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Bacillus thuringiensis* propagated in bio-urine media as a biological control of termite *Coptotermes curvignathus* and armyworm *Spodoptera litura

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Abstract Biological control using entomopathogenic bacteria *Bacillus thuringiensis* is in increasing demand because of its specific target toxicity, safe for natural enemies as well as pollinating insects. Propagation media for *B. thuringiensis* greatly determines the success of spores number produced and toxicity level to insect pests. This study aimed to record the spore density of indigenous isolates of South Sumatera propagated in bio-urine and 5% molasses media and their toxicity level against termites *Coptotermes curvignathus* and armyworm *Spodoptera litura*. The research was conducted at Biological Control Laboratory, Plant Protection Study Program, Faculty of Agriculture, UNSRI, from March to June 2019. The study was designed with Factorial Completely Randomized Design, using isolates as many as 3 isolates with 3 media treatments (bio-urine, bio-urine + 5% molasses, and Nutrient Broth) and 5 replications. The bio-assay used worker caste termites and 3rd instar armyworms. The results showed that a mixture of bio-urine and 5% molasses produced higher number of spores compared to bio-urine media only. The use of NB as a control showed higher spore production compared to the 2 other treatments. The mortality rate of *Coptotermes* termite was higher than the mortality rate of armyworms. Death symptoms of insect pests were indicated by the presence of wet rot and color change (blackish).

1. Introduction

Bacillus thuringiensis is an entomopathogenic bacterium that has been used to control insect pests. Bacteria work selectively towards targetted insect species. *B. thuringiensis* acts as a stomach-poison agent and can infect the tested insects using spores and protein crystals. Bacteria are isolated from soil, dead insects, water, and seeds [1, 2]. In the application, *B. thuringiensis* is used by producing bio-insecticides in which manufacture can use various media containing carbon and nitrogen elements. Some researchers use coconut water and soybean soaking water [3], tofu-filled laundry water [4], agricultural waste [5] and even food waste [6]. The use of bovine bio-urine was chosen because the media contained carbon and nitrogen as much as 21.29% and 1.67%, respectively. With the C / N ratio of 12.75 [7], it was very favorable for *B. thuringiensis* multiplication. Furthermore, the use of molasses containing sucrose, glucose, fructose, and raffinosa in large quantities and several non-sugar organic matters may support media to affect the growth of these bacteria [8, 9].



B. thuringiensis-based bio-insecticides can be used to control various species of insect pests, including the order of Lepidoptera [10], Diptera [11], Coleoptera [12], and Isoptera [3]. The effectiveness of *B. thuringiensis* to differentiate species of pests is mainly caused by the presence of different protein crystals in each isolates [13]. Therefore, each isolates found could be used to determine the level of pathogenicity of the test insects. The study aimed to investigate the spore density of indigenous isolates from South Sumatera propagated in bio-urine and 5% molasses media and their level of toxicity against termite *Coptotermes curvignathus* and armyworm *Spodoptera litura*.

2. Methods

The research was conducted at the Biological Control Laboratory, Plant Protection Study Program, Faculty of Agriculture, UNSRI, from March to June 2019. As many as 3 isolates of *B. thuringiensis* from the collection of Biological Control Laboratory was used. Armyworm larvae and termites were obtained from the experimental farm, Faculty of Agriculture, Indralaya Campus.

The research was arranged in a Factorial Complete Randomized Design (RALF) with 2 factors of treatment: isolate types (SMR4, DLM5 and KJ3R5) and propagation media types (Biourine, Biourine + 5% molasses, and Nutrient Broth). Each treatment was repeated 5 times. For the bio-assay, it was carried out by using 2nd instar larvae (10 larvae/repetition) and worker caste *Curvignathus* termites (20 termites/repetition).

2.1. Test insect preparation

Spodoptera litura larvae were taken from farmer's farmland in the Indralaya area of Ogan Ilir District, South Sumatera, then propagated and maintained in the Laboratory of Entomology, Faculty of Agriculture, Sriwijaya University. The size of 2nd instar larvae in the second offspring (F2) were used as samples for application. Meanwhile, termite *Coptotermes curvignathus* were obtained from oil palm plantations located in Indralaya experimental farm, UNSRI Campus. Then, termites were maintained in the laboratory with its original nest.

2.2. Bio-insecticide preparation

Seed culture was first made as a starter. Seed was taken by ooze needle from petri dish, then was put into 50 mL of Nutrient Broth (NB) in a glass bottle. The media was then put onto a shaker at 200 rpm for 2 hr. After that, 10 mL of the dilution were taken and put into 50 mL of NB, then the media was put onto a shaker at 200 rpm for 2 hr. Afterwards, seed culture was ready to use.

Fermentation medium (in the form of bio-urine, molasses and NB) were sterilized at 121 °C at 1 atm for 20 min in an autoclave. This sterilization process is intended to kill all microorganisms in the media and fermenters, so that it won't interfere with the growth process of *B. thuringiensis* during the fermentation. The treatments were: 1) 50 mL of bio-urine, 2) 45 mL of bio-urine + 5 mL molasses and 3) 50 mL of NB. Furthermore, 5 mL of seed culture was poured into a glass bottle for each treatments in laminar air flow to reduce contamination. Then all the glass bottles were put onto shaker at 200 rpm for 72 hr at room temperature. Bio-insecticide was ready to use.

2.3. Observation of spores density

Spore density calculation was done by sampling method: 1 mL of bio-insecticide from each treatment was taken by using a micropipette, then added into 9 mL of distilled water. The results of the last dilution were taken to calculate the spore density with a haemocytometer. Observation of bacterial spores was carried out at different times i.e. 3x observation (24, 48, and 72 hr).

2.4. Bioassay

The test insects being used were 2nd instar armyworm and worker caste termites. Bio-assay of *S. litura* larvae was carried out by a modified method [14]. Test insects were put into a plastic tray of 30 cm x 60 cm, then covered with gauze. Mustard green leaves were weighed as much as 7 g for each treatment. Bio-insecticide toxicity test on insects was carried out in a laboratory: 5 mL of bio-insecticide were taken and mixed with 100 mL of sterile water, then stirred until homogeneous. Mustard green leaves were immersed into each treatment for ± 3-5 min, then removed and left air-

dried. After that, the leaves were put into a plastic petri dish (15 x 15 cm) containing 10 larvae of *S. litura*. Each treatment was repeated 5 times.

The mortality test was carried out by using 20 termites for each treatment. Beforehand, 50 g of blended soil was weighed and put into a plastic cup. After that, 4 mL of *B. thuringiensis*-based bio-insecticide solution was applied by spraying. For further observations, soil was moistened with distilled water to prevent the soil from drying out. Observation of insect mortality was conducted every 24 hr for 7 d.

2.5. Calculation of insect mortality

Observation of larvae mortality was conducted every day in a duration of 7 d. The percentage of insects mortality was calculated by the following formula below:

$$P = \frac{a}{b} \times 100\%$$

In which:

P = Percentage of larvae or adults mortality (%)

a = Larvae or adults died infected by *B. thuringiensis*

b = total number of observed larvae and adults

2.6. Symptoms of infected larvae of *B. thuringiensis*

Observations were recorded by observing the symptoms that occurred in *S. litura* larvae and worker caste termites after application involved monitoring the symptoms and appearances of dead insects. Further observation of the metamorphosis process of alive insects was conducted after the observation period ended.

2.7. Data analysis

Data on spore density and mortality of test insects were analyzed using ANOVA. A significant difference results between treatments were further analyzed by Tukey HSD test (P = 0.05%). Symptoms of test insect infection were observed and presented descriptively.

3. Results and discussion

3.1. Spore density of *B. thuringiensis* in various growing media

Observations on spore density of 3 isolate treatments showed no significant difference statistically. However, it can be seen that spore density at biourine + 5% molasses was almost similar to those spore density in NB treatment (Fig. 1).

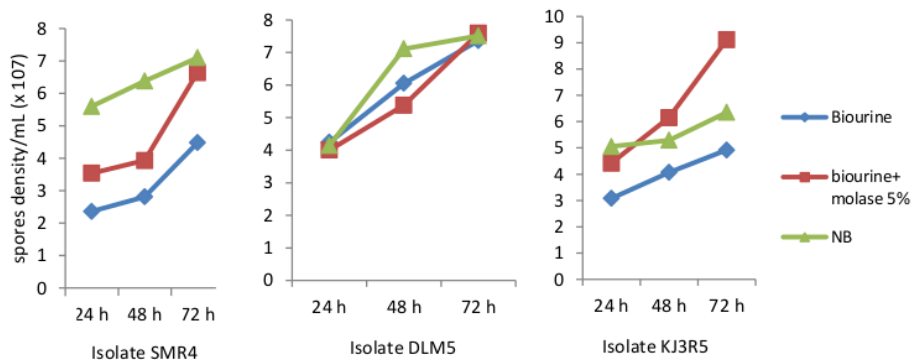


Figure 1. Spores density of *Bacillus thuringiensis* propagated on various growth media.

1 The use of bio-urine + molasses 5% produced high spore density values even exceeding NB media. In the mixed media, the constituent components have more variations. As reported by [15] growth media for *B. thuringiensis* must contain carbon and nitrogen elements of at least C / N = 5.2. In this research, cow bio-urine being used was C / N = 12.75. Thus the nutritional needs for *B. thuringiensis* growth were sufficient. Based from the time of growth, 3 isolates (SMR4, DLM5 and KJ3R5) have similar tendencies, which at the first 24 h was low and then increased at 48 hr and 72 hr. This result was consistent with the results of [5] research, which was stated that the growth of *B. thuringiensis* through suitable media will increase of spore density.

3.2. Test insect mortality

Mortality of *S. litura* in bio-urine treatment ranged from 50-86%, whilst *Coptotermes* termite's ranged from 64-90%. Mortality of *S. litura* in mixed bio-urine treatment + 5% molasses ranged 70-84%, whilst *Coptotermes* termite's ranged from 84-98%. The mortality of tested insects after 7 d of observation was presented in table 1.

Table 1. Mortality of *Spodoptera litura* and *Coptotermes curvignathus* caused by *B. thuringiensis* after 7 days.

Isolate	Treatments	Mortality ^a	
		<i>S.litura</i> (%)	Coptermestes (%)
SMR4	Biourine	60,00 ± 3,16bc	80,00 ± 3,4bc
	Biourine+molasses 5%	70,00 ± 3,16 bc	90,00 ± 2bc
	Nutrient Broth	80,00 ± 3,16c	94,00 ± 2,82c
	Control (aquadest)	8,00 ± 1,26 a	10,00 ± 1,41a
DLM5	Biourine	82,00 ± 2,28 bc	90,00 ± 2,28bc
	Biourine+molasses 5%	86,00 ± 2,82 c	98,00 ± 0,63c
	Nutrient Broth	90,00 ± 3,16c	96,00 ± 1,41c
	Control (aquadest)	22,00 ± 2,28a	10,00 ± 1,41a
KJ3R5	Biourine	50,00 ± 3,16	64,00 ± 4,04b
	Biourine+molasses 5%	76,00 ± 2,82bc	84,00 ± 2bc
	Nutrient Broth	80,00 ± 1,41c	94,00 ± 1,41c
	Control (aquadest)	10,00 ± 1,41a	12,00 ± 1,41a

^a Mortality followed by different letter in columns by trial were significantly different (P < 0.05) according to Tukey's test.

Test insect mortality in bio-urine and bio-urin + molasses mixture treatments on all isolates showed insignificant differences statistically. This is presumably because the spore density level was also not significantly different (Fig. 1). Compared to the control, all treatments showed significant differences. In NB treatment, the mortality of test insects, both armyworm and termite were also showed high mortality rate. In NB treatment, nutrition for bacterial growth was fulfilled as NB was a factory product. In the tested treatment, nutrient content for *B. thuringiensis* growth and development couldn't be measured in a laboratory manner. However, some researchers reported, that agricultural and livestock waste media have been tested as Bt propagation media and produced high mortality rates for tested insects [5, 16, 17].

3.3. Symptoms of test larval infection

Symptoms of infection on test insects were observed starting 24 hr after application. Symptoms of *S. litura* test insect mortality and *Coptotermes* termites were presented in Fig. 2.

Death of larvae began to occur 1 day after the application of *B. thuringiensis*. Some insectssustained mortality starting from the third day after application. The percentage of deaths

increased after 7 d after application. These results were supported by [18], which stated that on the first day of application, test insects experiencing symptoms of slow motion and decreased appetite. On the 2nd day, larvae began to inactive, dead and showed brownish-green appearance. Lastly, on the 3rd day to the 7th day of application, insect experiencing death. The symptoms of *B. thuringiensis* infected larvae were started from movement declining, then followed by decreasing appetite and if it was touched the larvae did not respond. All the larvae that died in this study had the same symptoms, such as the change of color to pale brown and gradually became darker, the body of the larvae shrank and gave off a foul odor. The body of larvae infected with *B. thuringiensis* bacteria will turn reddish-brown and the next day the larva's body will turn black [19].



Figure 2. Symptom of death on tested insects; (a) healthy *S.litura*; (b) dead *S.litura*; (c) healthy *C. curvignathus*; (d) dead *C. curvignathus*.

Infected worker termites changed its color from thorax to abdomen. At first, the body of infected termite would change to a pale brown color, then turned black and looked wet. Termites moved towards the edge of the plastic cup to avoid light exposure. The termite's body gradually became soft. If it was removed, the termite's body would easily be separated from 1 organ to the other. This result was consistent with the report of [20], which stated that termites that die due to infection with *B. thuringiensis* experience darker color changes throughout the body. Abdomen and thorax also turned black and released fluid, so the body looked like wrinkles soften and stink.

4. Conclusion

The use of bio-urine + 5% molasses as *B. thuringiensis* propagation material was promising because it produced high spore density. The waste material was easy to obtain and inexpensive, hence it could be used as a bio-insecticide. The mortality rate of *Coptotermes* termites was higher compared to armyworm's in all *B. thuringiensis* isolates treatments.

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