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Effectiveness of Proteins and Supernatants Isolated from *Bacillus Thuringiensis*-Based Bio-Insecticides Against Termites *Macrotermes Gilvus* (Isoptera: Termitidae)

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Abstract. *Bacillus thuringiensis* an entomopathogenic bacteria is widely used as an active bio-insecticide, including to control *Macrotermes gilvus* termites. The effectiveness of *B. thuringiensis* as a bio-insecticide can be derived from both its protein and spore activities. For this reason, research was carried out to investigate effectiveness of protein separated from spores in controlling these termites. The study was arranged in a factorial completely randomized design with two factors, namely ten isolates of *B. thuringiensis* (first factor) and bio-insecticide separation treatment (second factor). Propagation medium was bio-urine enriched with 5% molasses. Bio-insecticide treatment was protein and supernatant of *B. thuringiensis*. Isolates used were *B. thuringiensis* isolates indigenous South Sumatera. Results showed no differences effect among *B. thuringiensis* isolates. The highest spore density in a solution without separation was isolate SMR-04 (11.23 x10^{12} spores/ml). Average spore density in supernatant treatment was MSP isolates (6.00 x 10^{12} spores/ml). The highest mortality occurred in KJ3P1 isolates on supernatant application. LT_{50} value was in range of 0.921-1.025 days. To control *M. gilvus* termites with protein and supernatants caused high mortality and low LT_{50} value. This suggested B. *thurigiensis* could be a candidate for biological control agents of *M. gilvus* in the future.

1. Introduction

Macrotermes gilvus (Isoptera: Termitidae) is a wood-eating termite, an important pest on plantation crops. Generally, their attack cannot be detected at an early stage, because termites attack parts of plant under soil surface [1]. Controlling termites by chemical insecticides have to conduct very carefully due to unpredictable attack of termites. Therefore, it is necessary to carry out biological control by using soil-based bacteria *Bacillus thuringiensis*. These bacteria have been used to control various species of pests such as *Oryctes rhinoceros* [2], armyworm *Spodoptera* litura [3], [4].

As an entomopathogenic bacterium, *B. thuringiensis* is unique because it has a special target insect. This is due to the protein content in *B. thuringiensis* cells produced during sporulation [5]. When ingested by insects, this protein reaches midgut. Because of the presence of protease enzyme, it will dissolve and degrade into smaller, toxic protein molecules. In this process, high pH plays very important role. Therefore, if pH condition is low, the degradation process of protein molecules will not occur, and bacteria are not toxic [6] and [7] reported there are two factors which cause *B. thuringiensis* to be effective in killing insects, namely the presence of spores and protein. After ingestion, the spores grow in the midgut and eventually spread throughout the hemolymph leading to

insect death [8]. For this reason, it is necessary to conduct research on the effectiveness of spores and proteins against the insects tested for *M. gilvus*.

2. Methodology

The research was carried out at Entomological Laboratory of Plant Protection Department, Faculty of Agriculture, Sriwijaya University, from August to November 2019. *B. thuringiensis* isolates belong to Plant Protection Department [2]. Propagation media of *B. thuringiensis* were cattle's bio-urine and molasses. The study was arranged in a factorial completely randomized design (RALF) with two factors, namely ten isolates of B. thuringiensis (first factor) and bio-insecticide separation treatment (second factor).

2.1. Preparation of Test Insects

Termites were taken from the experimental farm of Plant Protection Department, Faculty of Agriculture, Sriwijaya University in weathered wood habitats. Termite maintenance was carried out using a plastic container (d = 15 cm, h = 30 cm), covered with gauze to keep air circulating. Humidity was maintained in order to resemble conditions in original habitat of termites. The test insects used were soldier caste termites. Feed of termite used was dry, weathered wood.

2.2. Bioinsecticide Preparation with Active Ingredient of Bacillus Thuringiensis

Preparation of seed culture used 10 isolates of *B. thuringiensis*, through a 24-hour fermentation process on Nutrient Broth (NB) media, room temperature and 200 rpm. The next process, the growth medium (bio-urine enriched with molasses) was transferred into fermenter flask. The fermentation process took at least 72 h. The next step was to separate the protein and supernatant. This process was carried out by transferring bio-insecticide solution in an erlenmeyer flask and centrifuged at a speed of 5000 rpm for 20 minutes. The results obtained were a white layer that settles at the bottom of the flask (protein) and an upper layer in the form of a supernatant liquid containing spores. Bio-insecticides in the form of proteins, supernatants and mixed solutions are ready for use in experimental applications.

2.3. Bio-Insecticide Application to Worker Caste of Termites

Application was carried out using 3 treatments i.e. protein, supernatant and a mixed solution of both. A total of 1 ml of test material was dissolved in 99 ml of distilled water. The solution was sprayed on the weathered wood as termite's feed prepared in 50 g of plastic petri dishes. Ten10 termites were put in each petri dish as part of the test. Observation of termite mortality was carried out every 24 hours for 7 days. Spores density of supernatant and mixture solution, symptoms of termite mortality and time of death (LT_{50}) were observed.

2.4. Data analysis

Data regarding spore density in mixed and supernatant solutions, and termite mortality were analyzed by analysis of variance (ANOVA), while the LT_{50} value was calculated by probit analysis.

3. Results and Discussion

B. thuringiensis-based bio-insecticide was propagated in bio-urine media enriched with molasses. Furthermore, the separation was carried out into supernatant and protein which was settled at the bottom of the tube. The results of the spore density calculations in the supernatant showed MSP isolates contained the highest spore density, namely 6.00 x 10^{12} spores / ml. On the other hand, bio-insecticide mixed solution showed the highest spore density was in SMR-04 isolate, namely 11.24 x 10^{12} spores / ml. Data was presented in Table 1.

Table1. Density of bio-insecticide spores with active ingredient Bacillus thuringiensis after separation between the supernatant from the protein and mix solution

***B. thuringiensis* Isolate**	**Average of spores density (10^{12} spores/ml)**	
	Mixed solution	**Supernatant**

***B. thuringiensis* Isolate**	**Average of spores density (10^{12} spores/ml)**	
	Mixed solution	**Supernatant**
SASU	7.37 ± 0.072 ab	4.47 ± 0.314 bcd
MSP	10.64 ± 0.100 b	6.00 ± 0.084 d
SMR4	11.24 ± 0.028 b	5.77 ± 0.491 cd
TPP	4.83 ± 0.166 a	3.13 ± 0.167 ab
KJ3R3	3.70 ± 0.094 a	2.28 ± 0.167 a
C14	8.67 ± 0.144 ab	4.08 ± 0.157 bcd
KJ3P1	4.77 ± 0.110 a	3.62 ± 0.157 bc
KJ3R5	7.13 ± 0.072 ab	4.42 ± 0.314 bcd
LK	5.11 ± 0.094 ab	2.97 ± 0.183 ab
MSKS	3.45 ± 0.075 ab	4.80 ± 0.909 bcd
F counted	286.05*	9.39*
F Table	2.39	2.39
P Value	4x 10^{-19}	0.00001853
Tukey's Value 5%	0.05	0.21

Notes *) Figures in the same column followed by the same letter indicate significant differences in Tukey's test with a confidence level of $\alpha = 5\%$

This showed each isolate had a different level of spore production. This property was a characteristic of each *B. thuringiensis* isolate itself. The origin of isolate and type of protein content were one of the characteristics of *B. thuringiensis* isolates [6]. Thus, *B. thuringiensis* isolates can be grouped based on their protein content in which impacts on their ability to kill target insect pests [9]. In supernatant, it appeared spores density was lower than spore density in the mix solution of *B. thuringiensis* without separation. There was a suspicion that separating protein and spores resulted in releasing protein from the cell, thereby reducing spore content in the cell [10] also reported cell content after sporulation was in the form of spores and protein. In this case, protein also contained toxic which can kill target insects [11].

Symptoms of Termite Death

Experiment was carried out on worker caste termites because they can feed themselves without any help of other castes. Death began on the first day, with symptoms of termites starting to stop eating. At second day, the colour of the body turned slightly blackish. At seventh day, tested worker caste termites died completely with symptoms of a blackish body and began to shrink (Figure 1).

Figure 1. Symptoms of termite death on the first day (a), second day (b), third day (c), fourth day (d), fifth day (e), sixth day (f), and seventh day (f), healthy termite (0)

Mortality of tested insects from the first to the fourth observation days tended to increase. However, the highest mortality was obtained in supernatant treatment compared to protein and mixed solution

treatment. The highest mortality on the fourth day of observation was 93.90%, namely supernatant treatment. Data was presented in Table 2.

Table 2. Average mortality of *Macrotermes gilvus* termites after application of *Bacillus thuringiensis*-based bio-insecticide with protein, supernatant and mixed solution treatment

Treatments	Mortality (%) observation day ...			
	1	2	3	4
Protein	30.94 ± 3.90 a	54.3 ± 4.66 a	76.42 ± 3.18 a	90.3 ± 2.66 a
Supernatant	43.45 ± 5.75 b	70.25 ± 5.59 b	87.24 ± 4.88 b	93.90 ± 3.06 a
Mixed solution	29.16 ± 4.64 a	55.4 ± 4.48 a	77.57 ± 3.41 a	89.99 ± 2.94 a
Control	0±0.00 c	0±0.00 c	0±0.00 c	0±0.00 c

Notes *) Figures in the same column followed by the same letter indicate significantly differences in Tukey's test with a confidence level of α = 5%

Supernatant still contain *B. thuringiensis* spores (2.97 - 6.00 x 10^{12} spores / ml) which are also capable in killing tested insects [6]. In the mixed solution, it was found number of spores was much higher than number of spores in the supernatant (Table 1). However, mortality rate of tested termites was not significantly different (Table 2). In protein treatment, mortality tends to be higher than that of supernatant and mixed solution. The mixed solution (supernatant and protein) will cause a lower mortality rate than supernatant solution or protein itself [11]. Calculation of the LT_{50} value in protein treatment showed SMR-04 isolate showed the shortest duration of time compared to other treatments, namely 1.025 days. The longest time was obtained in LK isolate (2.442 days). LT_{50} data was presented in Table 3 and Table 4.

Table 3. LT_{50} termite *Macrotermes gilvus* worker caste on protein treatment

B. thuringiensis Isolate	LT50 (day)	Confidencial Limit (day)		Regression Equations
		lower	upper	
KJ3P1	1.569	1.133	1.998	y= 0.803x-1.259
MSP	2.001	1.573	2.427	y= 0.803x-1.606
KJ3R5	2.133	1.695	2.573	y= 0.803x-1.712
KJ3R3	2.327	1.898	2.758	y= 0.803x-1.868
SMR4	1.025	0.546	1.495	y= 0.803x-0.823
C14	1.771	1.328	2.212	y= 0.803x-1.422
MSKS	2.182	1.746	2.618	y= 0.803x-1.751
TPP	2.084	1.651	2.515	y= 0.803x-1.673
SASU	2.187	1.751	2.624	y= 0.803x-1.756
LK	2.442	2.01	2.876	y= 0.803x-1.960

Table 4. LT_{50} *Macrotermes gilvus* termite worker caste in supernatant treatment

B. thuringiensis Isolate	LT50 (day)	Confidencial Limit (day)		Regression Equations
		lower	upper	
KJ3P1	0.947	0.402	1.482	y=1.028x-0.974
MSP	1.218	0.682	1.747	y=1.028x-1.252
KJ3R5	2.602	2.138	3.069	y=1.028x-2.675
KJ3R3	2.411	1.934	2.889	y=1.028x-2.479
SMR4	1.328	0.813	1.835	y=1.028x-1.366
C14	1.583	1.072	2.091	y=1.028x-1.628
MSKS	1.354	0.819	1.884	y=1.028x-1.392
TPP	1.524	0.998	2.048	y=1.028x-1.567
SASU	1.524	1.000	2.045	y=1.028x-1.567
LK	2.266	1.777	2.753	y=1.028x-2.330

LT_{50} value in protein treatment ranged from 1.025 to 2.442 days, while in supernatant treatment ranged from 0.947 to 2.602 days. This showed mortality of 50% of tested insects was quite fast because it was still in the same time range between protein treatment and supernatant treatment. However, supernatant treatment was faster than protein, in which supernatant treatment was 0.947 days. While protein treatment was 1.925 days. Protein contains active ingredients in the form of toxins that can cause poisoning (dehydration) in insect metabolic system. [6] reported their research on the effect of protein on insect mortality. In the process, protein molecules must be digested first in an alkaline environment (high pH). As a result, they changed into smaller molecules and were toxic [6], [12]. Therefore, tested insects usually took a longer time to die.

4. Conclusion

Controlling insects pests by *B. thuringiensis*-based bio-insecticide can be used. *B. thuringiensis* application might be done in the form of protein, supernatant and a mixture of the both. The use of protein lead to higher mortality compared to supernatant application. However, the effectiveness of a *B. thuringiensis* isolate was also affected by the protein content both in type and quantity.

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