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Exploration of *Bacillus thuringiensis* Berl. from soil and screening test its toxicity on insects of Lepidoptera order

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Abstract. Bacillus thuringiensis is a gram-positive bacterium that produces crystal proteins toxic (8-endotoxin) specific to the target insect, but is not toxic to humans and non-target organisms. This study aims to explore the origin of the soil bacterium B. thuringiensis subdistrict Sekayu, Banyuasin, South Sumatra and toxicity to larvae of lepidoptera. Fifty soil samples were taken from Musi Banyuasin District, namely 15 from Kayuare strip 2, 20 from Kayuare and 15 from Lumpatan. Isolation, characterization, identification and screening test were conducted in the laboratorium of Pest and Disease, Agricultural Faculty, Sriwijaya University. Isolat codes were given based on the area origin of the samples. Results of the study showed that from 50 isolates of bacteria that had been isolated, there were 15 bacterial isolates, characterized by morphology and physiology the same as B. thuringiensis, which has round colonies, white, wrinkled edges, slippery, elevation arise, aerobic and gram-positive. Of the 15 codes that contain positive isolates of B. thuringiensis, we have obtained several isolates of the following codes: KJ₂D₅, KJ₂N₁, KJ₂N₄, KJ₂B₃, KJ₃R₁, KJ₃R₂, KJ₃R₃, KJ₃R₅, KJ₃J₃, KJ₃J₄, KJ₃P₁, DLM₅, DLKK₁₂, and DLKK₂₃. Results of screening tests on insects of the Lepidoptera Order showed that there were six isolates that had toxic to Plutella xylostella and Spodoptera litura insects, ie bacterial isolate codes DLM5, KJ3R3, KJ3R5, KJ3J4, KJ3P1, and DLKK23.

Keywords: Exploration, Bacillus thuringiensis, Lepidoptera. isolate

1. Introduction

Bacillus thuringiensis is a type of aerobic bacteria, spore formers, which is gram positive and is an indigenous bacteria in soil, water, plant surface, and dead insects. B.thuringiensis has a specific target so that it does not kill insects that are not targeted and easily decompose, and do not accumulate and pollute the environment [1]. B. thuringiensis has a protein crystal that is toxic to insects. This toxin protein is first known as parasporal crystalline inclusion but hereinafter referred to as 8-endotoxin or otherwise known as Insecticidal Crystal Protein [2]. Strains of B. thuringiensis bacteria exhibit a wide range of specificity in various insect orders such as Lepidoptera, Diptera, Coleoptera. These bacteria produce crystalline proteins (cry proteins) during sporulation. Insects infected by B. thuringiensis bacteria will cause symptoms of no appetite, slow movement, and over time the insects will die, due to the crystals of proteins that dissolve in the insect's stomach.

In vegetable crops, especially plants belonging to the family brassicaceae, many types of pests that can attack the plant include *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Crocidolomia*

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pavonana Fab. (Lepidoptera: Pyralidae), Spodoptera litura Fab, (Lepidoptera: Noctuidae), Helicoverpa armigera Hubner (Lepidoptera: Noctuidae), Chrysodeixis orichalcea L. (Lepidoptera: Noctuidae), Liriomyza sp. (Diptera: agromyzidae) and Myzus persicae Sulz (Homoptera: Aphidoidae) [3]. P. xylostella and S. litura is a kind of leaf-eating caterpillar pests most commonly attack plants and vegetables can cause damage to approximately 12.5% [4]. In overcoming pest attacks, farmers often use pesticides that contain active synthetic. Many of the negative impacts arising from the unwise use of chemical pesticides. Among them is the occurrence of resurgence or blasting of pest populations, resistance, death of natural enemy population and high residue level on agricultural products so it is not safe for public consumption [5]. Therefore it is necessary to find alternatives in controlling crop pests. One alternative that can be done to replace a chemical insecticide is to use the biological control of the use of living things in the form of microorganisms.

Given the superiority of *B. thuringiensis* as a pest controlling agent, the search for new strains, specific to the pathogen of a particular pest is necessary. This study aims to explore the origin of the soil bacterium Bacillus thuringiensis sub-district Sekayu, Banyuasin, South Sumatra and toxicity to larvae of Lepidoptera. Utilization of microorganisms is very helpful in exploiting microbial agents biological controller is a breakthrough in enhancing the utilization of biological resources more intensively and save the environment from pollution.

2. Material and Methods

The research was conducted in Phytopathology Laboratory of Department of Plant Protection, Faculty of Agriculture Sriwijaya University, Indralaya, Ogan Ilir, South Sumatra, Indonesia, from March to May 2017. The materials used in this research are 50 soil samples taken from Musi Banyuasin district of Sekayu sub-district. The soil samples were 15 taken from Kayuare strip 2, 20 from Kayuare strip and 15 from Lumpatan village. Accordingly, codes of isolates were given on the basis of the origin locality names of samples. Other materials were larvae of *Plutella xylostella* instar III, larvae of *Spodoptera litura*, Nutrient Agar (NA) medium, 3% KOH solution, 5% H₂O₂ solution, Crystalline Violet, Safranin, 95% Alcohol, 5% malachit green solution, Aquadest. Leaves of Caisin as larvae feed. The tools used in this research are aluminum foil, autoclave, bunsen, petri dish, erlenmeyer, measuring cup, isolation, ose needle, digital camera, label paper, laminar air flow, microphone, microscope, analytic balance, oven, tweezers, shaker, Spatel Drygalski, test tube.

2.1 Isolation of bacteria from soil.

The isolation of *B. thuringiensis* from the soil sample from Sekayu subdistrict was then performed in accordance with [6] procedures., which was diluted by weighing 1 g of soil and then put into a reaction tube containing 9 ml of aquadest then dishaker for 5 minutes, the suspension was diluted Until 10-6 is then taken as much as 0.1ml to be grown in a Petri dish filled with Nutrien Agar (NA) medium and leveled on all surfaces of the medium using Splatel Drygalski. Incubation is carried out for 48 hours at room temperature of 28 ° C, until the colony appears to have the same morphology, color, similar to *B. thuringiensis*.

2.2 Characterization of bacteria.

Characterization of Bacillus thuringiensis bacteria was performed by conducting catalyst test, gram reaction test, and microscope observation. The results of the catalase test, and the gram reaction test are presented in the form of descriptive data.

2.2.1 Catalyst Test.

The catalyst test was performed by taking a colony using needle that has the same morphological characteristics as *B.thuringiensis*, scraped on the preparation and then dropped with 5% H₂O₂ solution then stirred. In bacteria that have gram positive properties will produce air bubbles (O₂).

2.2.2 Gram reaction test. Gram reaction test is done using a needle of bacterial colony ose then put on glass preparations that had been spilled with 3% KOH solution, if the bacteria is not slimy then the bacteria is a positive bacteria that is characteristic of bacterium *B. thuringiensis*.

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2.2.3 Gram staining. Gram staining is done to determine the characteristics of bacteria that are obtained gram positive bacteria or gram negative. Gram staining is done by taking 1 scratch of bacterial isolate and then placed on top of glass preparatory, flooding the bacteria with the primary dye that is crystal violet for 1 minute, then tilt the glass object and throw excess dye, then rinse with water. Next flush the odor of bacteria with iodine for 2 minutes remove excess dye, then rinse with water. Blanch with 95% alcohol then rinse with water. Then flush the bacteria with safranin for 30 seconds, remove the excess dyes and rinse with the last water to absorb the remains of water with absorbent paper, then observe under what staining microscope will be produced by the bacteria.

2.3 Selection of B. thuringiensis bacteria.

Of 10 replicated soil samples of 5 times that have been grown, which has the same characteristics as *B. thuringiensis* was selected as many as 15 isolates that have the same morphology and physiology of *B. thuringiensis* bacteria. Furthermore, in the screening test of lepidoptera larvae.

2.4 Test Screening on insects.

Screening test on exploratory bacterial strains that have been morphologically tested and microscopic observations based on spore form and spore staining. In each strain of bacteria that has demonstrated morphological properties as well as microscope observations as *B. thuringiensis*, then taken 1 ml then mixed with 20 ml aquadest. Furthermore the plant leaves are used as feed for tested insect larvae and soaked in a suspension containing 20 ml of aquadest and *B. thuringiensis* bacteria until the leaves are wet all then raised and dried. Plant leaves are put into plastic petri dish (15 cm x 15 cm) coated with paper. Furthermore, 5 larvae of insect test larvae were infected to plant leaves in plastic petri dish 15 cm x 15 cm which have been given treatment. To find isolates that are toxic to lepidoptera larvae.

3. Result and Discussion

3.1 Isolatation B. thuringiensis from soil samples in Musi Banyuasin Regency.

B. thuringiensis is a genus of bacteria capable of forming a dormant structure that is endospores that can be resistant to chemicals as well as physical treatments such as heat, UV and dry. Heating Treatment 800C For 10 minutes on this isolation method. It is intended that other microbes except the endospores will die. Thus only thermo-tolerant bacteria forming spores grow, and from these types of bacteria only aerobic bacteria alone can grow because the culture is incubated aerobically.

The isolation result from 50 soil samples from 3 urban villages in Sekayu subdistrict, found 15 bacterial isolates that have morphological characteristics of *B. thuringiensis*. Observations with a contrast phase microscope, these isolates show characteristics of bacteria known as biological agents (Table 1).

Table 1. Number of soil samples and B. thuringiensis isolate in sub-district Sekayu South Sumatra

Village	Soil sample amount	Isolat B. Thuringeinsis amount
1. Kayuare strip 2 *	15	5
2. Kayuare strip **	20	7
3. Lumpatan ***	15	3
Total	50	15

Legend: *= Desa kayuare Strip 2 (KJ2)

**= Desa kayuare Strip 3 (KJ3)

***=Lumpatan Village

3.2 Identification and Characterization of B. thuringiensis

From result of research which have been done result of isolation of *B. thuringiensis* from soil sample from Sekayu Subdistrict after done incubation process until emerge colony having similarity of morphology and physiology. The results showed the same morphological characteristics of white and yellow rounded colonies, wavy and slippery edges, and elevated elevations (Table 2).

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Table 2. Identification of morphology and bacterial physiology of *B. Thuringiensis*

	Morphological test		Biochemistry test		Miscroscopic test			
Isolate Code	Form	Colou r	Edge	Elevatio n	Reactio n test gram	Catalyst e test	P. Gram	P. Spora
			slipper					
KJ2D 5	Round	White	У	arise	positive	positive	Purple	Green
KJ2N 1	Round	White	Wavy	arise	positive	positive	Purple	Green
KJ2N 2	Round	White	Wavy	arise	positive	positive	Purple	Green
KJ2N 4	Round	White	Wavy	arise	positive	positive	Purple	Green
KJ3R 1	round	white	Wavy	arise	positive	positive	Purple	Green
		Yell-	slipper					
KJ3R 2	round	white	y	arise	positive	positive	Purple	Green
			slipper					
KJ3R 3	round	white	y	arise	positive	positive	Purple	Green
			slipper					
KJ3R 5	round	White	y	arise	positive	positive	Purple	Green
KJ3J3	round	White	Wavy	arise	positive	positive	Purple	Green
KJ3J4	round	White	Wavy	arise	positive	positive	Purple	Green
			slipper					
KJ3P 1	round	White	у	arise	positive	positive	Purple	Green
DLM 5	round	white	Wavy	arise	positive	positive	Purple	Green
			slipper				_	
DLKK1 2	round	white	y	arise	positive	positive	Purple	Green
			slipper		_	_	_	
DLKK2 3	round	white	У	arise	positive	positive	Purple	Green

B.thuringiensis bacterial colonies that grow on the media have a round, white, slimy shape, smooth edges and raised elevation (Fig. 1). According to [8], the bacterial colonies that grow on the media on a round shape, magnitude 5-10 mm, has a white color. At the edge of this bacteria is slightly shrunken or bumpy. The bacteria have an embossed elavance and a rough surface. The bacterial colonies that grow on the first day are very small and almost flat with the media, then on the second day the bacteria will enlarge and somewhat wide but not in touch with one another. On the third day bacteria that grow on the first day will enlarge. Enlargement of bacterial elevation will be evident in solid media.

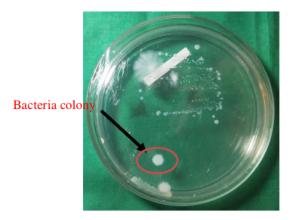


Figure 1. Morphology of colonies, colonies of bacteria growing on white medium such as bacillus colonies

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The result of bacterial isolation that has been grown on Sodium Agar (NA) medium of growing colony morphologically observed shows characteristic as B. thuringiensis that is round, white and yellowish white, wrinkled and slippery edge, and elevation of the colony arises. Gram reaction done in the study showed a gram-positive reaction. This is because if the bacteria given KOH 3% on bacterial cultures placed on the glass preparat not participate lifted on the needle ose. If the bacteria is lifted when lifting the needle ose then the bacteria is gram negative. In this case, the untreated B.thuringiensis bacteria not raised can be said to be gram positive bacteria. The catalase test is a test where when the bacteria tested produces froth after a 5% Hydrogen Peroxide (H_2O_2) solution is given, the bacteria is gram negative. If the bacteria after given a solution of Hydrogen Peroxide (H_2O_2) 5% does not produce froth the bacteria are gram positive.

The catalase test carried out by putting a needle of bacterial culture of *B.thuringiensis* on the preparatory glass which had been dropped by 5% Hydrogen Peroxide (H2O2) solution did not produce foam then the bacterium was positive. In this bacteria after testing does not produce foam means the bacteria is a positive bacteria. This bacterium is a bacterium belonging to the form of bacil. Based on the results of gram tests that have been done on bacteria B. thuringiensis in can if this bacteria is gram positive that is visible from the color of spores produced purple, this is because bacteria B. thuringiensis has a thick peptidoglycan so it can maintain the basic color when done gram staining (Figure 2).



Figure 2. Gram staining (Gram positive) on a 400 x magnification light microscope

B. thuringiensis bacteria are present in all purple isolates after coloring by using a violet crystal reagent. This is evidenced by the presence of the same bacterial cells in each of the diluted isolates. Basil-shaped bacterial cells. At the beginning of the growth of stem-shaped bacteria or bacillus cells then the bacterial cells will be split and oval-shaped. According to [7], bacteria B. thuringiensis will form spores aerobically and at the time of sporulation these bacteria will form protein crystals. The protein crystal formed is known as delta-endotoxin.

3.3 Screening test of Lepidoptera larvae.

Of the 15 codes of isolates that have been known morphology and physiology containing *B. thuringiensis*. Furthermore, a screening test was conducted to see the toxicity of Lepidoptera insects. Test results are shown on (Table 2).

Based on the results of research that has been done found that from 15 isolates that have been tested by taking bacterial cells as much as 10⁻⁶ from the results of dilution, there are 6 isolates that died due to treatment. According to [9], states that with bacterial cells *B. thuringiensis* 1 x 10⁶ cells / ml more virulence to targeted insects. Isolate B. thuringiensis in cells 1 x 106 cells / mL caused a high mortality in killing targeted insects. Cells owned by bacteria B. thuringiensis will enter the insect's large intestine causing the appetite to experience less appetite, the movement of the larvae becomes sluggish. Dead insects change color to black after B infected larvae thuringiensis, the average mortality of larval mortality occurs after 72 hours of application or 3 days. This is in line with the [10] which states that the symptoms of larvae infected by *B.thuringiensis* are test larvae changed the

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treatment to be slow and eventually stop moving and sometimes remove the green liquid from the mouth, then the dirt becomes watery (diarrhea), And finally the larvae will die. And the color of the larva turns dark or brownish black and the body becomes soft.

Table 3. Results of screening of toxicity of bacteria Isolate against Lepidoptera larvae for 168 hours (7 days)

Todata anda	Larva Lepidoptera				
Isolate code	P. xylostella	S. litura			
KJ_2D_5	Negative	Positive			
KJ_2N_1	Negative	Positive			
KJ_2N_2	Negative	Positive			
KJ_2N_4	Negative	Positive			
KJ_2B_3	Negative	Positive			
$KJ3R_1$	Negative	Positive			
KJ_3R_2	Negative	Positive			
KJ_3R_3	Positive	Positive			
KJ_3R_5	Positive	Positive			
KJ_3J_3	Negative	Positive			
KJ_3J_4	Positive	Positive			
$K_{J3}P_1$	Positive	Positive			
DLM_5	Positive	Positive			
$DLKK_{12}$	Negative	Positive			
$DLKK_{23}$	Positive	Positive			

Description: Negative (live insects / not toxic to treatment)

Positive (dead insects / toxic to treatment).

According to [11], the larvae infected by *B. thuringiensis* will die, the body color becomes blackish and the body is soft. When touched the skin of the larvae will break and remove the black liquid and foul-smelling. The color change in the body of the larvae that died from the B. thuringiensis fluid turned into black due to bacteria growing up to the haemokoel part so that the blood cells become poisoned [12,13].

4. Conclusion

From this study it can be concluded that from 50 isolates of bacteria which had been isolated, there were 15 bacterial isolates, characterized by morphology and physiology the same as B. *thuringiensis*, that had round colonies, white, wrinkled edges, slippery, elevation arise, aerobic and gram-positive. Of the 15 codes that contained positive isolates of B. *thuringiensis*, we obtained several isolates of the following codes: KJ₂D₅, KJ₂N₁, KJ₂N₄, KJ₂B₃, KJ₃R₁, KJ₃R₂, KJ₃R₃, KJ₃R₅, KJ₃J₄, KJ₃P₁, DLM₅, DLKK₁₂, and DLKK₂₃. Results of screening tests on insects of the Lepidoptera order showed that there were six isolates that had toxic to *Plutella xylostella* and *Spodoptera litura* insects, ie bacterial isolate codes DLM₅, KJ₃R₃, KJ₃R₅, KJ₃J₄, KJ₃P₁, and DLKK₂₃.

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