Prospect of Agricultural waste as media propagation of Bacillus thuringiensis and its pathogenicity against Oryctes rhinoceros larvae

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Abstract. Bacillus thuringiensis has been recognized as a prospective bio-insecticide. Various media are used to propagate B. thuringiensis and expected to be used to control insect pests. Oryctes rhinoceros (Coleoptera: Scarabaeidae) as one of the important pests in oil palm plantations must be controlled in an environmentally friendly and sustainable way. The aim of this research was to study various agricultural waste media in propagation of B. thuringiensis bacteria and their pathogenicity against O rhinoceros larvae. The experimental design was a completely randomized design (CRD) with 7 treatments and 4 replications. Observations were mainly carried out on the density of B. thuringiensis spores propagated in various types of agricultural waste, the pathogenicity of B. thuringiensis and the effect of treatment on Oryctes body length. Experimental results showed after 72 hours of fermentation, the highest spore density was found in the treatment of a mixture of rice bran water and biourine (1:1). The highest pathogenicity of this mixture reached 91.67%, as well as on the treatment of coconut water and 5% molasses. The body length of Oryctes decreased from day 12th. The use of B. thuringiensis bacteria has an opportunity to be used as biological control agents.

Keyword: Agricultural waste, Bacillus Thuringiensis, Pathogenicity, Oryctes Rhinoceros Larvae

1. Introduction

The horn beetle Oryctes rhinoceros is one of the important pests on oil palm plantations in Indonesia. If it is not controlled, this pest will cause damage to both immature and mature plants. Symptoms of attack appear on leaves forming a triangular cut [1], [2]. Stages of O. rhinoceros which causes damage to oilpalm are both larvae and adults [3]. Controlling this pest is carried out in various ways, including by installing Ferotrap [4], however, chemical insecticides is mostly chosen because it is easy and practical. Generally, due to negative impact of these chemicals on the environment and natural enemies [5], other methods are needed to control Oryctes pests. Therefore, control using B. thuringiensis is an alternative.

B. thuringiensis is an entomopathogenic bacterium, which produces spores and proteins during sporulation [6]. Both spores and proteins, if ingested by larval insects and entered to their midgut, will cause binding process in midgut layer and cause death of larvae [7]. To produce B. thuringiensis as a bio-insecticide, suitable materials or media are needed. Researchers from various countries have tried to use various materials such as agricultural

SRICOENV 2022, October 05-06, Palembang, Indonesia

DOI 10.4108/eai.5-10-2022.2328335

wastewater [8], restaurant waste materials [9], and industrial waste materials [10]. Selection of agricultural waste materials which contain carbon and nitrogen elements must be considered to determine the level of pathogenicity of B. thuringiensis [7]. Therefore, this research was aimed to study appropriate waste media for B. thuringiensis propagation and its pathogenicity against of Oryctes larvae.

2. Material and Methods

The research was carried out at Entomology Laboratory of Plant Protection Study Program, Faculty of Agriculture, Sriwijaya University from June to August 2022. B. thuringiensis isolate used was Bt TPP code (Entomology laboratory's collection), while waste materials used were rice bran water, cow bio-urine, coconut water, tofu liquid waste and molasses. The experiment was designed in a completely randomized design (CRD) with 8 treatments and 3 replications (in B. thuringiensis propagation) while bioassay used 9 treatments and 3 replications. In each replication, it was used 5 individues of 3rd Oryctes larvae.

2.1 Preparation of Oryctes rhinoceros

Oryctes larvae were obtained by scouting in various locations of oil palm plantations, especially those belonging to farmers. The habitat of Oryctes larvae was mainly on rotting oil palm trunks, trash bins and litter [2]. The larvae obtained were taken along with their feed (in the form of weathered stems), put them in plastic containers and labelled to be brought to laboratory. Sorting was done to separate healthy and sick larvae. Healthy larvae were put in plastic containers (d=15 cm h=20 cm) with sterile soil and fed with rotten palm trunks. The feed was changed every 7 days until Oryctes larvae were ready to be used as test insects for bioassays.

2.2 Propagation of B. thuringiensis on waste media

Initial preparation was done by producing seed culture on NB media. A total of one loupe of B. thuringiensis isolate from NGKG agar medium was put into 10 ml of NB and fermented on a shaker for 12 hours at a speed of 200 rpm. After this step, the solution was transferred into 10 ml of new NB and repeated to be shaken for 12 hours at a speed of 200 rpm. Seed culture was ready to be used for propagation on waste media.

Waste media used were : P1 (Rice bran water + 5% molasses) P2 (Bio-urine + 5% molasses) P3 (coconut water + 5% molasses) P4 (tofu liquid waste + 5% molasses) P5 (Rice bran water + bio-urine = 1:1) P6 (Bio-urine + Coconut Water = 1:1) P7 (Rice bran water + tofu liquid waste = 1:1). Separately, the waste media was sterilized using an autoclave. After cooling, seed culture as much as 10% of the waste volume was poured in the waste aseptically. Fermentation was carried out by shaking for 72 hours at room temperature and a speed of 200 rpm. Calculation of spore density was carried out at 24 hours, 48 hours and 72 hours.

2.3 Bioassay application on 3 instar Oryctes larvae

Preparation of bioassay was started by preparing of 300 g of sterile soil mixed with 10 g of weathered palm trunks. In this mixture, 30 ml of bio-insecticide solution (3 ml of bio-insecticide + 27 ml of water) was added. After mixed well, 5 larvae of 3rd instar Oryctes were put into this mixture. Observations began on the second day after application. Replacement of feed and soil was done every 7 days. Body length and mortality of larvae were observed every day until a 100% mortality rate was found.

2.4 Analysis of data

Data on larval mortality, and larval length were analysed by using 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 analysed using software of SAS University Edition 2.79.4M5.

3. Result and Discussion

3.1 B. thuringiensis spore density

Observations of spore density were carried out at 24, 48 and 72 hours to determine development of bacteria in waste media. In general, B. thuringiensis can grow on all waste media. At the end of the observation (72 hours) the highest spore density was obtained in water rice bran and bio-urine media, which was 15.28 x 109 spores/ml (Table 1).

Table 1. Density of Bacillus thuringiensis spores propagated in various waste media

Media	Spore density (109 spores/ml) on observation of		
	24 h	48 h	72 h
P1 (Rice bran water + Molassse 5%)	7.58	7.62	7.73
P2 (Bio-urine + Molasses 5%)	6.28	7.85	12.59
P3 (Coconut water + Molasses 5%)	7.30	6.76	11.73
P4 (Tofu liquid waste + Molasses 5%)	7.70	6.46	12.60
P5 (Rice bran water + Biourine=1:1)	6.08	8.03	15.28
P6 (Biourine + Air Kelapa=1:1)	5.18	7.75	14.30
P7 (Rice bran water + Tofu liquid waste = 1:1)	7.78	6.63	10.54
P8 (NB)	6.53	8.03	8.73

Within 24 hours B. thuringiensis grew on all media, as well as at 48 hours and 72 hours. This showed waste media used contains nutrients supporting the growth of B. thuringiensis [11]. The combination of mixed waste media resulted in sufficient C/N ratio content for the growth of B. thuringiensis. [12] reported a C/N ratio of 4 to 10 was a suitable condition for the growth of B. thuringiensis. [8] reported agricultural waste media in the form of rice bran was a suitable medium for propagation of B. thuringiensis.

3.2 Bioassay B. thuringiensis towards Oryctes larvae

Bioassay against oryctes larvae was carried out on B. thuringiensis which was propagated in waste media. After 36 days of application, the highest mortality was obtained in the treatment of a mixture of coconut water and molasses (1:1) and the treatment of a mixture of bran water and bio-urine, which was 91.67%. In all treatments, observations on days 6, 12 and 18 were not significantly different between treatments, but on observations on days 24, 30 and 36 there were significant differences between treatments (Table 2).

Mortality of Oryctes larvae generally started on 6th days after application (see P2, P3 and P4). This was due to the poisoning process in oryctes larvae. It was well known that B. thuringiensis was a stomach poison, and it took time to cause death. The poisoning process is usually caused by the presence of ingested spores or toxic proteins produced during sporulation [6]. Therefore, by increasing observation time, the death process also increases. If other insects such as armyworm Spodoptera litura need a short time of 12.5 days to die [13], mortality of Oryctes generally took a longer time. This may be due to Oryctes larvae own a larger body than Spodoptera and requiring higher doses to cause death.

Table 2. Mortality of O.rhinoceros larvae due to bio-insecticides application with active ingredient B. thuringiensis on various growth media

Treatments	Mortality of O. rhinoceros larvae (%) (day after application)					
	6	12	18	24	30	36
P1 (Rice bran water + Molassse 5%)	0.00	33.33	33.33	50.00 bc	50.00 abcde	66.67 abcde
P2 (Bio-urine + Molasses 5%)	8.33	50.00	50.00	58.33 bc	75.00 de	75.00 de
P3 (Coconut water + Molasses 5%)	8.33	25.00	41.67	75.00 d	83.33 de	91.67 de
P4 (Tofu liquid waste + Molasses 5%)	25.00	41.67	50.00	58.33 c	66.67 cde	75.00 cde
P5 (Rice bran water + Biourine=1:1)	0.00	16.67	33.33	75.00 d	83.33 de	91.67 de
P6 (Biourine + coconut water=1:1)	0.00	8.33	16.67	25.00 abc	50.00 abcde	66.67 abcde
P7 (Rice bran water + Tofu liquid waste = 1:1)	8.33	33.33	50.00	50.00 bc	66.67 cde	75.00 cde
P8 (NB)	8.33	50.00	58.33	75.00 d	91.67 e	91.67 e
9 (Control)	0.00	0.00	8.33	16.67 abc	16.67 abc	16.67 abc

Notes : Values within a column followed by the same letters were not significantly different at P 0.05 according to Tukey's HSD test.

3.3 Measurement of body length of Oryctes larvae

Body length of Oryctes larvae was measured since the beginning of observation. The aim was to determine the effect of B. thuringiensis application on the development of alive larvae. At the beginning of observation, larvae length was significantly different in each treatment, but at the end of observation (day 36), it was not significantly different in all treatments (Table 3).

Reduction in larval length was due to a large number of Oryctes larvae died since day 6 to day 36. This was found in treatment P2 (Bio-urine + Molasses 5%) where the final larvae length was only up to 1.5 cm. At P5 (bran water + Bio-urine=1:1) larvae length reached 2.96 cm. In relation to mortality of test larvae (Table 2), it appeared the higher mortality rate, the fewer number of surviving larvae. When proteins of B. thuringiensis entered larval digestive tract, a binding process occurred in midgut wall. If no binding occurred, larvae will continue to eat and alive. In this situation, larvae will survive in their condition in which the infection caused by B. thuringiensis did not cause death directly [6]. Symptoms of this infection will appear in the growth and development of Oryctes larvae, larvae will lose their appetite. The body will shrivel and change color to dark brown [14].

Table 3. Body length of O. rhinoceros larvae due to application of B. thuringiensis on various growth media

Treatments	Body Length of O. rhinoceros (cm) larvae days after application						
	0	6	12	18	24	30	36
P1 (Rice bran water + Molassse 5%)	8.58 def	8.58	5.82	5.76	5.05	5.05	5.05
P2 (Bio-urine + Molasses 5%)	8.58 def	7.75	4.96	5.00	5.00	3.63	2.29
P3 (Coconut water + Molasses 5%)	8.64 def	7.89	5.06	4.29	3.58	1.50	1.50
P4 (Tofu liquid waste + Molasses 5%)	9.00 f	7.38	5.88	4.42	2.17	1.42	1.42
P5 (Rice bran water + Biourine=1:1)	8.75 ef	8.75	7.08	5.78	4.36	2.13	2.13
P6 (Biourine + Coconut water=1:1)	8.75 ef	8.75	8.17	6.68	6.68	3.79	2.96
P7 (Rice bran water + Tofu liquid waste = 1:1)	8.07 abcde	7.41	5.26	3.84	3.84	1.76	1.76
P8 (NB)	7.48 a	6.85	3.67	3.06	1.82	1.19	0.58

Notes : Values within a column followed by the same letters were not significantly different at P 0.05 according to Tukey's HSD test.

3.4 Symptoms of infection and larval death

On the first day of bioassay observation, there were no symptoms. They appeared on the 6th day of observation. Infected Oryctes larvae changed color from abdomen to thorax. Body color of Oryctes larvae was pale brown then turned black. It has a soft texture, smells, and sometimes secreted water. Day by day, black color of dead body was getting darker and more fragile (Figure 1).

Figure 1. Symptoms of O. rhinoceros death on day 6 (a), day 12 (b), day 18 (c), day 24 (d), day 30 (e), and 36th day (f)

4. Conclusion

A high level of pathogenicity was shown in the treatment of a mixture of bran water and bio-urine as well as the treatment of coconut water and 5% molasses which reached 91.67%. Oryctes body length decreased from day 12. The use of B. thuringiensis bacteria has opportunity to be used as biological control agents.

Acknowledgment

The authors would like to thank to Rector of Universitas Sriwijaya who has provided financial support in implementation of this research, through a Competitive research grant in approval of Rector's Decree No. 0017/UN9.3.3/SK.LP2M.PT/2022, dated June 15, 2022.

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