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Bacillus thuringiensis berliner cells population growth in some naturally media and the patogenicity against *Plutella xylostella* Caterpilars

Akhmad Gazali[1*], Achmad Jaelani[2], Ilhamiyah Ilhamiyah[2], Siti Erlina[2]

[1]*Faculty of Agriculture, University of Lambung Mangkurat, Banjarbaru, Indonesia*

[2]*Faculty of Agriculture, Islam University of Muhammad Arsyad Al'Banjary, Banjarmasin, Indonesia*

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Abstract

Bacillus thuringiensis is an important biological control agent in nature. For farmers to utilize *B. thuringiensis*, it needs to be researched in media where it can be reproduced easily, quickly, and produce many cells with high pathogenicity. The study aimed to test the influence of some propagation media on the growth of *B. thuringiensis* Berliner cell populations that have a high pathogenicity and compares the pathogenicity between *B. thuringiensis* propagated in some media against *Plutella xylostella* caterpillars. *Bacillus thuringiensis* isolates were taken from the tidal land on the islands of Borneo. The research used a completely randomized design with five treatments a) J Media (corn extract; b) K Media (soybean extract); c) B Media (rice extract); d) C Media (extract mixture of corn, soybean, and rice ratio of 1: 1: 1); and e) Nutrien Broth Media (NB Media) and four replications. The parameter measured was the number of cells. Pathogenicity test between *B. thuringiensis* that is propagated in some media against *P. xylostella* caterpillars were determined by probit analysis. The results showed that, growth media that produced the most *B. thuringiensis* cells are J Media (corn extract), B Media (extract of rice), and C Media (extract mixture of rice, corn, and soybeans). The highest pathogenicity of *B. thuringiensis* which is propagated in the C Media (extract mixture of corn, rice and soybeans) with LC_{50} value is 7.96×10^5 cells/ml suspension. The media type which produce the most *B. thuringiensis* cells and have the highest pathogenicity is C media (extract mixture of corn, rice and soybeans).

* **Corresponding Author:** Akhmad Gazali ✉ akhmad_gazali@yahoo.com

Introduction

One of vegetable grown and consumed by people in Indonesia, and on Borneo in particular, is mustard greens. On the island of Borneo, mustard greens are grown in lowland areas. This plant is very easily cultivated and has a high selling price. However, many factors cause the low production of mustard plants in South Borneo, one of which is the disruption of insect pests. One of them is diamondback moth caterpillars, *P. xylostella*. Without controlled use of pesticides, in the dry season these pests can cause damage up to 100%.

Bacillus thuringiensis bacteria is one of the natural controlling microorganisms of *Plutella xyslotella* caterpillars. These microorganisms are always available in nature and can be isolated from the soil and from the remnants of organic matter. In South Borneo and Central Borneo the 6 isolates of *B. thuringiensis* with the highest LC_{50} value against *P. xylostella* caterpillars are *B. thuringiensis* isolated from gully of forest ecosystems (Gazali *et al.*, 2015). The next step is looking for media of mass propagation and then testing the influence of mass propagation media on the pathogenicity of *B. thuringiensis* against insect pests. Given the magnitude of the destructive ability of *P. xylostella* caterpillars of the mustard plant, it is necessary to find a suitable medium for the propagation of *B. thuringiensis* that can increase the pathogenicity against these pests, and is able to adapt to the environment of the tidal land.

The purpose of this study is: 1) to test the effect of several propagation media on the growth of *B. thuringiensis* cell populations that have the highest pathogenicity; 2) comparing between the pathogenicity of *B. thuringiensis* which were propagated in different media against *P. xylostella* caterpillars.

Materials and methods

Place and time

Tests of propagation media and pathogenicity were carried out in the laboratory of Plant Pests and Diseases Department of the Faculty of Agriculture, Lambung Mangkurat University, Banjarbaru. The study was conducted over eight months in 2015.

Materials and tools

Materials used consist of distilled water, Luria-Bertani Broth, 0.25 M sodium acetate pH 6.8, T3 medium (per liter: 3 g tryptone, 2 g Tryptose, 1.5 g yeast extract; 0.05 M sodium phosphate pH 6.8 and 0.005 g $MnCl_2$), Nutrient Agar, and Nutrient Broth, analytical alcohol, analytical methylated spirit. All materials were purchased from the laboratory of Plant Pests and Diseases, Faculty of Agriculture, laboratory of FMIPA University of Lambung Mangkurat, and chemicals store. The tools used include petri dishes, test tubes, Erlenmeyer glass, needles ose, phase contrast microscopes, Bunsen lamp.

Purification of Bacillus thuringiensis

To obtain pure *B. thuringiensis* the procedure was as follows: one ml culture of *B. thuringiensis* in Nutrient Broth media was resuspended into 9 ml of sterile distilled water and pasteurized at 80° C for 30 minutes. For the selection of *B. thuringiensis,* one milliliter of each suspension was added to 10 ml Luria-Bertani (Merck, Germany) broth (1.0% Tryptone, 0.5% Yeast Extract, 1.0% Sodium Chloride (NaCl), pH 7.0) by buffer with 0.25 M sodium acetate pH 6.8. The suspension was heated at 30°C for four hours and then heated at a temperature of 80 °C for 3 minutes. The suspension was diluted and cultured on the media T3 (per liter: 3 g tryptone, 2 g Tryptose; 1.5 g yeast extract; 0.05 M sodium phosphate pH 6.8 dan 0.005 g MnCl2), then incubated at 30 °C for 24 hours (Travers *et al.*, 1987). Colonies that showed the same morphology were selected and examined under a phase-contrast microscope to determine the presence of parasporal inclusion and spores. All isolates of *B. thuringiensis* were transferred into the medium nutrient to be slanted and prepared for further testing.

Test of propagation media

The research used a completely randomized design

with five treatments and four replications. The treatments were a) J Media (corn extract; b) K Media (soybean extract; c) B Media (rice extract); d) The C Media (mixture extract of corn, soybean, and rice ratio of 1: 1: 1); and e) NB Media. The parameter measured was the number of cells resulting from the procedure in the propagation medium according to the treatment.

These four types of each ingredient were finely ground, then filtered using a sieve, in order to obtain a solid powder. One hundred grams of powdered solid medium was boiled in 500 ml of distilled water to obtain a suspension medium, then filtered using a sieve to produce an extract of each ingredient and prepare them for experimentation. Bacteria used come from the isolation of the exploration activities that have the highest pathogenicity of *B. thuringiensis* among all the bacteria *B. thuringiensis* tested. Insect pathogens propagated using Nutrient Broth (NB) media was to move the bacteria in pure culture in slant NA media aged 2 days to NB media in Erlenmeyer using ose needle aseptically. Bacterial cultures were incubated for 48 hours at room temperature while shaken using a shaker. Two milliliters of cultured *B. thuringiensis* was put into 50 ml in each propagation medium tested.

Two days after inoculation of *B. thuringiensis* into each trial was observed by calculating the number of cells produced multiplied by using propagation medium which is treated using a dilution method. Observations were carried out every 3 days until the culture turned a month old.

Pathogenicity test of Bacillus thuringiensis which were propagated in Several Propagation Media Type against Plutella xylostella caterpillars

Implementation of the preliminary test carried out to determine the lethal concentration on test insects resulted in an effect between 20% to 95%. Implementation of the preliminary test was as follows:

Cell suspensions of all insect pathogens bacterial was prepared by using water with five different concentrations. Mustard leaf pieces the size of 5 × 5 mm by 5 pieces of spilled liquid with 1 ml bacterial suspension evenly spread on the leaf surface. The leaf pieces and one third instar larvae of *P. xylostella* who have fasted for 3 hours were put into a plastic cage that was covered with a damp paper towel and with gauze. When the leaves treated had been consumed by the test insects, additional leaves that were not treated with the bacterial suspension in plastic cages were added to prevent insect death caused by lack of food. In the treatment without *B. thuringiensis*, untreated leaf pieces were given to the test insects. Each concentration treatment was given to each of the 20 third instar larvae of *P. xylsotella*. Insect death was noted after 24 hours after treatment, every 24 hours until it forms a pupa.

Testing

Based on data of the death of insects on a preliminary test after 48 hours, set five different concentrations of bacterial suspensions with a range that can kill 20% - 95% of the population of *P. xylostella* larvae. Methods of treatment were as in the preliminary experiment, but the larvae used were as many as 30 larvae for each treatment concentration. The variable observed was the number of larvae that died every 24 hours until it formed a pupa. The level of pathogenicity was determined by calculating the value of LC_{50} at 48 hours after being treated using probit analysis.

Statistical analysis

The data were analyzed using analysis of variance in the completely randomized design. Differences between the treatment effect is determined using Duncan's Multiple Range Test. The software used was the IBM SPSS Version 17.0 Software.

Results and discussion

In this research, there were three growth media of *B. thuringiensis* which produce the most number of cells that were C media (mixed extract), B medium (extract of rice) and J medium (extract of corn). Bacterial cell population growth of *B. thuringinsis* of the life of cells 3 days after inoculation of cells in each medium to

grow and continue to grow until the age of cells 28 days after inoculation, the longer, the population of *B. thuringiensis cells* in increasing numbers (Table 1). The difference in treatment effect type of growing medium on the growth of bacterial cells of *B. thuringiensis*.

Table 1. Average number of *Bacillus thuringiensis* cells are propagated in some media.

Treatment	Observation time (days after inoculation)								
	3	6	9	12	15	19	22	25	28
	Number of *Bacillus thuringiensis* cells (cells / ml)								
	X 10^{11}	X 10^{15}	X10^{16}	X10^{19}	X10^{22}	X10^{25}	X10^{28}	X10^{31}	X10^{35}
C Media	6.805 a	2.135 a	8.185 a	14.75 a	4.355 a	8.04 a	7.065 a	3.825 a	1.50 a
B Media	8.60 b	3.425 b	13.94 b	8.50 b	5.375 b	8.64 b	6.09 b	4.450 b	1.45 a
J Media	5.945 c	2.125 ac	8.19 a	8.65 c	4.53 c	8.58 c	7.15 c	4.65 b	1.65 a
K Media	7.325 d	2.53 d	1.57 d	8.46 d	0.675 d	1.50 d	1.20 d	1.75 c	0.45 b
NB Media	9.84 e	3.475 e	6.755 e	3.49 e	6.70 e	6.27 e	6.07 be	4.75 b	0.80 c

Description: Average number of cells of *B. thuringiensis* on the same column followed by the same letter are not significantly different according DMRT with the confidence interval of 95%. C Media (Extract mixture of Corn, rice, and soybeans); B Media (Extract Rice); J Media (corn extract); NB Media (Nutrient Broth).

The average number of *B. thuringiensis* cells grow well on the three media- C Media (extract mixture), B Media (extract of rice), and J Media (corn extract). *Bacillus thuringiensis* grows more slowly in K Media (soybean extract). *Bacillus thuringiensis* cell growth in NB media increased quickly at 3-22 days after inoculation and slowed down in 25 to 28 days after inoculation (Table 1). This is due to C Media (extract mixture of rice, corn and soybeans), J Media (corn extract) and B media (rice extract) providing the availability of nutrients to support the growth of *B. thuringiensis* cells. This growth acceleration difference is due to the amount of carbon and energy content for biosynthesis, nitrogen, mineral elements and different substances between media types. This shows that the composition of the medium affects the cells produced. Some medium formulas produce the maximum number of cells and the time of the occurrence of different cell lysis.

This is due to the microbial cells need water, carbon and energy for biosynthesis, nitrogen, mineral elements and growth substances (Bhowmik *et al.*, 2015).

Microbial cells need water, carbon and energy for biosynthesis, as well as nitrogen, mineral elements and growth substances. Dosage forms of various substances depends on the fermentation process used (Gazali *et al.*, 2017a). The use and addition of materials derived from rice, corn and soybeans can increase the number of *B thuringiensis* cells and spores (Marzban, 2012; Hoa, 2014; Bhowmik *et al.*, 2015), Luria Bertani media enriched with nitrogen derived from soybean meal supports increased spore production by 28.5% (Bhowmik *et al.*, 2015). Rice flour is good for medium *B. thuringiensis* KON3 strain, and HO strain, intermediate for KN3 strain and KD2 strains (Marzban, 2012). *Bacillus thuringiensis* is ready to proliferate if environmental conditions such as temperature and nutrient availability support are appropriate. Formation of spores has proved to be triggered by internal and external factors including signals for hunger, nutrition, cell density, and cell cycle progression (Gazali *et al.*, 2017a).

Bacillus thuringiensis life cycle is divided into phases, namely Phase I: vegetative growth; Phase II: transition to sporulation; Phase III: Sporulation; Phase IV: the maturation of spores and cell lysis (Deist *et al.*, 2014; Berbert-Molina *et al.*, 2008). Production from the properties of the crystal protein stored in crystals on stem cells has proven especially

at the beginning of sporulation (Sedlak *et al.*,, 2000; Guidelli-thuler *et al.*, 2009). Some Cry-genes have been shown to be transcribed from two promoters overlap BTI and BtII by RNA polymerase containing sporulation depends on factor of sigma σE and σK (Sedlak *et al.*, 2000) and mutations in the consensus of σE has been shown to inhibit the transcription of a promoter BTI and BtII (Sedlak *et al.*, 2000). It has also been shown that some proteins of *B.thuringiensis* insecticide is produced and secreted into the culture medium during vegetative growth1 (Marzban, 2012; ; Milne *et al.*, 2008; Abdelkefi-Mesrati *et al.*, 2011).

Table 2. LC_{50} value of *Bacillus thuringiensis* is propagated in some media grows against *Plutella xylostella* caterpilars.

The growth media type of *Bacillus thuringiensis*	Value of LC_{50} (cells/ml)
C Media (Extracts mixture of corn, soybean, and rice)	7.96×10^5
B Media (rice extract)	2.36×10^7
J Media (corn extract)	2.79×10^7
K Media (soybean extract)	1.76×10^7
NB Media (Nutrient Broth)	2.41×10^7

Pathogenicity test results showed that the highest pathogenicity produced by *B. thuringiensis* are propagated from the C Media is extract mixture of rice, corn and soybeans, with the LC_{50} value is 7.96×10^5 cells/ml suspension. While low pathogenicity produced by *B. thuringiensis* which is propagated in the J Media (corn extract) with LC_{50} value is 2.79×10^7 cells/ml suspension (Table 2). The high pathogenicity of *B. thuringiensis* that propagated in the C Media caused mixed media provide enough nutrients to produce toxin of *B. thuringiensis* more so that it can increase pathogenicity.

The greater number of toxins and cells produced by *B. thuringiensis* causes the LC_{50} of *B. thuringiensis* paropagated in C media to be lower than that of LC_{50} propagated in other media. Thus, *B. thuringiensis* which is propagated on C medium, has higher toxicity than that propagated in other media.

Reproduction and virulence in entomopathogen *B. thuringiensis* (Bt) is influenced by bacterial density. We estimate that virulence will be affected by high pathogen density because long periods of death allow more host growth. We found that reproductive pathogens (spores produced per dead larva) peaked at the time between death and the lowest in the host who died earlier.

Insect-specific toxins from *B. thuringiensis* provide valuable resources for pest suppression (Deist *et al.*, 2014). *Bacillus thuringiensis* (Bt) protein can be divided into two main groups, the crystal-forming protein is included in *B. thuringiensis* spores (Cry and Cyt toxins) and proteins produced in vegetative cells (Vip Poison). Most of these toxins are produced as protoxins, only toxicity afterwards proteolysis (Van Der Hoeven *et al.*, 2014). Only cry genes that have high gene expression have a major role to determine the insecticidal activity of *B. thuringiensis* strains (Chen *et al.*, 2014). . Toxins released by *B. thuringiensis* the most common and noticeable effect on insect pathogenicity is beta-exotoxin and delta-endotoxin. Delta-endotoxin is only toxic if ingested by insects, ie after decomposed by a protease enzyme to be smaller toxic molecules (English and Slatin, 1992). The results of research in mustard crop, it was found that the most effective concentration decreased the intensity of leaf damages by pest was 6 cc / l and 8 cc / l (Gazali *et al.*, 2017b).

A few minutes after entering into the digestive tract of insects, toxins pass through the tropic membrane, and then will be bound to specific receptors found on mikrovilli cells of midgut epithelium. Once bound, the toxin will form small pores measuring 0.5 to 1.0 nm. Consequently osmotic balance of the cells to be

disrupted, thus ions and water easily enter the cell that causes the cells to swell and rupture, eventually causing lysis (destroyed) (Höfte and Whiteley, 1989). Epithelial cells that have been destroyed will be separated from the base membrane and released into the lumen. Damage and destruction of the epithelium cells causes the base membrane to be easily damaged by *B. thuriengiensis* (Nadu, 2015).

The toxin also inhibits the formation of ATP, damaging ion transport and glucose as well as the movement of the contraction of the muscles of the midgut, metabolic substances such as ions will come out of the lumen and into the hemolymph causing biochemical changes in the channel of digestion and hemolymph that causes paralysis and eventually death in insects (English and Slatin, 1992). Cokseys (1977) said that the main target of the workings of *B. thuringiensis* toxins in the midgut epithelium is "uncoupler" oxidative phosphorylation in the mitochondria, so the effect of ion transport of K to be disturbed[4]. *Bacillus thuringiensis* application is cultured on medium contain soybean meal and sugarcane molasses lead to high toxic effectiveness against *Aedes aegypti* (Soccol *et al*., 2009).

Conclusion

Bacillus thuringiensis is best cultured on C medium (mixture of corn, rice and soybean extracts) since *B. thuringiensis* cultured on C medium produces more cell counts and higher pathogensity to *P. xylostella* caterpillars.

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