica that had been cleaved. The sample holder was then put into the oven at 37°C to dry. After drying the sample metallized to form a 2.5 nm gold palladium deposit which allowed the crystals and spores to become conductive. Finally, the sample is placed in the SEM vacuum chamber and images are taken. Crystal and spore morphology of each isolate was analyzed individually.

3 Results and Discussion

This study used three isolates of *Bacillus* sp. in the collection of the Microbiology Laboratory of FMIPA Unila which had previously been isolated from the soil of the Liwa Botanical Garden, Lampung. Purity validation was carried out to ensure that the isolates that grew were pure colonies of *Bacillus thuringiensis*. This step was carried out by culturing the isolate on 0.2M acetate buffer Luria Bertani media pH 6.8 and transferring it back in a streak, as shown in Figure 1. According to Bahri et al. (2021), selective media is media that can grow certain bacteria and inhibit the growth of other bacteria (non-target bacteria), so the only bacteria that can live on the selective media used are *Bacillus thuringiensis. Bacillus thuringiensis* can grow in a medium with a pH range of 5.5 – 8.5 and grows optimally at a pH of 6.5 – 7.5. Acetate buffer LB media with a pH of 6.8 used is a medium with an optimum pH, so this solution is a selective medium for *Bacillus thuringiensis*. It is because *Bacillus thuringiensis* spores are unable to germinate in media containing high concentrations of sodium acetate, while *Bacillus* sp. non-*Bacillus thuringiensis* can still germinate.

Through macroscopic observation, the three isolates used showed white, creamy white, and yellowish colony colors with flat edges and flat and smooth colony surfaces. The culture results were then confirmed by rejuvenating the colonies into solid Luria Bertani media pH 6.8 that was previously heated at 80^{o}C for 5 minutes so that bacteria capable of germination would die and could not grow again on solid Luria Bertani media. The effect of temperature is essential for *Bacillus thuringiensis* bacteria. Each bacterial strain has a different growth temperature range, so the ambient temperature is an essential factor in determining the speed of bacterial growth. The Luria Bertani medium is a medium that is rich in carbon, nitrogen, and mineral content that is

sufficient for the survival of B. thuringiensis (Bahri et al., 2021). The rejuvenation is intended so that the bacteria can be more productive in producing protein crystals when transferred to sporulation media for the next stage.

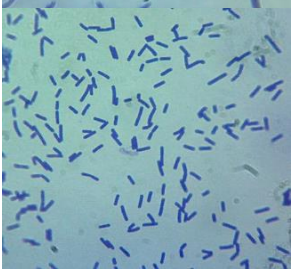
The culture results showed that the three isolates were *Bacillus thuringiensis* bacteria with characteristics of round, white, creamy white to yellowish-white colonies with flat edges and smooth colony surfaces with flat or elevated elevations. This feature is in line with (Astuti et al., 2018) who states that the characteristics of B. *thuringiensis* are round, white to yellowish white, wrinkled and smooth edges, and high elevation. After the macroscopic characteristics of the colonies were obtained, validation of the isolates was then followed by Gram staining to see the morphological forms as well as the Gram properties of the three isolates. The results showed that the three isolates were Gram-positive as evidenced by the purple staining color and cell morphology, namely bacilli or rods with different cell configurations as shown in Table 1. Bacteria can be grouped into Gram-positive or Gram-negative groups using the Gram staining technique. The Gram staining of *Bacillus thuringiensis* that was carried out in this study is presented in Table 1. where the three isolates showed positive Gram results with a color that appeared purplish. The cause of the blue or purplish Gram-positive bacteria is due to the dye complex. Violet-iodine crystals are retained even when given a bleaching solution such as alcohol while Gram-negative bacteria are red because the complex dissolves during the administration of the bleaching solution and then takes a second red dye. Gram staining is not only to determine the nature of Gram in bacteria but also to observe the morphology of bacterial cells. Based on the results of cell morphological observations, the three isolates obtained bacilli with three configurations and different cell sizes. The results of the Gram stain showed agreement with the literature that stated *Bacillus thuringiensis* was a Gram-positive bacterium with a *Bacillus* cell shape (Hamidah et al., 2019).

Detection of the presence of protein crystals was carried out by painting using Coomassie Brilliant Blue (CBB) dye and crystal violet as alternative dyes. The results of the microscopic observations in Figure 2. show the presence of protein crystals in isolates which is indicated by the presence of two different colored phases in the cells. Crystal violet can be an alternative dye for protein crystals. According to Nilles et al. (2022), crystal violet (also called gentian violet) is a triarylmethane dye that colors protein and DNA with a higher sensitivity than Coomassie blue or ethidium bromide. Under alkaline conditions, crystal violet stains DNA and proteins favorably.

Based on Coomassie Brilliant Blue (CBB) staining, it was accepted that the three isolates were able to produce protein crystals located at the end of the cell (terminal) side by side with the spores. Protein crystals can absorb dyes so they look blue, while spores are unable to absorb Coomassie Brilliant Blue, so they don't turn blue. It is due to the mechanism of the dye on protein crystals in the staining process, namely Coomassie Brilliant Blue (CBB) binds to the protein in the crystal through ionic interactions between the sulfonic acid groups and positive amino groups (Siallagan et al., 2020). The spores and protein crystals seen outside the cell were formed by the condition of the cell that has been lysis. In this study, the position of the protein crystals was seen adjacent to the spores which proved that the protein crystals were formed simultaneously with the sporulation phase as stated by (Palma et al., 2014). Unlike

fungi and ferns, bacterial spores do not play a role in reproduction. The type of spore in bacteria can be shaped of an endospore because formed inside the cell or the form of an exospore because located outside the cell (Bahri et al., 2021).

Table 1. Results of Microscopic Morphological Observations.

Isolate Code	Cell Image	Cell Shape	Characteristic Grams
Bt 4		Streptobacil	Positive
Bt 5		Streptobacil	Positive
Bt 6		Monobacil	Positive

Bacillus thuringiensis produces crystalline proteins that have a crucial role in insecticidal activity. These crystals have different shapes depending on the nature of the δ-endotoxin and the genes that encode it (Wang et al., 2013). In this study, to characterize each isolate in terms of its parasporal activity, crystal morphology was observed using an electron microscope. Based on the protein crystal morphology that has been observed, it is known that each isolate has various crystal forms with different combinations. Three isolates of *Bacillus thuringiensis* showed seven different crystal forms. The seven shapes that were successfully analyzed were ovoid, spherical, triangular bipyramidal, rectangle, elongate, cuboid, and geometrical. Regarding these differences, each crystal form is expressed by a different type of gene which makes it a unique trait of *Bacillus thuringiensis.* The cry2Ab gene is responsible for encoding the cuboid crystal

structure (Adalat et al., 2017), and the cry1Aa and cry1Ac genes encode the bipyramidal crystal structure (Evdokimov et al., 2014). Rectangle (flat rectangle) by Cry3A, irregular by Cry3B, rounded by Cry4A and Cry4B, and rhombus by Cry11A (Al-thani et al., 2022). The detailed morphology of protein crystals in each isolate code is further shown in Figure 3, Figure 4, and Figure 5.

In addition to the existence of genes that encode various crystal forms, it is also known that each of these forms is owned specifically by a definite *Bacillus thuringiensis* subspecies and determines its toxic activity against different insect orders. As described by (Jain et al., 2017). Bt israelensis (Bti) produces a combination of endotoxin (Cry) and hemolytic protein (Cyt) during the sporulation stage and crystallizes it into a spherical shape. On the other hand, Bt kurstaki (Btk) produces another combination of endotoxin (Cry) and crystallizes it into bipyramidal and cuboidal forms. The crystal structure of Bti is toxic to the insect order Diptera, while the bipyramidal and cuboidal crystals of Btk are toxic to Lepidoptera and Coleoptera (Adang et al., 2014). Also added by (Addiniyah, 2019) is that cuboid crystals are toxic to Diptera and flat rectangular shapes are present in the tenebrious subspecies toxic to Coleoptera. However, protein crystals are toxic to Diptera and are cubic, oval, and amorphous (Mafazah & Zulaika, 2017). Based on this relationship, it is concluded that each isolate observed in this study has the potential to have broad toxicity against various orders of insects because it has a variety of shapes, although the majority present ovoid (oval) and spherical (round) shapes. Isolate Bt 4 toxin against Diptera and Coleoptera, Bt 5 against Diptera and Lepidoptera, and Bt 6 against Diptera and Coleoptera.

The large number of variations that appear where there is more than one form in each isolate indicates a wide diversity of genes from the isolates. However, based on (Ma et al., 2023) wide diversity of cry and cyt genes in *Bacillus thuringiensis* has no link with bigger toxicity activity. However, the existence of multiple cry and cyt genes can be associated with a greater diversity of Cry and Cyt protein production itself. It can help to avoid the occurrence of insect resistance and increase the spectrum of toxicity to various types of insects. This profile variation might be related to the high frequency of exchange of genetic information between B. *thuringiensis* bacteria which allows for variations in genes within one isolate.

The initial formation of protein crystals in each isolate was observed by staining with Coomassie Brilliant Blue every 3 hours for 48 hours. After observations were made during cell growth every three hours, it was found that overall all isolates showed differences in the time of formation of protein crystals. Bt 4 isolates at 12-15 hours, Bt 5 and Bt 6 isolates at 0-3 hours (Figure 6). This proves that there is a discrepancy between the results obtained and related literature which explains that *Bacillus thuringiensis* protein crystals are formed during the sporulation phase, which should have occurred during the stationary growth phase (Khorramnejad et al*.,* 2020). The gap that occurs can be caused by various factors such as the use of T3 sporulation media which induces sporulation and the difference in speed when incubating the shaker which results in aeration instability, where aeration can affect the growth rate and sporulation of bacteria as stated by (Rahbani et al., 2018). In addition, it can be caused by technical errors where the cells with visible crystals are cells that have just been inoculated from

the previous media and have not yet germinated and deficiencies of the direct counting method used.

Apart from the gap that occurred above, overall all isolates showed that protein crystals had been found after a culture age of over 18 hours followed by a large number of cells that showed lysis and spores that were outside the vegetative cells. It turned out to be correct if it was matched with related literature, where there were research results that said that the stationary phase had several stages that had started at the age of 9 hours of growth. It was explained in detail by (Wang et al., 2013) that a culture age of 7 hours (mid-log growth phase) represents the vegetative growth stage, 9 hours (early stationary growth phase) may cover stages 0 to II, 13 hours (middle stationary the growth phase, the proportion of sporulated cells reaching about 30%) probably includes stages IV to VI, and 22 hours (spore maturation and stem cell lysis phase) mainly represents stages VI and VII, due to the heterogeneity of the sporulating population.

4 Conclusion

Based on the results of the research that has been done, it is concluded that the isolate used is *Bacillus thuringiensis* with the characteristics obtained in the form of bacilli, Gram-positive, circular and irregular colony shapes, white to cream in color, can produce spores and can form protein crystals. Then the SEM results showed that there were seven morphological forms of protein crystals with different variations in the three isolates, namely ovoid (oval), spherical (round), triangular bipyramidal, rectangle (rectangular), cuboid (box), and geometrical. The seven forms provided specific activity against certain insect orders, namely isolate Bt 4 toxin against Diptera and Coleoptera, Bt 5 against Diptera and Lepidoptera, and Bt 6 against Diptera and Coleoptera. Observation of the time of formation of protein crystals in isolates showed different results. Overall protein crystals were seen at the 3rd to 18th hour of growth. These results can be used as a reference for testing the toxicity activity of isolates in terms of the time of crystal formation and the growth time used.

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Attachment

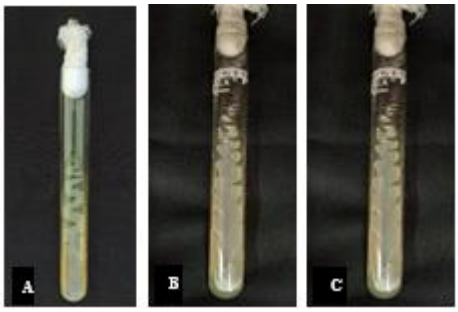

Fig. 1. Pure Colonies on Luria Bertani Agar Medium pH 6.8. with (A) Bt 4 Isolate, (B) Bt 5 Isolate, and (C) Bt 6 Isolate.

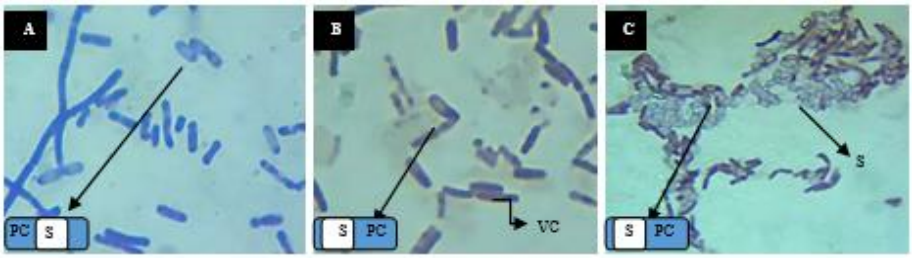

Fig. 2. Protein Crystals Found in (A) Bt 4 Isolates, (B) Bt 5, and (C) Bt 6. Description: (PC) Protein Crystals, (S) Spores, (VC) *Bacillus thuringiensis* Vegetative Cells. You Can Also See the Distribution of Spores Outside the Vegetative Cells in Bt 6.

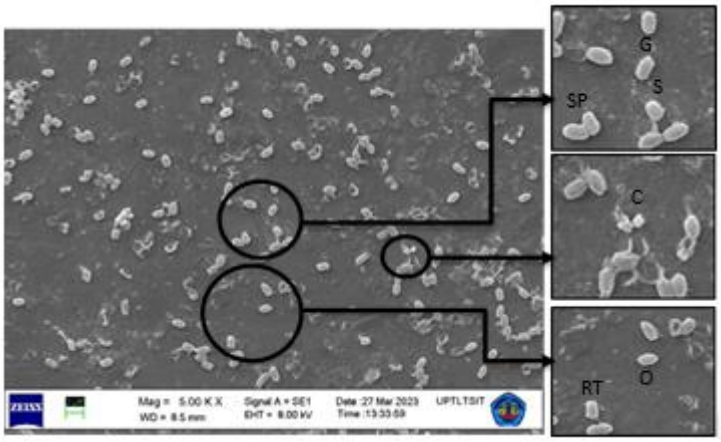

Fig. 3. SEM Results on Bt 4 Isolates Showed 5 Variations in Crystal Morphology. Description: (G) Geometrical, (O) Ovoid and (SP) Spores, (C) Cuboid, (S) Spherical, and (RT) Rectangle.

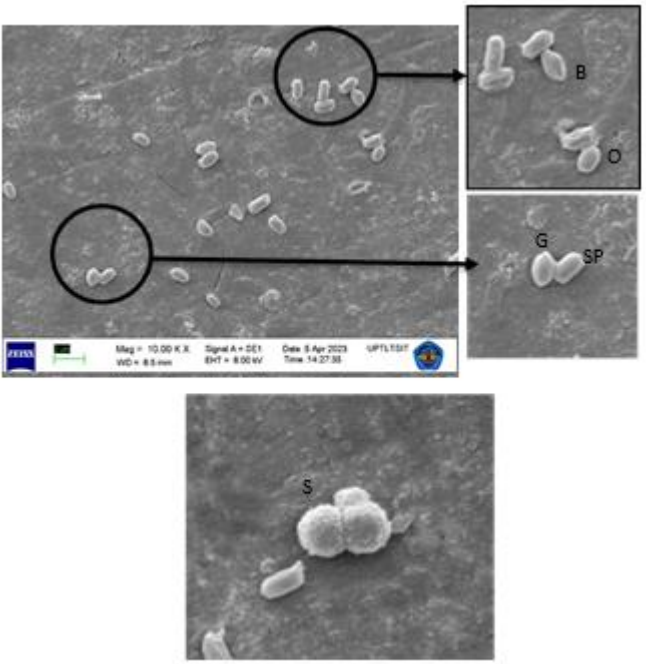

Fig. 4. SEM Results on Bt 5 Isolates Showed 4 Variations in Crystal Morphology. Description: (B) Bipyramidal, (O) Ovoid, (G) Geometrical and (SP) Spore, and (S) Spherical.

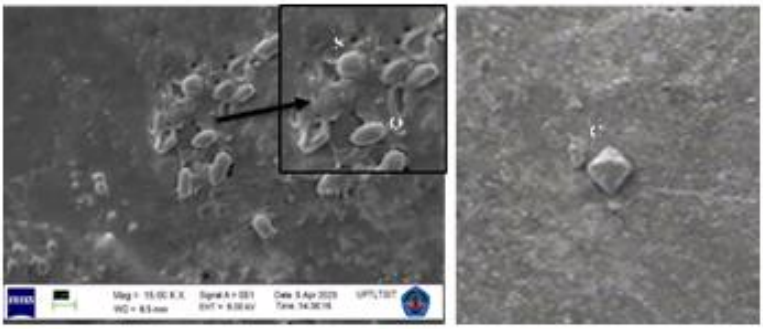

Fig. 5. SEM Results on Bt 6 Isolates Showed 3 Variations of Crystal Morphology. Description : (S) Spherical, (O) Ovoid, and (C) Cuboid.

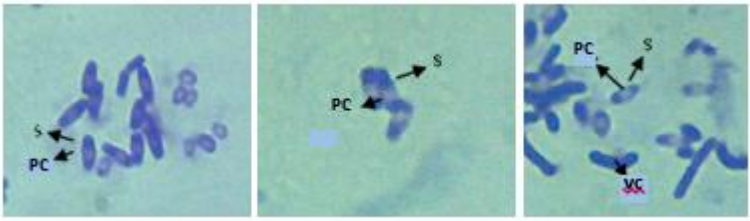

Fig. 6. Formation of Bt 4 Protein Isolate Crystals at 15th Hour, Bt 5 at 3rd Hour, and Bt 6 at 3rd Hour. Description: (PC) Protein Crystals, (S) Spores, (VC) Vegetative Cells of *Bacillus thuringiensis.*