Comparison Study Of Toxicity *Bacillus thuringensis* from Soil Isolate and Mulberry Leaves in Indonesia

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Abstract: Background and Objective: Most strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to lepidopteran insects such moth. But some strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to dipteran insects such as mosquitoes and blackflies. To compare *B. thuringiensis* isolate from soil and mulberry leaves. Material and Methods:One gram of soil samples was suspended in 9 ml of sterile distilled water and shaken for 5 min. the upper layer of the soil suspension was transferred to a test tube and heated at 80°C for 5 min in water bath to kill non-spore-forming organism and vegetative cells to prepare the sporulated culture, bacteria were grown on nutrient agar pH 7.2, at 30°C for 4 days. The isolates were obtained from mulberry leaves collected in West Java, Indonesia, using the leaf-lift technique. Leaves were trimmed to fit inside a 100 mm petri dish. Abaxial leaf surfaces were placed in contact with nutrient agar, and a sterile, per forated stainless steel disk was placed on the leaf sections to ensure maximum contact with the agar. After the sample was coated with carbon and gold, it was observed and photographed with (SEM). However they had differences form crystal protein, but they had unique as insecticidal to control the same orders (mosquitocidal). Results:The study found that *B. thuringiensis* serovar *entomocidus* INA288 produced large cuboidal-form crystals and *B. thuringiensis* serovar *aizawai* BUN 1-14 a little homology with serovar *entomocidus* INA288 but, *B. thuringiensis israelensis* ONR60A has irregular shaped crystal protein. Toxicity results that serovar *entomocidus* INA288, serovar *aizawai* BUN 1-14 and serovar *israelensis* ONR60A showed toxic to mosquito insects. which was composed of major protein of 130 kDa peptides. *B. thuringiensis* serovar *entomocidus* INA288 has 70 kDa and *aizawai* BUN 1-14 had 69 kDa. Conclusion: *B. thuringiensis* strain had different crystal protein form but had same toxicity.

Keywords: *B. Thuringiensis*, Soil, Leaves

1. Introduction

Numerous chemical insecticides have been used to control some insects. While chemical insecticides have knock down effect, they are too expensive harmfull to both humans and the environment. In addition, target insect pests develop biological resistance rapidly especially at higher rates of application. Thus, the increase in pesticidal application to control this pest has urged to researcher to search for biological control alternatives that would be a good component of Integrated Pest Management [1, 32, 33].

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produce parasporal crystal during the sporulation stage. The crystal is made of one or more proteins toxic to some insect species. Most strains of *B. thuringiensis* produce delta-endotoxin crystal toxic to lepidopteran insect such as moth [2, 28, 29].

The name proposed of *Bacillus thuringiensis* for a species of *bacillus* which was isolated from diseased larvae of Mediterranean flour moth Angasta (Ephestia) kuhniella Zell [3]. Later, noted infection of the larvae after the ingestion of the *bacillus* or its spores, described and named it *Bacillus thuringiensis* [3, 4]. It isolated the same *bacillus* from the same insect host [4], which had found earlier. This strain is now maintained as *B. thuringiensis* serovar *thuringiensis* (serotype H-1). They noticed that the vegetative remains of sporulating cells assumed a rhomboid shape [3, 4]. He described this crystalline inclusion in the sporangium of the organism and made further interpretations of the data being accumulated on this bacillus at that time. Neither Berliner nor Mattes attributed those parasporal bodies any role in the the disease process caused by the ingestion of sporalating *B. thuringiensis* [5].

B. thuringiensis is a gram-positive soil bacterium, and

produce a crystalline inclusion body during sporalation [6, 27, 28]. This parasporal body is composed of proteins termed "delta-endotoxin", and specifically toxic to insects. In addition, *B. thuringiensis* produce another toxins namely: alpha-exotoxin, beta-exotoxin, and gamma-exotoxin. All of the toxic substances may not be present in the bacterium [7, 20, 21]. In another hand, Krieg [8] has defined various toxic substance produced by *B. thuringiensis* as follow: (a) thermolabile endotoxin; (b) thermostable exotoxin; (c) bacillogenic antibiotic; (d) lecithinase; (e) proteinase.

B. thuringiensis has been studied world wide over the past decades, mainly because this gram-positive bacterium produce significant amount of crystal proteins with toxic activity against economically important insect larvae [9, 18, 28]. The most attractive characteristics of the *B. thuringiensis* proteins for insect control are their specificity and high unit activity. Members of non-target insect orders are not susceptible to the potent effects of the lepidoptern-specific and dipteran-specific insecticidal proteins [10, 27, 29]. Most strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to lepidopteran insects such moth [11, 23, 25], But some strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to dipteran insects such as mosquitoes [12, 22-24] and blackflies.. Lepidopteran-specific delta-endotoxin are composed 130 kDa proteins [13, 15-17] while dipteran-specific delta endotoxin are composed of several protein [13, 18-20].

Bacillus strains possessing a high larvacidal activity, specific for mosquitoes, from the soil of mosquitoes-breeding site in Israel [11, 25-27]. The objective of the studies to compare *B.thuringie*nsis strains serovar *entomocidus*I NA288 (soil isolated), serovar *aizawai* Bun 1-14 (leaves isolated) and *israelensis*ONR60A (standard isolate).

2. Materials and Methods

Bacterial stains. The strain of *B. thuringiensis* used in the present study were *B. thuringiensis* serovar *entomocidus* INA 288, serovar *aizawai* BUN 1-14 and *israelenis* ONR60A (standard isolate).

2.1. Isolation and Identification

B. thuringiensis serovar *entomocidus* INA288 which had been isolated from Indonesia soil, was prepared according to the method [14]. One gram of soil samples was suspended in 9 ml of sterile distilled water and shaken for 5 min. the upper layer of the soil suspension was transferred to a test tube and heated at 80°C for 5 min in water bath to kill non-spore-forming organism and vegetative cells to prepare the sporulated culture, bacteria were grown on nutrient agar pH 7.2, at 30°C for 4 days. Formation of spores and parasporal inclusion were monitored with a phase-contrast microscope. The culture was scratched on the agar slant as a stock. *B. thuringiensis* serovar *aizawai* BUN 1-14 which had been isolated from mulberry leaves. Isolates were obtained from mulberry leaves collected in West Java, Indonesia, using the leaf-lift technique [15, 16]. Leaves were trimmed to fit inside a 100 mm petri dish. Abaxial leaf surfaces were placed in contact with nutrient agar, and a sterile, per forated stainless steel disk was placed on the leaf sections to ensure maximum contact with the agar. The lid was replaced, and samples were incubated at 30°C overnight. To prepare the sporulated culture, bacteria were grown on nutrient agar, pH 7,0, at 30°C for 4 days. Formation of spores and parasporal inclusion were monitored with a phase-contrast microscope.

2.2. Morphology of Parasporal Inclusion

Isolates were examine with a HITACHI S-800 Scanning Electron Microscope (SEM) at a magnification of 10,000x, according to the method presented by [23, 24]. *B. turingiensis* serovar *entomocidus* INA288 were cultured on N-broth agar at 30^0C until almost all cells lysed (overnight). The crystal and spores (about 100 mg wet weight) were washed in 10 ml of 50 mMTris-HCL (pH 8.0). The final precipitate was resuspended in 1 ml of distilled water, and 20 ul of the suspension was air-dried on a glass disk (O 10 mm). After the sample was coated with carbon and gold, it was observed and photographed with SEM.

2.3. Biological Activity

The strain were examined for their larvicidal against the larvae of the silkworm, *Plutella xylostella* and *Spodoptera litura*. The insect cultures were maintained in this laboratory. Toxicity test with the Leppidopteran insect, *B. mori*, *P. xyloetella* and *S. litura*, were done by introducing ten 3rd-intar larvae were fed on an artificial diet dropped with 0.3 ml of the bacterial suspension and rear at 25°C for 48 hr to determine mortality. The *B. thuringiensis* isolates were examined for oral insecticidal activity against the insects were prepared by the following procedures. Overnight culture of serovar *entomocidus* INA288, BUN 1-14 and *israelensis* ONR60A were grown on 2 ml of nutrient broth at 30°C using tube glass. Then, 200 ul of the overnight culture was plating on nutrient agar, reincubated for 4 days at 30°C. Sporulated cultures were harvested by centrifugation at 10,000 g for min at 4°C. The pellet was washed three times by centrifugation in mMTris-HCL and 1 M NaCL at 4°C, the bacterial suspensions were finally suspended in 500 ul of sterile distilled water. The bacteria were also tested against larvae of the mosquitoes, *Aedes aegypti*, *Aedes japonicus* and *Culex quinquefasciatus*. Ten 2nd-instar larvae were placed in a test tube containing 10 ml of the spore-parasporal inclusion suspension, respectively, under levels 1 ul/ml. The tubes were kept at 22°C for 24 hr without feeding.

3. Result and Discussion

In order to identify serovar *entomocidus* INA288 strains by H-serotype cell with broth, they were dropped to glass slide and the motility of cells was observed under phase-contrast microscope. Since isolate of serovar *entomocidus* INA288 gave positive reaction in the H 6 serum agglutination test, it was identified as *B. thuringeisis* serovar *entomocidus*

INA28*8*. interestingly, *entomocidus* INA288 produced large cuboidal-form crystals (Figure 1). On the other hand, *B. thuringiensis* serovar *aizawai* BUN 1-14 a little homology with serovar *entomocidus* INA288 (Figure 2). Interestingly, *B. thuringiensis* serovar *israelensis* ONR60A [23, 24] has irregular shaped crystal protein.
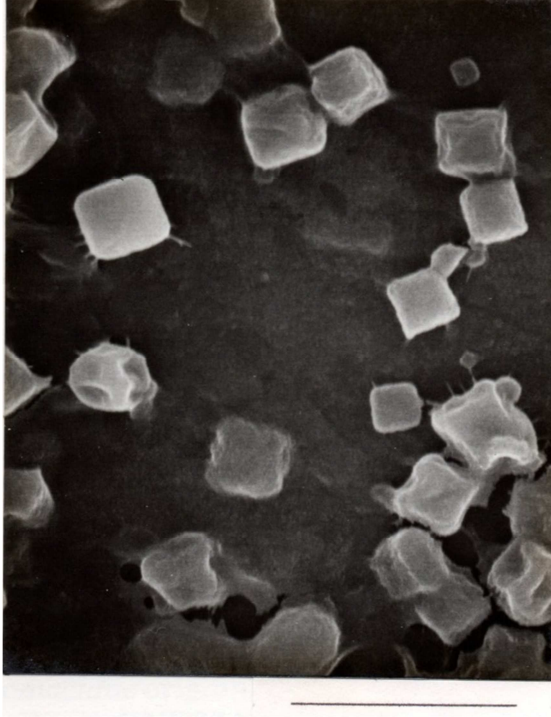

Figure 1. Scanning electron microscopy (SEM) showing spores and parasporal crystal of Bacillus thuringiensis serovar entomocidus INA288. Bar indicates 3 um.

B. thuringiensis serovar *israelensis* ONR60A had mosquitocidal activity, which was composed of major protein of 130 kDa peptides. On the other hand, *B. thuringiensis* serovar *entomocidus* INA288 and *aizawai* BUN 1-14 had mosquitocidal activity, while *aizawai* Bun 2-1 both did not have. In addition, neither serovar *entomocidus* original strain nor serovar *aizawai* IPL had lepidopteracidal activity. SDS-PAGE profiles of *enotmocidus* INA288 crystals indicated that the polypeptide of 70 kDa was dominant, while *entomocidus* original strain was constituted of 130-65 kDa. However, *B. thuringiensis* serovar *aizawai* Bun 1-14 was composed of polypeptides of 69 kDa, while *entomicidu*s Bun 2-1 was constituted of 130-65 kDa. Interestingly, using antibody of *B. thuringiensis* serovar *entomocidu*s INA288 crystal protein demonstrated that there is cross-reactivity among the parasporal inclusion proteins of *B. thuringiensis* serovar

aizawai Bun 1-14, *israelensis* ONR60A, and *fukoukaensi*s. However, [27] demonstrated that using antibodies of *B. thuringiensis* serovar *israelensis* ONR60A showed weakly cross-reactivity to serovar *jegathesan*. Indeed, *B. thuringiensis* serovar *jegathesan* included *cryIVD* and showed immunological similarity with anbodies of serovar *israelensis*. While, *entomocidus* INA288 contained a novel crystal protein gene cryINA288, on plasmid. In addition, the similarity of amino acid sequence between cryINA288 and *cry4Aa* was 38%. On the other hand, serovar *aizawai* Bun 1-14 a little homology with serovar *entomocidus* INA288 (Figure 2).
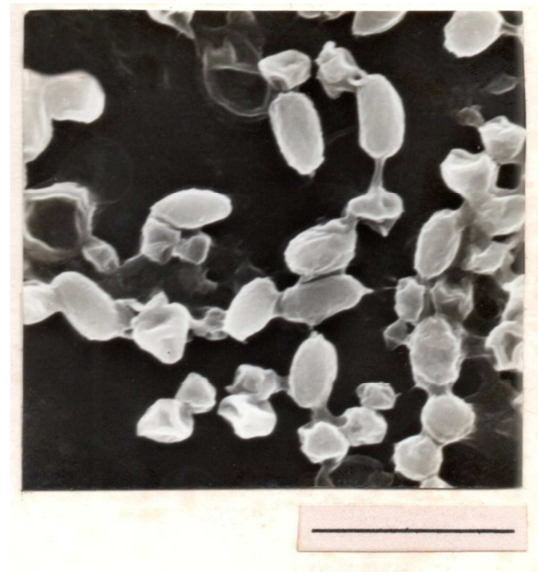

Figure 2. Scanning electron microscopy (SEM) showing spores and parasporal crystal of Bacillus thuringiensis serovar entomocidus INA288. Bar indicates 3 um.

Quantitative toxicity test isolate

The serovar entomocidus INA288, serovar aizawai BUN 1-14 and israelensisONR60A were bioassayed against 4th-instar larvae of B. mori, P. xylostella, S. litura, A. japonicas, A. aegypti and C. quiquefasciatus. Respectively, serovar entomocidus INA288, aizawai BUN 1-14 showed not toxic activity against latter 3 former lepidoteran species, while serovar entomocidus INA288, aizawaiBUN1-14 and israelensis ONR 60A had the toxicity against latter 3 dipteran species (A. aegypti, A. japonicas and C. quinquefasciatus) (Table 1). However they had differences form crystal protein, but they had unique as insecticidal to control the same orders (Mosquitoes).

Table 1. Toxic activity of three B.thuringiensis strains against some insect species.

Strain	Lepidopteracidal			Dipteracidal		
	B, m,	*P.x.*	*S.l.*	*A.j.*	*A.a.*	*C.q.*
Serovar *entomocidus* INA288	-	-	-	+	+	+
Serovar *aizawai* BUN 1-14	-	-	-	+	+	+
Serovar *israelensis* ONR60A	-	-	-	+	+	+

B, m: Bombyx mori, P.x: Plutella xylostella, S.l: Spodoptera litura
A.j: Aedes japonicas, A.a: Aedes aegypty, C.q: culex quinquefasciatus

4. Conclusion

In the search for potential alternatives to the application of *B. thuringiensis* serovar *israelensis* ONR60, *entomocidus* INA288 and *aizawai* BUN 1-14 isolation of novel mosquitocidal strains. The crystal proteins form of *B. thuringiensis* serovar *entomocidus* INA288 has cuboidal shaped, serovar *aizawai* BUN 1-14 has homology shaped ones and *B. thuringiensis* serovar *israelensis* ONR60A has irregular., which was composed of major protein of 130 kDa peptides. *B. thuringiensis* serovar *entomocidus* INA288 has 70 kDa and *aizawai* BUN 1-14 had 69 kDa.

Acknowledgements

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