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The production of bioinsecticide using *Bacillus thuringiensis*

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Abstract

This study aims at determining the ability of *Bacillus thuringiensis* to induce a biocidal effect on mosquito larvae. The *B. thuringiensis* used in this work was isolated from soil samples obtained from the root of plantain trees in a garden at Umuoma community in Uli Town. Soil samples were subjected to high temperature of about 50°C in order to exclude other microbes that may contaminate the culture, *B. thuringiensis* was able to survive the heat condition because they are spore formers. To further enhance purity of the culture, the organism was cultured in Luria Bertani medium which is selective for *Bacillus spp.* Before the toxicity tests were carried out, various morphological tests such as endospore stain, Gram stain, macroscopic examination and biochemical tests such as starch hydrolysis, oxidase test, motility test, citrate test, methyl red test, indole test, sugar fermentation test and catalase test were carried out to identify the organism. The results showed the isolates to be *Bacillus thuringiensis*. To ensure the ability of the isolate to produce spores needed for toxicity testing, the isolate was subcultured in nutrient agar and incubated at 37°C for 7 days after which there was formation of spores in the growth medium. The isolate was then inoculated in sterile water containing mosquito larvae in order to test for its toxicity against the mosquito larvae. At intervals, observations were made to determine the rate at which the Isolates induce the lethal effects on the larvae by using the sterile loop to check for viable larva remaining at each set up. The viability is deduced from the ability of the viable larva to retain their motility when agitated with the sterile wire loop. The result for toxicity testing showed that set-up Pr1 had the highest number of dead larvae at 1 – 4 hours respectively, while Gr1 had the lowest number of dead larvae at 1. Statistical representation of the results showed that there was significant difference of $p \geq 0.05$ between time and the parasporal crystals of *Bacillus thuringiensis* against mosquito larva. Two- factor ANOVA without replication also showed that there was a significant difference between the set-ups Pr1, Pr2, Gr1 and Db2, and the positive control used gave significant difference of $p < 0.05$. This study successfully demonstrated the toxicity of the parasporal crystals of *Bacillus thuringiensis* against mosquito larvae.

Keywords: *Bacillus thuringiensis*; Mosquito larvae; Toxicity; Lethal effects

1. Introduction

Chemical insecticides provide many benefits to food production and human health and have proven very effective at increasing agriculture and forestry productivities. They also pose some hazards as contaminants of water and food, fauna and flora (Wojciech and Korsten, 2012S). It is well documented that chemical pesticides reduced natural-enemy populations and chemical applications can disrupt biological control and may cause outbreaks of secondary pests previously suppressed by natural enemies (Bartlet, 2014).

The use of synthetic organic pesticides has caused serious economic, social and environmental problems. Economically, the rapidly increasing cost of development and production of petrochemically-derived insecticides, together with the declining effectiveness due to widespread insect resistance is alarming. As a result, the chemical pesticide industry continues to develop new more expensive compounds thus increasing pesticide prices. Socially and ecologically they have caused deaths and diseases in humans and damaged the environment. It is estimated that only a minute fraction

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of the insecticides applied is required for suppression of the target pest. The remainder, more than 99.9%, enters the environment through soil, water and food cycles (Metcalf, 2002). Alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of microbial insecticides that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low. Compared to other commonly used insecticides, these are safe for both the pesticide user and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. Chemical insecticides are far more commonly used in the world than microbial control, however some microbial control agents, at least in part, can be used to replace some hazardous chemical pest control agents. A number of biological control agents formulated with bacteria, fungi, virus, pheromones, and plant extracts have been in use mainly for the control of insects responsible for the destruction of forests and agriculture crops (McDonald and Linde, 2002).

Global use of insecticides for mosquito vector control in recent decades has caused environmental pollution of aqueous ecosystem and resulted in insecticide resistance in many mosquito species. The last decade has witnessed an increased interest in biological control agents. Biological means to control vectors, based on entomopathogenic bacteria has been studied for more than 20 years. More number of biological control agents was screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. Only a few spore-forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of bacteria like *B. thuringiensis*, which is highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs all over the world (Poopathi and Tyagi, 2002). The larvicidal substances of these preparations are based on endotoxins and other proteins that have accumulated as parasporal crystals produced by the bacterial cells during the sporulation growth phase. These biological preparations have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organisms including human beings. Also, it is harmless to the environment (Prabakaran and Balaraman, 2006). The *B. thuringiensis* serovar israelensis has been used operationally for the control of mosquitoes for over two decades and its formulations are highly effective against *Anopheles*, *Aedes* and *Culex* mosquitoes (Mahmood, 2010).

This study seeks to test the ability of *B. thuringiensis* in the destruction of mosquito larva.

2. Material and Methods

2.1. Sources of Sample

In this research a total of 2 samples were used and they were obtained from agricultural and domestic sources. They include the following: rhizosphere soil of tree and garden soil samples plantain.

2.2. Sample Collection

2.3. Soil samples were collected from the two locations as described above from Umuoma Uli in Anambra State, using well labeled sterile sample containers and transported to the Microbiology laboratory of Chukwuemeka Odumegwu Ojukwu University, Uli Campus within 30 minutes of collection for further analysis.

2.4. Sterilization of Petri Dishes and other Plastic Wares

The plastic Petri dishes and other plastic wares e.g. the test tube covers, and others were washed with detergent and water, rinsed with 70% ethanol and sterilized using a bacteriological hood with laminar air flow compartment which functions by the use of ultra-violet and electric current to create uniform velocity along parallel lines which helps in the removal of any form of contamination.

2.5. Sterilization of Test Tubes and other Glass Wares

The test tubes as well as other glass wares e.g. conical flask, beakers were washed with detergent and water and sterilized using hot air oven.

2.6. Formulation of LB broth

The LB broth was formulated using modified methods of Travers *et al.* (2008). The modified broth contained tryptone: 4g, NaCl: 4g, yeast extract: 2g and was buffered with 0.25M sodium acetate: 3.42g.

2.6.1. Preparation

The above listed compounds were measured and placed into a clean conical flask containing 100 ml of distilled water which was measured with a sterile measuring cylinder; 10g of each of the soil sample was added. The conical flask containing the mixture was properly covered with cotton wool. The mixture was shaken for 4 hours on an electric shaker having speed of 150rpm. At the end of time, 2ml of the soil suspension was taken and heat shocked in a water bath at 80°C for 20 minutes.

2.7. Serial Dilution of Sample

Ten-fold (10) serial dilution was used according to the methods of Cheesbrough, 2006.

2.8. Formulation of T3 medium

The T3 medium was formulated using modified methods of Travers *et al.* (2008), which contained tryptone: 4g, NaCl: 4g, yeast extract: 2g, MnCl: 0.001g, Agar: 5g and peptone water: 0.2g was used in place of tryptose. The broth was buffered with 0.05M sodium phosphate: 3.42g.

2.9. Formulation of LB medium

The LB medium was formulated using modified methods of Travers *et al.* (2008), which contained tryptone: 4g, NaCl: 4g, yeast extract: 2g, MnCl: 0.001g, Agar: 5g and peptone water: 0.2g was used in place of tryptose, NaH₂PO₄ 0.69g, Na₂HPO₄ 0.89g.

2.10. Isolation of *B. thuringiensis*

The isolate was enriched on LB broth and isolated on T3 agar and incubated for 2 days for visual evidence on growth and re-isolated on LB agar for pure culture. Careful observation was made from zero-hour till when there was visual evidence of growth on the agar plates by colony formation. See plate 1-2 for growth on T3 medium and LB medium respectively.

2.11. Identification of the Bacterial Isolates

The bacterial isolates were identified based on morphological, and biochemical tests characteristics.

2.11.1. Gram staining techniques

A thin smear of each of the pure 1-hour old cultures were prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 seconds and rinsed with water. The smear was again flooded with Lugol's iodine for 30 seconds and rinsed with water, decolorized with 70% alcohol for 15 seconds and were rinsed with distilled water. They were then counter stained with 2 drops of safranin for 60 seconds and finally rinsed with water. This was allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple (Fawole and Oso, 2004).

2.11.2. Motility Test

Hanging drop method was utilized using a bright field microscope.

2.11.3. Spore staining technique

Heat-fixed smears of the organisms were prepared on separate slides and flooded with 5% malachite green solution and steamed for a minute. The stain was washed off with water and counter stained with 2 drops of safranin solutions for 20 seconds. The slides were allowed to air dry and examined under oil immersion objective lens. Endospores stained green while vegetative cells stained pink (Cheesbrough, 2006).

2.12. Preparation of Sterile Nutrient Agar for Sporulation

Fresh nutrient agar plates were prepared for sporulation and the plates were inoculated with *B. thuringiensis*. This was incubated at 37°C for 7 days for observation of spores.\

2.13. Toxicity Testing

Aseptically, 120 mosquito larvae were collected from stagnant water. Colonies of *B. thuringiensis* from the nutrient agar plate were transferred using a sterile inoculating wire loop into test tubes containing 10 ml of sterile water. 20 larvae of mosquitoes, 5 ml suspension of *B. thuringiensis* spores and 100 ml of normal saline were set up in duplicate 250 ml conical flask. A replicate with no bacteria was prepared as negative control; 1 ml of sniper insecticide was introduced into another flask containing 20 larvae and 100 ml of normal saline this served as a positive control. The set up was allowed to stand for 1, 2 and 48 hours respectively. Larval motility was verified by counting living versus the dead larva. The larvae that did not move when touched with a sterile stick were considered dead.

3. Results

3.1. Isolation *Bacillus thuringiensis*

Bacillus thuringiensis was isolated on T3 medium and LB medium respectively.

3.2. Identification of *Bacillus thuringiensis*

Table 1, 2 and Figure 1-2 shows the results for the biochemical and morphological tests conducted.

Table 1 Biochemical tests

Biochemical Tests	Pr1	Gs1
Oxidase	+VE	+VE
Catalase	+VE	+VE
Citrate	+VE	+VE
Gas	-VE	-VE
H ₂ S	-VE	-VE
Indole	-VE	-VE
Voges-proskauers test	-VE	-VE
Starch hydrolysis test	+Ve	+Ve

Key: +VE = Positive; -VE = Negative; Pr1= Plantain root sample; Gs1= Garden soil sample



Figure 1 Simmons' citrate Agar containing the Isolate showing positive after 24h

Table 2 Morphological characteristics of isolates

Morphology	Pr1	Gs1
Aerobic growth	+VE	+VE
Gram staining	+VE	+VE
Structure	Rods	Rods
Motility	Motile	Motile
Spore	+VE	+VE

Key: +VE = Positive; -VE = Negative; Pr1= Plantain root sample; Gs1= Garden soil sample



Figure 2 Biochemical test set up including indole, motility and sugar fermentation showing positive after 24h

3.3. Toxicity Testing

Tables 3 -4 and Figures 3-6 show the results of toxicity tests. The tests were monitored for 1, 2 and 4 hours' interval respectively.

Table 3 Toxicity test for different set-ups at 1 hours

Set up	Frequency of the dead mosquito Larvae	Frequency of live mosquito larvae	Percentage frequency of dead mosquito larvae %
Pr1	15	5	75
Pr2	7	13	35
Gs1	5	15	25
Gs2	4	16	20

Key: Pr1= Plantain plantation sample; Gs1= Garden soil sample; Note: n=20

Table 4 Toxicity test for different set-ups at 2 hours

Set-up	Frequency of the dead mosquito larvae	Frequency of live mosquito larvae	Percentage frequency of dead mosquito larvae %
Pr1	16	4	80
Pr2	8	12	40
Gs1	7	13	35
Gs2	5	15	25

Key: Pr1= Plantain plantation sample; Gs1= Garden soil sample; Note: n=20

Table 5 Toxicity test for different set-ups at 4 hours

Experimental design	Frequency of the dead mosquito larvae	Frequency of live mosquito larvae	Percentage frequency of dead mosquito larvae %
Pr1	18	2	90
Pr2	15	3	75
Gs1	13	7	65
Gs2	11	9	55

Key: Pr1= Plantain plantation sample; Gs1= Garden soil sample; Note: n=20

3.4. Statistical Analysis

A Two- factor ANOVA without replication was done and the result showed that there was a significant difference between the experiments (Pp1, Pr2, Gs1 and Gs2) and the positive control used i.e. $p < 0.05$.

Table 6 Two- Factor ANOVA without replication table

SUMMARY	Count	Sum	Average	Variance		
Pr1	3	245	81.66667	58.33333		
Pr2	3	150	50	475		
Gs1	3	125	41.66667	433.3333		
Gs2	3	100	33.33333	358.3333		
Column 1	4	155	38.75	622.9167		
Column 2	4	180	45	583.3333		
Column 3	4	285	71.25	222.9167		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	4016.667	3	1338.889	29.66154	0.000538	4.757063
Columns	2379.167	2	1189.583	26.35385	0.001068	5.143253
Error	270.8333	6	45.13889			
Total	6666.667	11				

Table 7 The ANOVA table showing the methods used in analyzing data

Sum of squares	DF	MS	F-ratio	
Factor A	SS-A	A-1	SS-A/DF-A	MS-A/MS-W
Factor B	SS-B	B-1	SS-B/DF-B	MS-B/MS-W
I.E	SS-I	(A-1)*(B-1)	SS-I/DF-I	MS-I/MS-W
W.G	SS-W	N-AB	SS-W/DF-W	
Total	SS-T	N-1		

Where I.E=Interaction Effect; W.G=Within Group; Percentage frequency of dead Mosquito larva= $X / N * 100/1$
 Where N = total number of larva for a specific isolate; X = number of dead larva for a

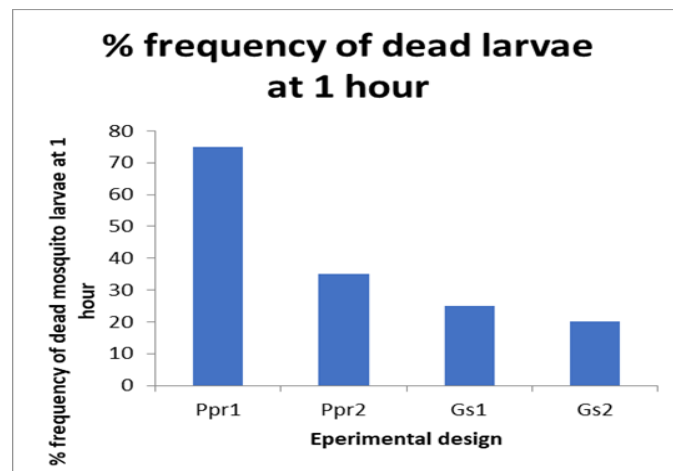


Figure 3 Percentage frequency of dead larva at each experimental design at 1 hour interval

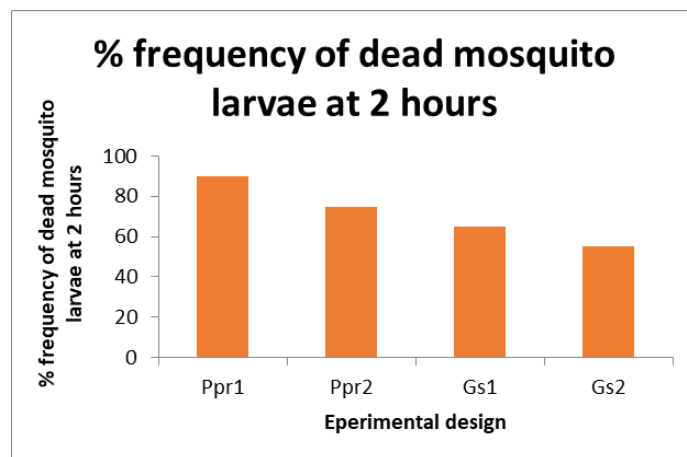


Figure 4 Percentage frequency of dead larva at each experimental design at 2 hour interval

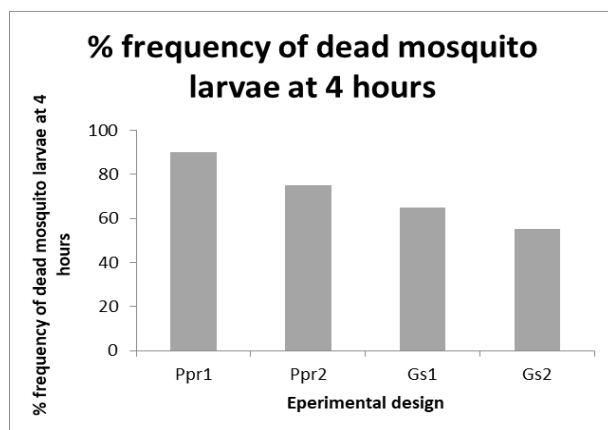


Figure 5 Percentage frequency of dead larva at each experimental design at 4 hours interval

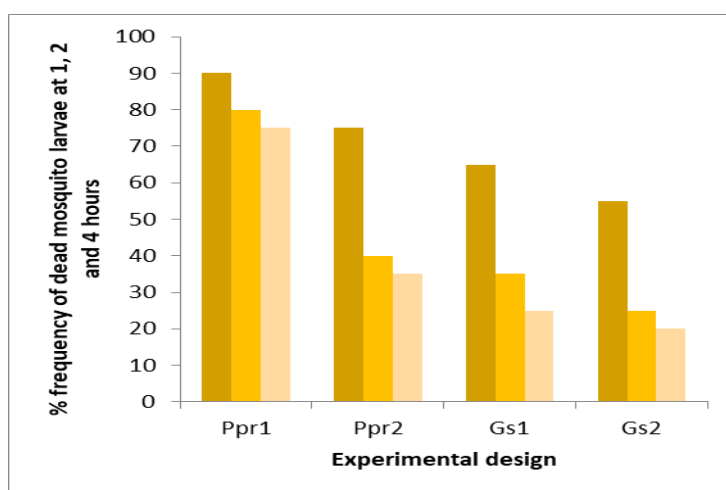


Figure 6 Frequency of dead larva at each experimental design at 1, 2 and 4 hour's interval

4. Discussion

Mosquitoes are vectors of the etiology of Malaria disease. Studies carried out by Bartlet (2014) on the chemical control of Mosquitoes revealed that the chemical methods induce the formation of recalcitrant, resistance strains and environmental hazards. The study also revealed that the chemical factors are often difficult to procure hence, giving rise to the need to establish a safer and cheap biological control.

The presence of *Bacillus thuringiensis* in the studied soil samples gotten from the root soil of plantain tree and Garden plants could be attributed to the secretion of root exudates in the plant Rhizosphere which contains nutrients required for growth of *Bacillus thuringiensis* (Kreig, 2009).

In accordance to the results established by Johnson *et al.* (2008) work on this topic, the highest occurrence of this organism in the plantain root soil sample collected is attributed to the greater secretion of exudates that favours the growth of *Bacillus thuringiensis* in the rhizosphere.

Similar findings were reported by many researchers (Baum *et al.*, 2001; Bernhard *et al.*, 2002; Chilcott., 2003; Konwles *et al.*, 2008). Chilcott (2003) reported the presence of *Bacillus thuringiensis* from soil sample gotten from the root of plantain trees when they studied Biocontrol potentials of the organism against insects. They equally discussed the chemical components of plant exudates in the rhizosphere which is the sole factor bringing about the growth of the organism in that region the he carried out an experiment to evaluate the difference between the biomass of the organism in the rhizosphere and that in the edaphosphere. They discovered that the biomass is more in the rhizosphere than in

the edaphosphere. They went further to attribute this result to the difference in amount of plant exudates present in both sides.

The significant mortality rate caused by *Bacillus thuringiensis* on the insect larva can be attributed to the secretion of parasporal crystals by the *Bacillus thuringiensis* which are highly toxic to the larva of mosquito, the toxicity is due to presence of polypeptide toxins in the crystals (Travers *et al.*, 2008).

The highest toxicity observed after 4h of exposure can be attributed to the high accumulation of the parasporal crystals, due to the fact that increased concentration of the crystal will induce increase in mortality rate.

5. Conclusion

The present study was successful in detecting the toxicity of *Bacillus thuringiensis* toxin on mosquito larvae, and to formulate a biopesticide from a cheap (free) source which is soil containing nutrient and presence of this *Bacillus thuringiensis* which can be used to produce large scale of biopesticides. So the results obtained from this study are very promising and will be very useful in developing efficient *Bacillus thuringiensis* biopesticides in Nigeria.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest amongst the authors

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