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Submission date: 24-Mar-2023 03:43PM (UTC+0700)
Submission ID: 2045266732
File name: Prodising_internasional_Suparman_2.pdf (5.26M)
Word count: 2672
Character count: 13530

E3S Web of Conferences **373**, 07010 (2023) https://doi.org/10.1051/e3sconf/202337307010
ISEPROLOCAL 2022

Potency of *Bacillus thuringiensis* in liquid formulation as a biological agent in controlling larvae of *Oryctes rhinoceros* (Coleoptera:Scarabaeidae)

Yulia Pujiastuti[1*], *Suparman* SHK[1], and *Abu* Umayah[1]

[1]Sriwijaya University, Department of Plant Protection Faculty of Agriculture, South Sumatera, Indonesia

Abstract. Propagation of *B. thuringiensis* in agricultural liquid waste media can be done because the waste still contains nutritional components which contain carbon and nitrogen. The aim of research was to study *B. thuringiensis* in a liquid formulation with high toxicity against Oryctes larvae. Parameters of observation were spore density, larval mortality and symptoms of both infection or death larvae. Experimental was designed as a completely randomized design with 6 treatments and 4 replications. Oryctes larvae used were 3rd-instar. The treatments were 1). Tofu liquid waste; 2) Tofu Liquid Waste + Bio-urine (1:2); 3) Tofu Liquid Waste + Bio-urine (1:1); 4) Tofu Liquid Waste + Bio-urine (2:1); 5) Bio-urine and 6) NB media (as control). The results showed the highest spore density was found in tofu liquid waste propagation media and biourine (1:2). The highest larval mortality was shown in treatment of Tofu Liquid Waste + Bio-urine (1:2), which was 91.67%. Observations on the symptoms of infection and death in tested larvae were indicated by changes in color of integument and fragility of the larva's body. Bio-insecticide with active ingredient of *B. thuringiensis* in liquid formulation has potency to be used as biological agent for controlling *O. rhinoceros* larvae.

1 Introduction

Oryctes rhinoceros has been known as a major pest on oil palm plantations. The pest attack stage is adult stage, but larval stage must also be controlled because it is a series of life cycles which related to one another [1,2]. Oryctes larval control can be carried out biologically, among others, by applying *Bacillus thuringiensis* [3]. This bacterium is one of entomopathogenic bacteria which is used as an active ingredient in manufacture of bio-insecticides. One of the uniqueness of *B. thuringiensis* is the presence of spores and proteins produced during sporulation. *B. thuringiensis* works as stomach poison where spores and proteins must be eaten by target insect [4]. In propagation of *B. thuringiensis,* it needs a growth media with adequate nutrition. Materials containing carbon and nitrogen are media

* Corresponding author : ypujiastuti@unsri.ac.id

supporting the growth of *B. thuringiensis* [5]. Some liquid byproducts of agricultural production can be used to produce bio-insecticides with *B. thuringiensis* active ingredient. Several researchers have conducted research on agricultural waste, including solid waste [5], commercial lab medium and agricultural by-products as nutrient sources [6], foodwaste [7], industrial-by product [8] and agro-industrial waste [9]. Bio-insecticide formulation is important to determine toxicity level to tested insect. It is known several types of formulations including solid and liquid formulations. Research on liquid formulations has been carried out [6] by using waste materials proven to be effective in controlling several types of pests. Wahyuono [10] reported a bio-insecticide liquid formulation in the treatment of palm oil mill effluent (LCPKS 100% + 0.4 g brown sugar + 30 ml coconut water + *B. thuringiensis*) because it able to increase mortality value higher (namely 66.6%), speed of mortality (4.6 days), change in population (66.6%), and feeding resistance (41.1%) against nettle caterpillar *Setora nitens*. This paper reported a research aimed to study probability of *B. thuringiensis* in a liquid formulation against *O. rhinoceros* larvae.

2 Materials and methods

This study was conducted at the Entomological Laboratory of Plant Protection Department, Faculty of Agriculture, Sriwijaya University (Inderalaya Campus). Experiment of *B. thuringiensis* propagation was carried out with 6 treatments (including control) and repeated 4 times. The treatments were 1). Tofu liquid waste; 2) Tofu Liquid Waste + Bio-urine (1:2); 3) Tofu Liquid Waste + Bio-urine (1:1); 4) Tofu Liquid Waste + Bio-urine (2:1); 5) Bio-urine and 6) NB media (as control). Tested insects were 3rd-instar Oryctes larvae. *B. thuringiensis* (TPP code) isolate tested was one of collection from Laboratory of Entomology Plant Protection Department, Faculty of Agriculture, Sriwijaya University (unpublished data).

2.1 Preparation of test insects

Larvae were obtained from various oil palm plantations owned by farmers around Indralaya District, Kab. Ogan Ilir South Sumatra. They were taken and placed in a rearing container (d=15 cm, h=20 cm) with sterile soil and feed with rotten oil palm trunks. Rearing area was cleaned every 7 days, as well as the soil and feed were replaced to maintain the fulfilment of their life needs. Larvae were reared until they were used for bioassay assays.

2.2 Preparation of liquid waste as a medium for B. thuringiensis propagation

Liquid waste media used were tofu liquid waste and cow bio-urine. Tofu liquid waste was obtained from the home-based tofu industry in Permata Village, North Indaralaya District, Kab. Ogan Ilir while bio-urine was obtained from Lab. Of Cowshed, Department of Animal Husbandry, Faculty of Agriculture, UNSRI. These liquid wastes were prepared in certain comparison and then sterilized.

2.3 Preparation of seed culture

Seed culture was a source for producing bio-insecticides. One loop of *B. thuringiensis* isolate was put into 10 ml of Nutrient Broth (NB), fermented for 12 hours, speed of 200 rpm at room temperature. After 12 hours, another 10 ml of NB was added and fermented again for 12 hours at 200 rpm and room temperature. Seed culture was ready to use.

2.4 Production of B. thuringiensis-based bio-insecticides

Liquid formulation of bio-insecticide was made by preparing the following treatments: 1) Tofu liquid waste; 2) Tofu Liquid Waste + Bio-urine (1:2); 3) Tofu Liquid Waste + Bio-urine (1:1); 4) Tofu Liquid Waste + Bio-urine (2:1); 5) Bio-urine and 6) NB media (as control). Culture media were enriched with mineral salts (MnSO4, MgSO4, ZnSO4, and FeSO4) and pH adjusted to 7.0. Each treatment was prepared as much as 100 ml and aseptically added 10% seed culture. It was incubated in Erlenmeyer flask for 72 hrs in shaker-incubator.

2.5 Bioassay toward 3rd instar Oryctes larvae

Application of bio-insecticides was started to place 300 g of sterilized soil and 10 g of weathered oil palm trunks in a plastic container (20x15x10 cm). Both ingredients were mixed evenly, then 5 ml of bio-insecticide (dissolved in 45 ml of water) was sprayed on mixture. A total of 5 individues of 3rd-instar Oryctes larvae were put into each of these containers, and then tightly closed. At 7th day, soil and feed were replaced with new ones. Observations of tested larvae were carried out from the first day until about one month. Symptoms of infection and larval death were observed. Number of larval mortality and the weight of remained alive larvae were counted.

2.6 Data analyses

Data on spore density, larval mortality, and larval weight were analyzed by analysis of variance (ANOVA). Tukey's Honesty Significant Difference (HSD) Test was employed to test for significant differences among treatments at P=0.05. All data were analyzed using software of SAS University Edition 2.79.4M5.

3 Results and discussions

3.1 Bacillus thuringiensis spore density

Fermentation process of *B. thuringiensis* in liquid waste media caused growth and development of spores and bacterial cells. This can be seen in the first 24 hours. *B. thuringiensis* has grown in various treatments and increased in the next 24 hours. At the end of observation (72 hours) it appeared that Tofu Liquid Waste + Bio-urine (1:2) media treatment showed the highest number of spores, namely 6.5 x x108 spores / ml (Table 1). This showed this mixture was a better comparison among other treatments, while other treatments produce fewer spores. This was inseparable from the components contained in each of these materials. Tofu liquid waste contained 40-60% protein, 25-50% carbohydrates, and 10% fat, while bio-urine contained N (1.4 - 2.2 %), P (0.6 - 0.7%), K (1.6 – 2.1%). When the amount of bio-urine was added, number of protein components increased [11]. However, [5] also reported the growth of *B. thuringiensis* was highly dependent on the C/N ratio**.**

Table 1. Spore density of *B.thuringiensis* propagated in liquid waste.

Treatments	Spore density (x10^8 spores ml^{-1})		
	24 h	**48 h**	**72 h**
Tofu liquid waste	2,73 bc	3,77 d	2,28 b
Tofu Liquid Waste + Bio-urine (1:2)	3,78 e	4,69 e	6,50 d
Tofu Liquid Waste + Bio-urine (1:1);	2,45 ab	3,01 c	2,69 b
Tofu Liquid Waste + Bio-urine (2:1)	3,24 d	3,07 c	2,15 a
Bio-urine	2,96 cd	2,49 a	2,48 b
NB media (as control).	2,53 ab	2,52 a	2,91 b
F Value	2,79*	42,93*	3,65*
F Table	2,66	2,66	3,11
BNJ 5%	0,06	0,02	0,02

Notes : *) significantly different

3.2 Oryctes larval mortality on bioassay

Mortality was known since 6th day after application. This is indicated that *B. thuringiensis* (both protein and spores) had been eaten by tested larvae and ingested into their midgut. In the midgut there was a process of protein breakdown from large proteins into smaller particles which were toxic [12]. Thus, toxic proteins will bind to midgut wall causing lysis process. From this process, a porus will be formed. As a results, it caused exchange of hemolymph fluid out of midgut wall. Finally, larvae will undergo lysis and then die [13]. Mortality occurred gradually and cumulatively occurred on day 36, in which the highest peak of mortality was 91.37% in the mixed treatment of Tofu Liquid Waste + Bio-urine (1:2) (Figure 1). This proved that poisoning process in midgut took a long time and a large number of spores. It was possible because Oryctes larvae have a large body posture consequently processes in their body also took longer time compared to other insects [3].

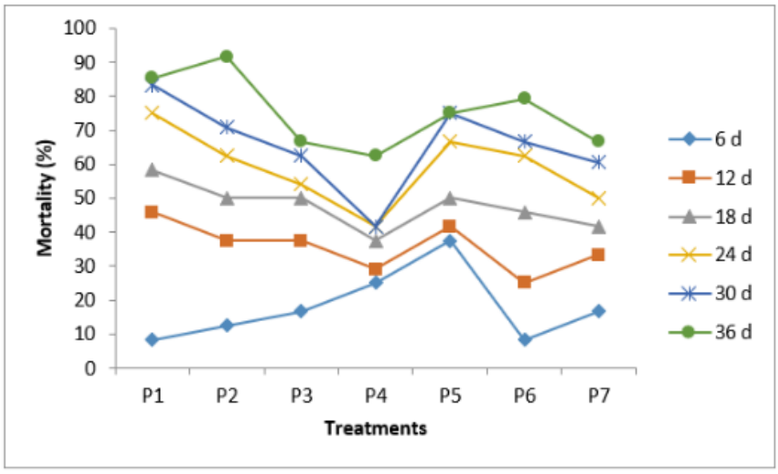

Fig. 1. Mortality of Oryctes larvae applicated with *B.thuringiensis* -based bio-insecticide in liquid formulation.

Notes :
P1.Tofu liquid waste; P2.Tofu Liquid Waste + Bio-urine (1:2); P3.Tofu Liquid Waste + Bio-urine (1:1);
P4.Tofu Liquid Waste + Bio-urine (2:1); P5.Bio-urine; P6.NB media (as control).; P7. Control (water)

3.3 Symptoms of infection and larval death

O. rhinoceros larvae infected by *B. thuringiensis* bacteria showed early symptoms of decreased insect activity, color changed from white to light brown or dark brown, decreased appetite of larvae and decreased body weight. Furthermore, body color becomes dark brown and when it died, larva's body color changed to black (Figure 2). This was indicated by the presence of a fragile, watery and foul-smelling body [14].

Fig. 2. Symptoms of dead larvae of Oryctes; a. healthy larvae; b. larval cadaver.

3.4 Oryctes rhinoceros larvae weight

Alive Oryctes larvae were weighed to determine their body weight. In addition to small number of tested larvae which alive, larval weight was also experienced a drastic decrease. This is the impact of *B. thuringiensis* infection on larval body. There was a decrease amount of feed consumed by larvae. This will result in slower growth and development of larvae [14]. At the beginning of application (0 d), there was a significantly difference among treatments, as well as on the 6^{th} and 12^{th} days. However, from day 18 onwards, there was no significant difference (Table 2). Toxic effect of *B. thuringiensis* for 12 days had an effect on larval body, in which number of died larvae reached 60-91% (Figure 1). Therefore, surviving larvae were generally in an unhealthy condition leading a lower level of feed consumption. Process of mortality larvae was different length depending on insect species [13].

Table 2. Effect of *B.thuringiensis* -based bio-insecticide in liquid formulation application on *O. rhinoceros* larval weight.

Treatments	Weight (g) days to-						
	0	**6**	**12**	**18**	**24**	**30**	**36**
Tofu liquid waste	9,33 c	6,17 c	4,50 b	3,50	2,00	0,83	0,67
Tofu Liquid Waste + Bio-urine (1:2)	7,00 ab	4,00 b	3,00 a	1,50	1,17	0,50	0,50
Tofu Liquid Waste + Bio-urine (1:1);	8,00 b	4,67 b	2,83 a	2,58	1,92	1,33	1,00
Tofu Liquid Waste + Bio-urine (2:1)	5,83 a	3,17 a	3,00 a	1,75	1,87	2,08	2,27
Bio-urine	6,00 a	2,67 a	2,50 a	2,33	1,22	1,00	0,68
NB media (as control).	11,67 c	9,33 d	6,33 bc	3,08	1,62	1,42	0,50
F Value	9,33*	15,75*	3,35*	1,25^{tn}	1,32^{tn}	0,69^{tn}	1,10^{tn}
F Tabel	2,31	2,31	2,31	2,31	2,31	2,31	2,31
Tukeys HSD test 5%	0,24	0,20	0,35	0,50	0,49	0,44	0,44

Notes: *) significantly different, tn) not-significantly different

4 Conclusion

The highest spore density was found in tofu liquid waste propagation media and biourine (1:2) and its larval mortality as well (91.67%). Observations on the symptoms of infection and death in tested larvae were indicated by changes in color of integument and fragility of the larva's body. Bio-insecticide with active ingredient of *B. thuringiensis* in liquid formulation has potency to be used as biological agent for controlling *O. rhinoceros* larvae.

The authors would like to convey their gratitude to the Ministry of Technology and Higher Education for the full financial support of this research through the PDUPT Research Grant Number 142/E5/PG.02.00.PT/22.

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