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Isolation and Identification of *Bacillus thuringiensis* from *Harpaphe Haydeniana* and its Entomotoxic evaluation against *Aedes* and *culex* larvae

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Abstract

This study was designed to isolate and identify Bacillus thuringiensis from dead Diplopods (Harpaphe havdeniana) and to evaluate its entomotoxicity to Culicidae larvae, Aedes and Culex. Twenty-three (23) viable colonies were selected and characterized through morphological, physiological, cellular, biochemical and antibiotic sensitivity reactions and observations. Only six (6) isolates were presumptively identified as B. thuringiensis as indicated by its endospores that causes toxicity against various kinds of insects. Entomotoxicity activity of the isolates was evaluated by selective bioassay through dose mortality response of different bacterial densities to Aedes and Culex. McFarland Turbidity standards (0.5, 1, 2, and 3) was used to indicate cell count concentrations. Average mortality rate of both larvae was determined through 50% mortality. Only two (2) presumptively identified strains labeled as IF7 and IF15 were entomotoxic. Determination of toxicity was measured by the assessment o f lethal median concentration (LC50) to both larvae in the minimum hour of observation. Results showed that both isolates exhibited higher mortality rate to Culex than on Aedes larvae. Similarly, lethality rate was more evident to Culex than Aedes based on LC_{50}. Final evaluation was done through tests between subject effects (ANOVA). Significant differences between the different bacterial densities and control was indicated and showed that isolate IF7 and IF15 shows efficacy only to Culex larvae.

Keywords: Bioassay, entomotoxicity, lethality, mortality.

Introduction

The current interest in the development of biocontrol agents for the control of vectors, especially mosquitoes is an indication of the concern and sheer helplessness felt by the scientific community in the wake of the recurrence mosquito-carrying diseases in epidemic and proportions to the global society[1].

Bacillus thuringiensis (*Bt)* is a soil-dwelling, Gram-positive, spore-forming, rod-shaped bacteria which grows at body temperature and produces polymorphic crystal proteins[2]. The corresponding cultures of *Bt* are found in nature in one of the two states as either vegetative cells that are actively growing and dividing or can be found as a spore. These endospores are resistant to any environmental stress. They are metabolically inactive and a resting form of the bacterium which has a completely different structural form, chemical composition and enzymatic constitution from its vegetative state.

B. thuringiensis has been used as a biocontrol agent and considered as uniquely specific, safe and effective tool for the control of a wide variety of insec pests. Intensive screening programs all over the world have identified *Bt* strains from soil samples, plant surfaces, dead insects and stored grains. Moreover, it was indicated that *Bt* strains isolated from several insects of Coleoptera *Stromatium fulvum* was toxic to *Culex quinquefasciatus.* Isolation also of *Bt* from *Simulium* larvae shows entomotoxicity to *Aedes aegypti*[3]. A review of the related literature on host range and effect on non-target organisms indicates that several strains of *Bt* are relatively specific to the Nematocera suborder Diptera, in particular the Culicidae or filter-feeding mosquitoes[4].

The aim of this study is to identify *B.thuringiensis* from dead *H. haydeniana* by morphological and physiological characterization, determine the dose mortality response of different bacterial densities by selective bioaasay and to evaluate the lethal median concentration (LC50) of *B. thuringiensis* through minimum hour of observation between *Aedes* and *Culex* larvae.

Material and Methods

Collection of Diplopod Samples: Sixteen (16) dead diplopod species *H. haydeniana* which is locally known as "labud" was collected from Manresa Farm, College of Agriculture, Xavier University, Cagayan de Oro City. The collected dry samples were placed in a sterile plastic bag and was immediately brought to the laboratory.

Selective Isolation and Cultural Characterization of ***B. thuringiensis*****:** One gram of pulverized *H. haydeniana* was diluted to each of the three tubes of Luria-Bertani broth. Samples were suspended in a digital water bath to thoroughly mixed and pasteurized at 80°C for 30 minutes to kill vegetative cells. After heating, tubes were incubated at 30°C for 4 hours

and at 80°C for 3 minutes for a quick pasteurization. Each tube of primary cultures was then serially diluted (from 10^{-1} to 10^{-3}). Spread plate method was performed in order to obtain a pure isolate and was transferred periodically to fresh agar slant and kept at 4^0C inside the refrigerator.

Growth patterns on solid culture medium and in broth were used to distinguish *B.thuringiensis* for identification. Observation of growth was done in nutrient agar plate, nutrient agar slant, and nutrient broth.

Cellular and Biochemical Characterization: Gram staining and endospore staining were conducted to distinguish cell wall characteristics, spores, nuclear bodies, and capsules. For the biochemical characterization, the following were conducted: determination of oxygen requirement, catalase test, triple sugar iron agar test, hydrogen sulfide production test, methyl red and Voges- Proskauer test, starch hydrolysis agar test, and citric acid utilization test.

Disk Diffusion Antibiotic Sensitivity Test: Kirby-Bauer Disk diffusion method was used to test the antibiotic sensitivity in a Mueller Hinton agar plates. Two common antibiotics were used, Amoxicillin and Ampicillin. This method was done in triplicates. Plates were incubated and were observed after 16 hours for zone inhibition. The average of the three replicates was calculated and evaluated based on the Standard Diameter Inhibition of Antibiotics[5].

Selective Biological Assay of *Aedes* and *Culex* larvae to *B.thuringiensis:* Identification was based upon the mosquitoes' unique morphological characteristics[6]. For the dose mortality response, all strains of *Bt* were grown in sporulation medium for 48 hours at 30^0C. Four densities were prepared using the McFarland turbidity standard (Standards number 0.5, 1, 2, and 3). Each standard was added to a 200ml cups with 100 ml of sterile distilled water and thirty (30) larvae as test organisms. After 48 hours of observation, the number of dead larvae was recorded. A mortality count of more than 50% of the larvae after 48 hours of observation was considered toxic.

Entomotoxic activity of each isolates was also evaluated after four (4) hours which is the minimum hour of observation. The mortality rate of the earliest hours of observation was considered to assess the severity of the toxic. Comparison between the two isolates to *Culex* and *Aedes* larvae were determined.

Lethal Median Concentration (LC50) and Statistical Tool Analysis: Isolates that were considered entomotoxic were subjected to its LC50 between the two larvae using ratio and proportion formula:

$$LC_{50}=\frac{\text{Bacterial Density}}{\text{Mortality \%}}\ (50\%)$$

A relationship between the different bacterial densities was considered for the evaluation and was done on a statistical tool analysis through the analysis of variance (ANOVA).

Results and Discussion

Identification of *B.thuringiensis*: Cultural, cellular, and physiological characterization of isolates is a conventional method in identifying specific species of bacteria. A total of thirty-five (35) viable isolates were determined through spread plate method. Of these, only twenty-three (23) colonies were selected based on its diameter, colony color, and colony formation. *B.thuringiensis* colonies were described to be around 3-7 mm in diameter, cream in color, and circular in form[7,8]. Thus, endospore formation was only observed in six (6) isolates[7]. Among the six isolates, only two were considered entomotoxic and labeled as IF7 and IF15.

Antibiotic Sensitivity Test: *Bt* which has been long considered as non-pathogenic for humans and used extensively for pest control were found to be resistant to the ß-lactams, amoxicillin and ampicillin[9,10]. Figure 1 shows the resistance of two isolates to the two antibiotics mentioned, thereby confirming the presumption that the isolates are *B.thuringiensis*[10].

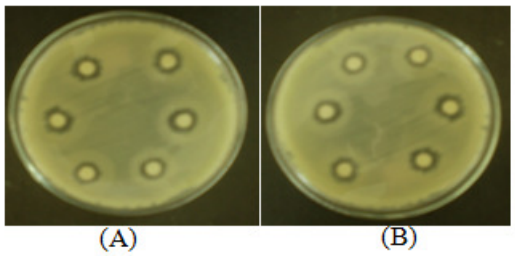

Figure-1
(A) Isolate IF7 and (B) Isolate IF15

Dose Mortality Response of *Aedes* and *Culex:* The six isolates were measured for its entomotoxic activity. Figure-2 shows the dose mortality response of larvae to isolate IF7. Both genera exhibit a 100% mortality rate which indicates that the total population has been killed after 48 hours. The same result goes for isolate IF15. Both isolates are determined as entomotoxic since all bacterial densities to each larva exceeds 50% mortality rate. The different toxic potentials of *B.thuringiensis* can be related to its genetic diversity due to plasmid exchange between strains[11].

Evaluation After Minimum Time of Observation: The toxicity to both larvae was determined in the minimum time of observation which considered only the first four hours of observation. Percentage comparison of the average rate of mortality among the four bacterial densities is illustrated in figure-3. The two isolates, IF7 and IF15, were assessed to determine its rate of mortality using the minimum time of observation. From figure-3, highest rate of mortality was noticeable to 9.0 x 10^8 cfu/ml. It also shows that both isolates exhibits higher mortality rate to *Culex* than on *Aedes* larvae. Comparison of percentage mortality rate to *Culex* larvae is higher on isolate IF15 to all densities except in 1.5 x 10^8 cfu/ml.

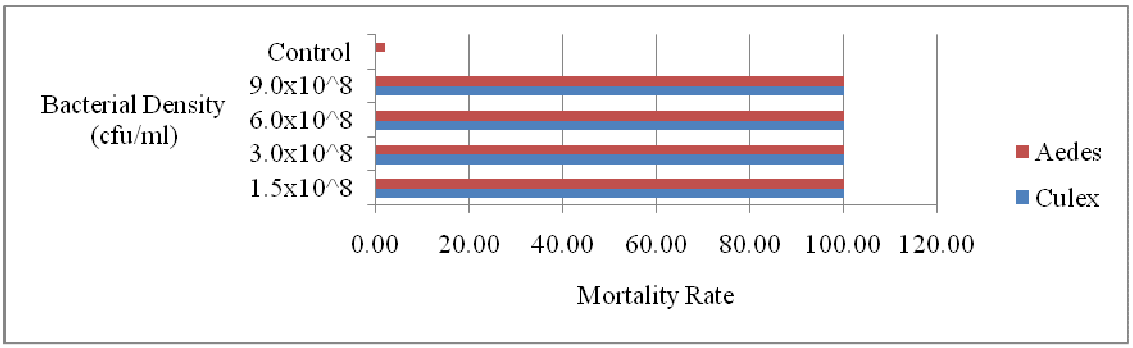

Figure-2
Dose mortality response of *Aedes* and *Culex* larvae on isolate IF7

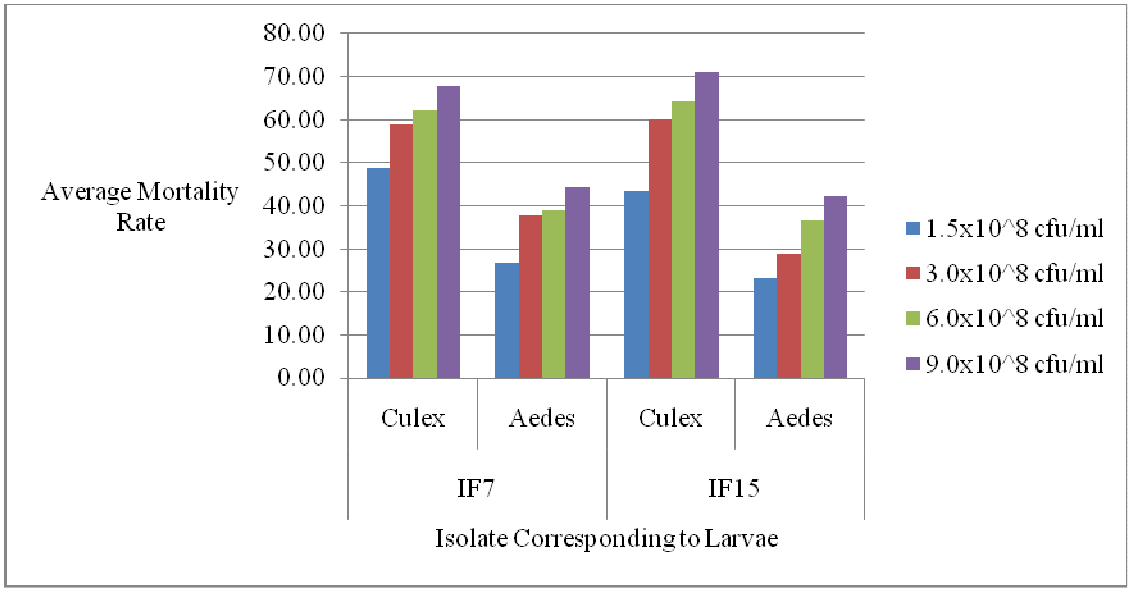

Figure-3
Comparison of mortality rate between *Culex* and *Aede s*larvae after four (4) hours of observation

Table-1 shows the specific bacterial densities in which the mortality rates of both larvae were determined through LC50. Isolates with their corresponding cell concentrations shows that at 50% lethality, determination of bacterial density which is entomotoxic to *Culex* and *Aedes* larvae was evaluated. For isolate IF7, it showed that a bacterial density of 3.89 x 10^8 cfu/ml is lethal to *Culex* larvae. However, 6.16 x 10^8 cfu/ml is the cell concentration which is 50% lethal to *Aedes* larvae. On the other hand, 3.81 x 10^8 cfu/ml is lethal to *Culex* larvae for isolate IF15 while a density of 6.81x10^8 cfu/ml is lethal to *Aedes* larvae.

From figure-4, it illustrates that *Culex* larvae are more susceptible than *Aedes* to both isolates. Based from the LC_{50} values of bacterial densities, lethality rate is more evident to *Culex* larvae than *Aedes* because it only required lesser cell concentration of bacteria. An implication is determined that both isolates are more entomotoxic to *Culex* are than on *Aedes* larvae.

Multiple Comparisons of the Different Bacterial Densities of Each Isolates: The purpose of multiple-comparisons procedure is to control the overall significance level for some set of interferences performed as a follow-up to ANOVA. Table-2 and 3 shows that the two isolates exhibit a significant difference between the different bacterial densities and the control. Similarly, both isolates shows significant difference in the rate of mortality in both *Culex* and *Aedes* larvae between the different bacterial densities.

Table-1
Lethal median concentration (LC_{50}) of isolates from the minimum hour of observation

Bacterial Density	Isolate IF7		Isolate IF15	
	Culex	***Aedes***	***Culex***	***Aedes***
1.5x10^8 cfu/ml	1.53x10^8	2.81x10^8	1.73x10^8	3.21x10^8
3.0x10^8 cfu/ml	2.55x10^8	3.97x10^8	2.50x10^8	5.19x10^8
6.0x10^8 cfu/ml	4.82x10^8	7.71x10^8	4.66x10^8	8.18x10^8
9.0x10^8 cfu/ml	6.64x10^8	1.01x10^9	6.33x10^8	1.07x10^9

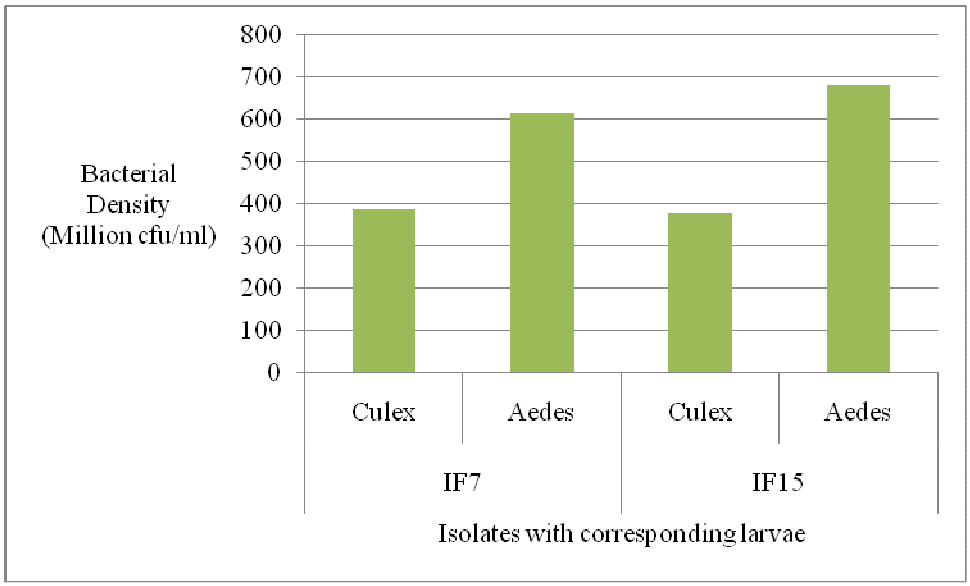

Figure-4
Bacterial densities at LC_{50} of *Culex* and *Aedes* larvae

Table-2
Multiple Comparisons of isolates to *Aedes* larvae using the tests of between-subjects effects

LSD						
	Isolate IF15			Isolate IF7		
(I) Density	(J) Density	Significance	Decision	(J) Density	Significance	Decision
MFS 0.5	MFS1	0.454	Not Significant	MFS1	0.004	Significant
	MFS2	0.005	Significant	MFS2	0.001	Significant
	MFS3	0.008	Significant	MFS3	0.004	Significant
	Negative Control	0	Significant	NEGATIVE CONTROL	0	Significant
MFS 1	MFS0.5	0.454	Not Significant	MFS0.5	0.004	Significant
	MFS2	0.025	Significant	MFS2	0.426	Not Significant
	MFS3	0.043	Significant	MFS3	1	Not Significant
	Negative Control	0	Significant	NEGATIVE CONTROL	0	Significant
MFS 2	MFS0.5	0.005	Significant	MFS0.5	0.001	Significant
	MFS1	0.025	Significant	MFS1	0.426	Not Significant
	MFS3	0.801	Not Significant	MFS3	0.426	Not Significant
	Negative Control	0	Significant	NEGATIVE CONTROL	0	Significant
MFS 3	MFS0.5	0.008	Significant	MFS0.5	0.004	Significant
	MFS1	0.043	Significant	MFS1	1	Not Significant
	MFS2	0.801	Not Significant	MFS2	0.426	Not Significant
	Negative Control	0	Significant	NEGATIVE CONTROL	0	Significant
Negative Control	MFS0.5	0	Significant	MFS0.5	0	Significant
	MFS1	0	Significant	MFS1	0	Significant
	MFS2	0	Significant	MFS2	0	Significant
	MFS3	0	Significant	MFS3	0	Significant
Based on observed means.				Based on observed means.		
The error term is Mean Square (Error) = 5.133.				The error term is Mean Square (Error) = 4.533.		
*. The mean difference is significant at the 0.05 level.				*. The mean difference is significant at the 0.05 level.		

Table-3
Multiple Comparisons of isolates to *Culex* larvae using the tests of between-subjects effects

LSD						
	Isolate IF15			**Isolate IF7**		
(I) Density	(J) Density	Significance	Decision	(J) Density	Significance	Decision
MFS0.5	MFS1	0.191	Not Significant	MFS1	0.122	Not significant
	MFS2	0.061	Not Significant	MFS2	0.071	Not significant
	MFS3	0	Significant	MFS3	0.04	Significant
	Negative Control	0	Significant	Negative Control	0	Significant
MFS1	MFS0.5	0.191	Not Significant	MFS0.5	0.122	Not significant
	MFS2	0.535	Not Significant	MFS2	0.772	Not significant
	MFS3	0	Significant	MFS3	0.564	Not significant
	Negative Control	0	Significant	Negative control	0	Significant
MFS2	MFS0.5	0.061	Not Significant	Mfs0.5	0.071	Not significant
	MFS1	0.535	Not Significant	MFS1	0.772	Not significant
	MFS3	0.001	Significant	MFS3	0.772	Not significant
	Negative Control	0	Significant	Negative Control	0	Significant
MFS3	MFS0.5	0	Significant	MFS0.5	0.04	Significant
	MFS1	0	Significant	MFS1	0.564	Not significant
	MFS2	0.001	Significant	MFS2	0.772	Not significant
	Negative Control	0	Significant	Negative Control	0	Significant
Negative Control	MFS0.5	0	Significant	MFS0.5	0	Significant
	MFS1	0	Significant	MFS1	0	Significant
	MFS2	0	Significant	MFS2	0	Significant
	MFS3	0	Significant	MFS3	0	Significant
Based on observed means.				Based on observed means.		
The error term is Mean Square (Error) = 10.233.				The error term is Mean Square (Error) = 3.867.		
*.The mean difference is significant at the 0.05 level.				*. The mean difference is significant at the 0.05 level.		

Conclusion

Two isolates from the *Harpaphe haydeniana* were presumptively identified as *B.thuringiensis* based on the traditional and conventional method of identification. The presence of endospores and resistance to beta lactam antibiotics also confirmed the results. Thus, only two isolates were considered entomotoxic and were labeled as IF7 and IF15. The results show that from the mortality rate of the two isolates, it illustrates that both exhibits higher rate of lethality to *Culex* larvae than on *Aedes* larvae.

Similarly, lethal median concentration (LC_{50}) rate is more evident to *Culex* larvae which imply that a lesser amount of cell concentration can provide entomotoxicity to the larvae. Also, Multiple Comparison tests through the use of ANOVA indicate significant differences between the different bacterial densities for both isolates (IF7 and IF15) to *Culex* and *Aedes* larvae.

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