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Mosquito Larvae Biocontrol Potential of Bacillus Thuringiensis Isolated from Soil Samples

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ABSTRACT

Overcoming pesticide resistance is a fundamental barrier for effective mosquito control. This study used established methods to examine the larvicidal activity of Bacillus thuringiensis isolated from diverse soil samples on the Nnamdi Azikiwe University main campus. Biochemical characterisation and microscopic inspection were used to confirm the organism's identity. In a bioassay, the larvicidal activity of Bacillus thuringiensis isolated was tested against mosquito larvae using three dilutions of the Bacillus culture (10-1, 10-3, and 10-5). Bacillus thuringiensis was identified in the isolated organisms. The bioassay results revealed that the amount of efficiency of the bacteria varied depending on the period of exposure. After 6 hours, the mortality rate was greater than 20% and climbed to 100% when the time of exposure was increased for all B. thuringiensis dilutions utilized. Bacillus thuringiensis toxins were found to be bacteriocidal to mosquito larvae in a matter of hours, depending on the dosage swallowed by the larvae. In summary, this demonstrated that Bacillus thuringiensis is an excellent biolarvicide that may be used to minimize and possibly eliminate the annoyance of disease-causing mosquitoes and aid in the control of malaria and other mosquito-borne disorders.

Keywords: Bacillus thuringiesis, Mosquito, Larva, Biocontrol,

INTRODUCTION

Mosquitoes are disease vectors, responsible for the transfer of infections that cause more life-threatening and severe human diseases than any other organism. Every year, over one million people die from mosquito-borne diseases such as malaria, filariasis, yellow fever, chikungunya, and dengue fever in disease-endemic countries (Nareshkumar et al., 2012).

The extensive use of pesticides is one of the major causes of pollution of soil and water environments (Ifediegwu *et al.*, 2015; Agu *et al.*, 2016). Chemical pesticides help food production and human health, and they have proven to be quite effective at increasing agricultural and forestry productivity. However, unrestricted use of chemical insecticides has caused irreversible environmental damage (El-Kersh et al., 2012). According to Radhika et al. (2011), the repeated use of man-made insecticides for mosquito control alters natural ecosystems, resulting in the reemergence and expansion of mosquito populations. Zhang et al. (2011) also stated in their investigations that the continued use of chemical-based insecticides has resulted in the development of resistance, negative impacts on non-target creatures, and human health issues. As a result, they advocated for other control methods, such as biological control, which is a feasible alternative to chemical control. In some circumstances, biological control has been favoured since it is selective, has no adverse effects, and is inexpensive. Resistance to biological control is uncommon, as biological control agents propagate and self-perpetuate. (Agu et al., 2015; Agu et al., 2016; Okigbo et al., 2015).

Microbial pesticides are particularly helpful because of their low toxicity to non-target animals and people, and they are an important component of integrated pest control (El-kersh et al., 2012). They are safer than other regularly used insecticides for both pesticide users and consumers of treated crops. Bacillus thuringiensis, for example, is an important insect pathogen that is particularly toxic to mosquito larvae and similar dipterans (Zulfaidah et al., 2013). Bacillus thuringiensis is selectively active on pests and less likely to generate resistance, hence it is regarded as helpful to humans, animals, and plants, as well as a viable alternative to chemical pesticides in many countries.

Bacillus thuringiensis is a saprophytic soil bacterium that forms spores and is a Gram-positive facultative anaerobe. The toxicity is related to an endotoxin, which is composed of proteins generated and synthesised during the sporulation process of bacteria (Sanahuja et al., 2011). During sporulation, *Bacillus* thuringiensis creates one or more proteinaceous parasporal crystals, known as delta-endotoxin. Under the alkaline conditions of an insect's midgut, this crystal protein is solubilized and subsequently activated by intrinsic protease into an active toxin that preferentially binds a specific receptor in the cell membrane, resulting in pore development and the death of the insect larvae (El-kersh *et al.*, 2012). The majority of Bacillus thuringiensis preparations on

the market contain spores with parasporal inclusion bodies made up of δ (delta) -endotoxins. In commercial production, the fermented crystals and spores are concentrated and prepared for spray application using traditional agricultural practises. Many Bacillus thuringiensis strains have insecticidal activity against insect orders such as Lepidoptera, Diptera, Homoptera, Mollaphage, and Coloptera. Only a few of these have seen commercialization.

The aim of this research is to study the bio-larvicidal activity of *Bacillus thuringiensis* isolated from different soil samples in Nnamdi Azikiwe University, Awka, against the larvae of *Aedes aegypti*; as well as to study the mortality rate of varying concentrations of the suspension of *Bacillus thuringiensis* on the *Aedes aegypti* larvae and finally, to estimate the death rate of the *Aedes aegypti* larvae exposed to *Bacillus thuringiensis.*

MATERIALS AND METHODS

Sample Collection

A total of four soil samples were taken from Nnamdi Azikiwe University Awka's Science Village, Management Science Faculty, Mass Communication Department, and Boys' Hostel, all of which have no prior record of application of Bacillus thuringiensis-based insecticides. Soil samples were taken aseptically from the top of a 5cm depth. The samples were immediately packed in plastic bags and labelled. The soil samples were brought to the lab and kept at room temperature. Mosquito eggs were also received from the Parasitology and Entomology laboratory.

Isolation of *Bacillus thuringiensis*

One gram (1g) of the soil samples were weighed out aseptically and introduced into 9ml of sterile distilled water for bacteria, it was properly shaken to homogenize the sample. The set-up was incubated at 80°C for 10 minutes to allow the growth of *Bacillus thuringensis* spores. 0.1ml of appropriate dilutions (10^{-2}) of the sample were pour plated in sterile plates of Nutrient agar (NA) agar plates for the culture of bacteria. The culture plates were incubated at 37°C aerobically for 24-48 hours for bacteria. Developing colonies on Nutrient agar were counted to obtain total viable. Discrete colonies for the bacteria were obtained by subculturing into Nutrient agar plates and were subsequently identified using standard methods**.**

Total *Bacillus thuringensis* Count:

$$TBC = \frac{(N)}{VD} \times 10$$

Where TBC: Total Bacterial Count

V: Volume plated

D: Dilution Factor

N: Number of colonies

A representative colony from the Nutrient agar (NA) plate was cultured into Luria Bertani (LB) agar plate and Mannitol Egg Yolk Polymyxin (MYP) agar plate simultaneously, which are selective media for *Bacillus* species and incubated at 37°C for 24 hours. The colony was sub-cultured into a Nutrient agar plate for further identification and characterization.

Media Preparation

The media to be used to culture the bacteria such as Nutrient agar, Luria-Bertani agar, Mannitol Yolk Polymyxin agar will be prepared according to the manufacturer's instruction.

Nutrient Agar

In a sterile conical flask, two grammes (2g) of nutritional agar were weighed and dissolved in 70ml of distilled water. To dissolve the medium, the mixture was heated with a Bunsen burner and tripod stand. Cotton wool stoppers were used to seal the conical flask, which was then wrapped in aluminium foil. After that, it was sterilised in an autoclave at 121°C for 15 minutes. After sterilisation, the medium was chilled to 45-50°C before being placed into sterile petri dishes (approximately 20ml per plate) under aseptic conditions. The plates were allowed to harden before being incubated at 37°C for 24 hours to ensure that the medium was sterile.

Luria-Bertani Agar

This media is made up of Peptone, Yeast extract, NaCl, and Bacto agar for jelling. One-half gram (0.5g) of Peptone, one-half gram (0.5g) of NaCl, and one-half gram (0.5g) of yeast extract were weighed and dissolved in 50ml of distilled water in a sterile conical flask. The mixture was heated using a bunsen burner and tripod stand to dissolve. One gram (1g) of Bacto agar was added. The conical flask was plugged with cotton wool stoppers and wrapped with aluminium foil. It was then sterilized using an autoclave at 121°C for 15minutes. The medium was cooled at about 45–50°C after sterilization and then poured into sterile petri dishes (about 20ml per plate) under aseptic conditions. The plates were allowed to solidify and incubated at 37°C for 24hours and the sterility of the medium was checked.

Isolation of *Bacillus thuringiensis* from Soil

Each soil sample was weighed and one gramme (1g) was added to 9ml of distilled water. The samples were heated in an 80°C water bath for 10 minutes to kill any bacteria that could not produce endospores. Given that Bacillus thuringiensis generates spores, we may safely conclude that if it was present in the soil, it would be present in our heated sample.

Culturing of *Bacillus thuringiensis*

The diluted samples were cultivated on nutrient agar plates for 24 hours at 37°C to allow the spores to germinate on media with appropriate nutrients and optimal temperature. The media, on the other hand, promotes the growth of a diverse spectrum of microorganisms, including Bacillus thuringiensis. The colonies were subcultured onto Luria-Bertani plates and incubated at 37°C for 24 hours to achieve pure cultures of B. thuringiensis. The colonies were then subcultured onto Mannitol Yolk Polymyxin agar and incubated at 37°C for 24 hours to ensure that they were pure B. thuringiensis cultures. Following the growth of colonies with a smooth round shape and an earthy odour, a series of assays, including Gram staining and biochemical tests, were used to identify Bacillus thuringiensis.

Characterization and Identification of bacteria

Identification of the bacterial isolates was accomplished by the observation of colonial characteristics, Gram reaction and biochemical tests (Chessbrough, 1984). The characterization of the isolates were performed, by employing Gram staining reaction, Catalase test, Citrate test, Sugar fermentation test, Coagulase test, Motility test, Oxidase test, Urease test, Indole test, Methyl Red and Voges-proskauer test as described by Bergey's Manual of Determinative Bacteriology, 9th edition (1994).

Gram reaction

Thin smear of the isolate was made on clean, non-greasy, dust-free slides, air dried and heat fixed. The smear was flooded with crystal violet and allowed to remain on the slide for 60 seconds. Thereafter, the crystal violet was washed off with gentle running water. Again, the slide was flooded with slide with Gram's iodine, allowed to remain for 60 seconds and washed off. The slide was decolourized with acetone-alcohol mixture. The slide was counter-stained with safranin for 60 seconds and rinsed with tap water and allow to air dry. The slide was then viewed under oil immersion lens microscope (×100). Purple colour indicated Gram–positive organisms while red or pink colour indicated Gram-negative organisms.

Catalase test

Exactly 3ml of 3% solution of hydrogen peroxide (H_2O_2) was transferred into a sterile test tube. Then, 3 loopful of a 24 hour pure culture of the test bacteria were inoculated into the test tube. The tube was observed for immediate bubbling indicative of a Positive, while no bubbling indicated a negative reaction.

Motility test (Hanging Drop Method)

A loopful of 18-24 hour broth culture of the test bacteria was placed at the centre of a clean grease-free cover-slip. Carefully, the coverslip was inverted and placed over the concave portion of a hanging drop slide. The cover-slip/slide arrangement was observed for motility at X100 magnification on a compound microscope. Care was taken to not interprete "drift" or "Brownian motion" as motility. Results were recorded as motile or non-motile.

Oxidase Test

All bacteria that are oxidase positive are aerobic, and can use oxygen as a terminal electron accept or in respiration. This does NOT mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport.

Whatmann No.1 filter paper was soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. The filter paper was moistened with sterile distilled water. Then the test colony was picked with wooden or platinum loop and smeared in the filter paper. The inoculums was observe the area around the inoculated paper for a color change to deep blue or purple within 10-30 seconds. Positive and negative quality controls were also setup (Positive control: *Pseudomonas aeruginosa*; Negative control: *Escherichia coli*). **Positive** was indicated by development of dark purple color (indophenols) within 10 seconds. **Negative**: Absence of color.

Urease Test using Christensen's Urea Agar

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbondioxide. It is primarily used to distinguish urease-positive *Proteeae* from other *Enterobacteriaceae*.

Heavy inoculum from an 18-to24-hour pure culture was used to streak the entire **Christensen's Urea Agar** slant surface. Adequate care was taken not to stab the butt as it will serve as a color control. The tubes were incubated loosened caps at 35^{o}C. The slants were observed for a color change at 6 hours, 24 hours, and everyday for up to 6 days. Urease production would be indicated by a bright pink (fuchsia) color on the slant that may extend into the butt. Note that any degree of pink is considered a positive reaction. Prolonged incubation may result in a false-positive test due to hydrolysis of proteins in the medium. To eliminate protein hydrolysis as the cause of a positive test, a control medium lacking urea was also setup. Rapidly urease-positive *Proteeae* (*Proteus* spp., *Morganella morganii*, and some *Providencia stuartii* strains) will produce a strong positive reaction within 1 to 6 hours of incubation. Delayed-positive organisms (e.g.,*Klebsiella* or *Enterobacter*) will typically produce a weak positive reaction on the slant after 6 hours, but there action will intensify and spread to the button prolonged incubation (up to 6 days). The culture medium will remain a yellow color if the organism is urease negative

Indole Test

A loopful of an 18-24 hour culture was used to inoculate the test tube containing 3ml of sterile tryptone water. Incubation was done at 35–37ºC first for 24 hours and further for up to 48 hours. Test for indole was done by adding 0.5ml of Kovac's reagent, shaken gently and then examined for a ring of red colour in the surface layer within 10 minutes, indicative of a positive reaction. Absence of red color indicated a negative reaction.

Methyl Red test

Exactly 5drops of methyl red indicator were added to an equal volume of a 48 hours culture of the isolate in Methyl red – Voges Proskauer (MR-VP) broth. The production of a bright red color indicates a positive test while yellow color indicates a negative test after vigorous shaking.

Voges-Prausker test

Exactly 2ml of the 18-24 hours culture of the test organism growing on MR-VP broth was asceptically transferred into a sterile test tube. Then 0.6ml of 5% α-naphthol was added, followed by 0.2ml of 40% KOH (NB: It was essential that this reagents were added in this order). The tube was shaken gently to expose the medium to atmospheric oxygen and then allowed to stand undisturbed for 15-30 minutes. A positive test was indicated by the presence ofaredcolourafter15-30 minutes, indicative of the presence of diacetyl, the oxidation product of acetoin (Test was always considered invalid after one hour because VP-negative cultures may produce a copper-like color, false positive), lack of pink-red color denoted a negative reaction.

Citrate test

A 24 hold culture was inoculated into test tubes containing sterile Simmons Citrate agar slant and then incubated for 24 hours. A positive test was indicated by a change from green to blue color on the surface of the Simmons Citrate agar slant. No color change indicated a negative reaction.

Sugar Fermentation Test

Each of the isolate was tested for its ability to ferment a specific sugar. 1g of the sugar and 1g of peptone water were dissolved in 100ml of water. 5ml of the solution were transferred into clean test-tubes using sterile pipettes. The test-tubes containing peptone water and sugar were added Durham's tube which were placed inversely and bromothymol blue as an indicator. These were sterilized for 10 minutes and allowed to cool before inoculating the inocula. The test-tubes were incubated for 3days. The production of acid and gas or acid only indicated utilization of sugars. Acid production was indicated by change in color of the medium from green to yellow while gas production was observed by presence of gas in the Durham's tubes.

Culturing of mosquito larvae

For three days, one ribbon of fresh Aedes aegypti eggs hatched in a basin of rain water. At the end of the third day, the second larval batch was taken and used in the experiment.

Bioassay

The Bacillus thuringiensis isolates chosen were tested against mosquito larvae. Bacillus thuringiensis stock cultures from slant bottles were collected with a sterile wire loop and diluted five-fold 10-1-10-5 in sterile distilled water in five test tubes. Five (5)ml of each culture from the first, third, and fifth test tubes was put to three (3) disposable cups containing 45ml of sterile distilled water, with each cup having a different dilution factor. Each disposable cup received twenty-five (25) larvae. The cups were stored at 25°C-30°C for 6 hours. Each cup was checked for the existence of larvae at 30 minute intervals, and the larval mortality rate was calculated.

RESULTS

Table1: Total *Bacillus thuringensis* Count

	Total Bacteria count(Cfuml^{-1})	
Samples	**No. Of Bacterial colonies on plate**	**Total Bacterial Count (cfuml^{1})**
Management science faculty	263	$2.63x10^3$
Boys' Hostel	56	$5.60x10^2$
Science Village	110	$1.10x10^3$
Mass Communication department	69	$6.90x10^2$

Table2: Morphological and Biochemical Identifications of the Various Bacterial Isolates.

Isolate	Form	Surface	Color	Margin	Elevation	Opacity	Gram	Cat	Mot	Ind	MR	VP	Cit	Lac	Glu	Suc	Fru	Mal	Oxi	Ure	Identity
A	Circular	Smooth	Whitish	Even	Raised	Opaque	+Rod	+	+	-	+	+	+	+	+	+	+	+	var	-	*Bacillus thuringensis*
B	Circular	Smooth	Whitish	Even	Raised	Opaque	+Rod	+	+	-	+	+	+	+	+	+	+	+		+	*Bacillus thuringiensis*
C	Circular	Smooth	Creamy	Even	Raised	Opaque	+Rod	+	+	-	+	+	+	+	+	+	+	+		+	*Bacillus thuringiensis*
D	Circular	Smooth	Whitish	Even	Raised	Opaque	+Rod	+	+	-	+	+	+	+	+	+	+	+		+	*Bacillus thuringiensis*

Key:
Gram: Gram reaction
Cat: Catalase test
Mot: Motility test
Ind: Indole test
MR: Methyl-red test
VP: Voges-Proskauer test
Cit: Citrate Utilization test
Sugar Fermentation Tests:
Lac: Lactose Fermentation
Glu: Glucose Fermentation
Suc: Sucrose Fermentation
Fru: Fructose Fermentation
Mal: Maltose Fermentation
Oxi: Oxidase
Ure: Urease

Table3: Bioactivity of *Bacillus thuringiensis* of 10^{-1}, 10^{-3}, and 10^{-5} dilutions against mosquito larvae at 2hours, 4hours, 6hours, 8hours and 10hours

Conc	T-0	T-2	T-4	T-6	T-8	T-10
0.1	25	17	12	7	4	0
0.001	25	20	16	9	6	1
0.00001	25	21	17	10	7	3

DISCUSSION

Mosquitoes are a major pest and a serious hazard to human health in modern culture. In recent years, many chemical pesticides have been developed for mosquito control, some of which have proven to be quite successful while others have proven to be ineffective. The majority of pesticides used are synthetic compounds that have been shown to have a harmful impact on the diversity of numerous insects, as well as the health of humans and the environment. As a result, there is an urgent need for the development of biological control approaches that are less harmful to human health, insect variety, and the environment. Bacillus thuringiensis was found to be an effective larvicide for suppressing mosquito larvae. The organisms' microscopic and biochemical properties, as given in Table 2, corroborated the basic characteristics of Bacillus thuringiensis, which is Gram-positive and has a rod shape. Among other things, the biochemical properties revealed the organism's motility.

Bacillus thuringiensis was found in all four soil samples examined. Bacillus thuringiensis was found in abundance in soil samples, according to the findings of this investigation.

Different diluents employed in the bioassay for mosquito larvae control exhibited variable degrees of efficiency, with 100% mortality rate recorded after 10 hours. This could be linked to the organism's ability to create a binary toxin (Bin), which is a primary insecticidal component produced during the sporulation and vegetative stages of B. thuringiensis in inhibiting mosquito larvae growth. This is consistent with the findings of Oei et al. (2012). After 2 hours of incubation, extremely minimal mortality was observed in all diluents, which might be related to the period of exposure of the larvae as well as

the number of organisms present in the container. However, within 8 hours, more than half of the larvae were dead, with a high death rate of more than 70% documented in all B. *thuringiensis* diluents. This could also be due to the period of exposure of the larvae, as well as an increase in the number of cells in the medium, which could be ascribed to an increase in the number of organisms swallowed by the mosquito larvae, causing damage in the larvae's mid-gut Wei et al. (2013). As a result, the crystallised binary toxins are released, which are then solubilized in the mid-gut, releasing two proteins that are cleaved by endogenous proteins to create active toxins. This is consistent with the findings of Aissaoui and Boudjelida (2014).

CONCLUSION

Bacillus thuringiensis, which is found naturally in soil, has been shown in the laboratory to be an effective larvicidal agent against mosquito larvae. This organism and its product can be explored further in order to find novel chemicals that can be utilised to combat mosquito-borne diseases such as malaria.

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