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Research Article

A Native *Bacillus Thuringiensis* Strain with Higher Insecticidal Activity Against Adult *Aedes Aegypti* Than the Larvae

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Abstract:

Mosquitoes of several species cause the deaths of millions of people annually, making them the major disease vectors. Bioinsecticide formulations derived from *Bacillus thuringiensis* are widely acknowledged as highly efficient, sustainable, environmentally friendly, and safe means of controlling insect pests. In this study, a strain of *B. thuringiensis* exhibiting significant efficacy in controlling *Aedes aegypti* was isolated from the local environment, identified, and physiologically characterized. The isolated *B. thuringiensis* strain exhibited positive insecticidal activity against the larvae and adult *Aedes aegypti*, causing mortality of the insect larvae at a rate of 3.33% and adult at 53.33 – 93.33% in 48 hours. Experiments conducted in a controlled laboratory setting showed that *B. thuringiensis* toxin exhibited higher levels of activity against the adult mosquito than the larvae. This study demonstrates that bioinsecticide formulation using *B. thuringiensis* as *the* active agent has the potential to effectively and sustainably manage larvae and adult *Aedes aegypti*.

Keywords: Bioinsecticide, Bacillus thuringiensis, Aedes aegypti, mosquito, Larvae, Mortality

Introduction

Mosquitoes are well recognized as a vital group of insects that are responsible for transmitting many devastating infectious diseases, such as malaria, yellow fever filariasis, dengue, Japanese encephalitis, Rift Valley, chikungunya fever, and Zika fever (WHO 2020). Collectively, these diseases cause millions of deaths each year (Ma et al. 2023). The mosquito exhibits a strong attraction towards humans and is frequently encountered in domestic environments. The insect consumes blood regularly, and its capacity to thrive is associated with favourable conditions such as water-rich environments for mating, fast development, and eggs that can endure desiccation (De Obaldia et al. 2022). These attributes render the management of this insect challenging, therefore exacerbating a global public health concern.

The widespread and intense use of chemical insecticides for mosquito control has resulted in a range of environmental and human health issues, such as disturbance of natural-biological control systems, the development of mosquito resistance, and adverse impacts on beneficial species (Demirak and Canpolat 2022). As of 2019, the mosquito species *Aedes aegypti* has been notably widespread in Nigeria, which is one of the most densely populated countries in Africa (Otu et al. 2019). According to Emeribe et al. (2021), it was identified as the underlying cause of the hyperendemic dengue fever infection in the country. Unfortunately, this specific species has developed immunological resistance to insecticides, thereby impeding its capacity to efficiently manage them (Sene et al. 2021). Numerous approaches have been utilized thus far to manage mosquitoes (Weeratunga et al. 2017; Yakob et al. 2017; Roiz et al. 2018). Although considerable progress has been made, there remains a necessity to develop more efficient, enduring, and environmentally friendly methods. The existing biodiversity on Earth offers the possibility to identify methods for development and use in tackling this significant problem, or at the very least, significantly reducing the negative effects of disease transmission by these vectors (Oladipo et al. 2022).

One of the feasible alternatives to conventional chemical agents for vector control is the use of a combination of *Bacillus thuringiensis* (Bt) spore and crystal (Ma et al. 2023; Jouzani et al. 2017). These organisms have been obtained from many sources including soil, aquatic habitats such as sewage, dead insects, herbivore dung, stored grains, phylloplane (the outer layer of plant leaves), and wood (Belousova et al. 2021). Over more than six decades, this soil bacterium, which possesses aerobic, Gram-positive, spore-forming, and saprophyte properties, has been employed as a biological insecticide due to its safety, efficacy, and selectivity against a diverse array of pests and diseases that impact human health. Approximately 95% of all commercial bioinsecticides are attributed to it (Kumar et al. 2021). The efficacy of *B. thuringiensis* as a bioinsecticidal agent is well recognized. It demonstrates toxicity against a diverse range of insect species, encompassing Dipteran, Lepidopteran, and Coleopteran (Bravo et al. 2011).

Bacillus thuringiensis usually produces insecticidal proteins and δ-endotoxins during the sporulation phase (Argolo-Filho and Loguercio 2013). Following the consumption of bacterial crystals, a concise summary of the mode of action of *Aedes aegypti* is

provided. Subsequently, these crystals are dissolved in the alkaline pH of the larval midgut, resulting in the liberation of biotoxins. Different proteases catalyze the activation of protoxins synthesised by *B. thuringiensis*, thereby transforming them into toxic variants. Upon reaching their active state, these toxins bind to receptors on the intestinal epithelium of several insects (Ma et al. 2023; Tetreau et al. 2021; Bravo et al. 2007). This interaction triggers the creation of pores in the cell membrane, resulting in an anion imbalance that finally leads to the rupture, disintegration of the cell and death of the insect.

Bacillus thuringiensis (Bt) strains may exhibit variations in genetic diversity and toxic potential across different countries and locations. Identifying Bt strains indigenous to different locales with insecticidal potency against commonly found mosquito species is of utmost importance.

The objective of this work is to isolate indigenous *B. thuringiensis* that possess inherent increased toxicity to control both larvae and adult *Aedes aegypti*.

Materials and Methods

Isolation, Identification, and Characterization of *B. thuringiensis* Isolates

Soil samples were collected in sterile plastic tubes from three different farmlands in Awka South LGA Anambra State Nigeria, located at 6°12′25″N 7°04′04″E. The collected samples were transported to the Microbiology laboratory of Nnamdi Azikiwe University, Awka, and stored at 4 °C until processing for *B. thuringiensis* isolation.

Ten grams of each soil sample was wrapped in sterile aluminium foil and placed in a water bath at 100 °C for 20 minutes to remove vegetative cells. After allowing to cool for 3 hours, the soil sample was introduced into sterile 50 ml Luria-Bertani (LB) broth (yeast extract, 2 g/L; tryptone, 4 g/L; sodium chloride, 4 g/L; sodium acetate, 3.42g; pH 7) in a 100 ml Erlenmeyer flask and incubated in a rotary shaker at 150 rpm for 24 hours. After incubation, 1 ml of the medium was spread-inoculated on sterile T3 agar (tryptone, 4 g; NaCl, 4 g; yeast extract, 2 g; MnCl, 0.001 g; agar, 5 g; peptone: 0.2 g and $NaHPO_4$, 0.05 M) plates and incubated for 48 h at 30 °C. After that, each colony was sub-cultured on nutrient agar (yeast extract (3 g/L), peptone (5 g/L), and sodium chloride (5 g/L) (Sangon-Biotech) and incubated at 30 °C for 24 h. *B. thuringiensis*-like colonies, white, large, and nearly circular with fine irregular margins and glossy, less glossy, or rough were selected.

Biochemical tests were carried out on the selected colonies for the identification of *Bacillus thuringiensis*. Biochemical tests conducted include Gram's reaction, motility test, catalase test, citrate utilization test, hemolysis on blood agar, and sugar fermentation test. Isolates that align with the characteristics of *Bacillus thuringiensis* described in Rabinovitch et al. (2017) were grown on mannitol yolk polymyxin (MYP) agar plates for confirmation.

Fermentation and Extraction of *B. thuringiensis* Endotoxin

We used one of the identified *Bacillus thuringiensis* strains confirmed on MYP agar to produce Bt toxin. The submerged fermentation process was employed following the procedure outlined by Okafor and Okeke (2017). The liquid fermentation medium utilized consisted of maize starch (6.8%), sucrose (0.64%), casein (9.94%), corn steep liquor (4.7%), yeast extract (0.6%), and phosphate buffer (0.6%). A 50 ml sterile fermentation medium was placed in a 100 ml Erlenmeyer flask. Then, a loop full of a 24-hour-old culture of the isolate was added to the flask. The flask was placed in a rotary shaker and subjected to agitation at a speed of 150 revolutions per minute for 72 hours.

Following the fermentation period, the fermentation broth was subjected to centrifugation at a speed of 4,000 revolutions per minute for 1 hour. The toxin component of the isolate was recovered using a solvent extraction procedure, following the approach reported by Buss and Butler (2010). The supernatant, measuring 50 ml, was combined with an equal volume of acetonitrile in a 1:1 ratio by volume. The mixture was incubated for 10 minutes and centrifuged at a speed of 5000 rpm for 1 hour afterwards. The supernatant was subjected to evaporation until complete dryness in a pre-weighed. The beaker's final weight was determined. The crude toxin content was calculated gravimetrically and represented in grams by subtracting the initial weight of the beaker from the final weight.

Toxicity Bioassay Against the Larvae and Adult *Aedes aegypti*

Larvae assay:

The supernatant of the fermentations of one of the Bt strain with the highest endotoxin production was used for the assay. The bioassay was designed according to the guidelines for the laboratory testing of mosquito larvicide from the World Health Organization (WHO 2005) with slight modifications. The *Aedes aegypti* species was used in the experiments, it was reared in the Insectary of the Department of Parasitology and Entomology, Nnamdi Azikiwe University, Awka. The Bt fermentation supernatant was diluted using sterile distilled water to obtain 3.12 %, 6.5 %, 12.5 %, 25 % and 50 % concentrations. One milliliter (1 ml) of the different concentrations was added to fifty milliliter (50 ml) of chlorine-free water in separate plastic containers of uniform size. To each container, twenty fourth-instar larvae were added. A positive and negative control were also set up containing chemical insecticide (Altocid) and distilled water respectively. Each set-up was replicated three times. The bioassays were evaluated by checking for mortality of the larvae at 24 h and 48 h. Mortality rate, expressed in percentage was calculated using the formula:

$$\text{Mortality rate (\%)} = \frac{No.of\ insect\ deaths}{Total\ no.\ of\ insects} \times 100$$

Adult assay:

Adult toxicity bioassays were done with 3-day-old adult *Aedes aegypti*. Whatman filter papers No.1 was cut to Sixty-five millimeter (65 mm) in diameter and each paper was impregnated with 1 ml of the different concentrations (50%, 25 %, 12.5 %, 6.25 % and 3.12%) of the *Bacillus thuringiensis* fermentation supernatant. The positive and negative controls comprised the same size of filter paper impregnated with 1 ml of chemical insecticides Repel® and distilled water respectively. The papers were allowed to air dry, and each was transferred into clean, dried 250 ml conical flasks. The papers were spread to ensure they completely covered the base area of the flask. Each concentration, as well as the controls, were replicated three times (WHO 2005). Twenty adults *Aedes aegypti* were transferred to each concentration in the conical flasks. The top of the flask was covered with a soft net and the mosquitoes were fed with a sugar solution (10%). The bioassay was monitored at 24 h and 48 h for the death of the mosquitoes. The mortality rate, expressed in percentage, was calculated using the formula:

Mortality rate (%) = $\frac{No.of\ insect\ deaths}{Total\ no.\ of\ insects} \times 100$

Data Analysis

All the assays were performed in replicates, and the values presented in the graphs /tables are means ± standard errors of the means. Statistically significant differences among the mean values were determined using analysis of variance (ANOVA) from the mean at $P < 0.05$. P values of < 0.05 were considered statistically significant. The data were analyzed and processed using MS Excel 365 and IBM SPSS version 21.

Results and Discussion

Isolation and Identification of *B. thuringiensis* Isolates

A total of fourteen bacterial colonies were obtained from the soil samples collected in the three different farmlands in Awka. Out of the 19 colonies that were isolated, only three colonies (C1, C3 and B4) were identified as *B. thuringiensis*. The percentage of *B. thuringiensis* colonies was quite low, accounting for only 21.43 % of the total (Table 1). The morphology of the three strains of *Bacillus thuringiensis* can be described as circular form, irregular margin, large, glossy and white colonies. The three strains showed similar biochemical characteristics (Table 2) that agree with the characteristics described by Rabinovitch et al. (2017). They showed spore formation under the microscope, Gram-positive, rod-shaped, motile, catalase and citrate positive and produced beta hemolysis on blood agar plate. The organisms were confirmed *Bacillus thuringiensis* when they appeared as yellowish colonies on MYP agar indicating the ability to ferment mannitol and lecithinase activity.

The Mannitol Yolk Polymyxin (MYP) agar medium is used to distinguish between the *B. thuringiensis* and *B. cereus* groups (Rabha et al. 2017). This medium contains D-mannitol and phenol red, which is a pH indicator. When mannitol is fermented, it produces acidic by-products that lower the pH of the medium (Rabinovitch et al. 2017). *B. thuringiensis* can ferment D-mannitol, releasing organic acids and lowering the pH of the medium. At the low medium pH, phenol red turns yellow, causing Bt colonies to appear yellowish as seen in this study. On the other hand, *B. cereus* is unable to ferment D-mannitol, resulting in an increase in pH and a change in the color of phenol red to pinkish red. The Polymyxin B sulfate in the medium inhibits the growth of Gram-negative bacteria. Lecithinase activity was detected by the presence of precipitation at the edge of the colonies resulting from the breakdown of lecithin in the egg yolk by lecithinase enzyme produced by *Bacillus thuringiensis*. The isolation and identification of *Bacillus thuringiensis* from agricultural soil in this study corroborates the work of other researchers. Hassan et al. (2021) isolated and characterized 12 Bt strains from cultivated and uncultivated agricultural soils in Saudi Arabia, Rabha et al. (2017) isolated and characterized 42 *Bacillus thuringiensis* strains based on Mannitol Egg Yolk Polymyxin (MYP) agar and 16S rDNA analyses from Northeast Indian soil, while out of the 50 bacterial isolates recovered from the soil by Astuti et al. (2018), 15 were characterized as *Bacillus thuringiensis* based on morphology and biochemical characteristics.

Table 1. Distribution of *Bacillus thuringiensis* isolates in the soil samples from different farmlands in Awka

Samples	Number of bacterial colonies	Number of B. thuringiensis isolates
A	5	0
B	4	1 (B4)
C	5	2 (C1 and C3)

Table 2: Biochemical test result of the bacterial organisms isolated from soil samples.

Isolate	Gram reaction	Motility	Catalase	Citrate	Blood agar test	Growth on MYP	Sugar fermentation					
							Glu	Mal	Suc	Man	Lac	Fru
A1	+ve rod	+	+	+	B	-	+	-	+	+	-	+
A2	+ve cocci	+	+	-	B	-	+	-	+	-	-	+

A3	+ve cocci	+	+	-	B	-	+	-	+	+	-	+
A4	+ve rod	+	+	-	B	-	+	-	+	-	-	+
A5	+ve cocci	+	+	-	B	-	+	-	+	-	-	+
B1	+ve rod	+	+	-	B	-	+	-	+	+	-	+
B2	+ve cocci	+	+	-	A	-	+	-	+	+	-	+
B3	+ve rod	-	+	-	A	-	+	-	+	+	-	+
B4	+ve rod	+	+	+	B	+	+	-	+	+	-	+
C1	+ve rod	+	+	+	B	+	-	-	-	+	-	-
C2	+ve rod	+	+	-	B	-	-	+	+	+	+	+
C3	+ve rod	+	+	+	B	+	-	-	-	+	-	-
C4	+ve rod	+	+	+	B	-	-	-	-	+	-	-
C6	+ve rod	+	+	+	A	-	-	-	-	+	-	-

Key: + = positive, - = negative, Glu = Glucose, Mal = maltose, Suc = Sucrose, Man = Mannitol, Lac = Lactose, Fru = Fructose, α = Alpha, β = Beta

Fermentation and Extraction of *B. thuringiensis* Endotoxin

A fermentation medium composed of corn starch and casein as carbon and nitrogen sources was provided for the three Bt isolates for endotoxin production. Media formulation is a key consideration in the development of bioprocesses that can produce affordable biological agents, yet limited progress has been made in this area to satisfy market opportunities for affordable commercial biological insecticide products (Patil et al. 2014). It has been well documented that nutrient sources like carbon, nitrogen sources, and macronutrients strongly influence the growth, toxin production, and toxicity associated with parasporal proteinaceous crystalline inclusions and synthesis of commercially useful metabolites in *Bacillus* species (Yan et al. 2007). Endotoxin is an active insecticidal component of the organism. The toxin concentrations produced in the medium by the isolates in this study were 0.52g/L, 1.02g/L and 0.88g/L for C1, C3 and B4 respectively (Figure 1). This is dissimilar to the report of Ndao et al. (2021) and Fayad et al. (2022); while Ndao et al. (2021) used effluent from the starch industry as the substrate for the production by *Bacillus thuringiensis* var *kurstaki* HDI and recorded maximum toxin production of 435 µg/ml, Fayad et al. (2022) used wheat bran as the primary substrate and recorded endotoxin concentrations ranging from 0.04 – 1.93 mg/mL. The endotoxin yield in this study is fair but not encouraging enough for commercialization, however, optimization of fermentation conditions is recommended for improved yield.

Toxicity Bioassay Against the Larvae and Adult *Aedes aegypti*

Bacillus thuringiensis strain (isolate C3) which produced the highest toxin concentration in the fermentation medium was used for the toxicity bioassay. The *B. thuringiensis* toxin in this study expressed poor larvicidal activities at 48 h. Mortality of *A. aegypti* larvae was observed only at 50 % concentration of the toxin at 3.33 % rate (Figure 2). The toxicity of the chemical insecticide was significantly higher (p-value < 0.05) than the Bt toxin. Interestingly, insecticidal activity against the adult was above 50 % for all the toxin concentrations with the highest observed at 50 % concentration (Figure 3). The result shows an interesting alternative to adult mosquito control. It also took 48 hours for an observable toxicity against the adult *Aedes aegypti.* This can be attributed to the mode of action of the toxins. The toxicity of Bt toxin is based on the ingestion of the proteinaceous crystal toxin which needs to be solubilized in the mosquito larvae's alkaline midgut lumen for activation of the protoxin by midgut proteases ending in pore formation into the midgut cells (Silva-Filha et al. 2021). The time for the process of crystal protein solubilization and protoxin activation differ for various Bt toxins, and hence, the time it took for observable mortality of the mosquito larvae in this study. Additionally, the higher mortality recorded against adult *A. aegypti* than the larvae can be attributed to enhanced solubilization and activation of the crystal protoxin in the relatively developed midgut of the adult mosquito. Genomic analysis has identified the presence of lectin in the gut of adult *Aedes aegypti* which supports the binding of Bt cry toxin (Lee et al. 2014).

The use of *B. thuringiensis* as a mosquito larvicide is widely known, and previous research on its biological effects on adult mosquitos has shown interesting and contradictory results (Davis et al. 2021; Terbot et al. 2015; Futami et al. 2011). Larvicidal activity is low compared with previous works where larvicidal activities were higher (El-Kersh et al. 2016; Rajendran et al. 2018; Alves et al. 2020). The result of the insecticidal activity against adult *Aedes aegypti* is consistent with the report of previous researchers. For example, Davis et al. (2021) reported 97 % and 98 % mortality for *Aedes aegypti* and *Aedes albopictus* after 48 hours of exposure to *Bacillus thuringiensis israelensis.* Zaki et al. (2020) recorded a 93.8 % reduction in the *Aedes* species population upon 24-hour exposure to *Bacillus thuringiensis* during their work on the efficacy of *Bacillus thuringiensis* treatment on *Aedes* population using different application methods. In a recent study, the endotoxin protein of *Bacillus thuringiensis* was expressed in fungus against adult *Aedes aegypti* by recombinant technology, and it recorded an enhanced virulence of the fungus against *Aedes aegypti* and *Aedes albopictus* mosquitoes (Deng et al. 2024). We believe that the locally isolated *Bacillus thuringiensis* is crucial for future bioinsecticide production for the control of adult *Aedes aegypti.*

This study aimed to isolate and characterize new indigenous Bt isolates in a controlled laboratory setting. Nevertheless, these findings merely yield a potential commercial product. Natural conditions will necessitate the implementation of assays on small,

medium, and large scales, and downstream processes for a manufactured final product. We anticipate further research on *Bacillus thuringiensis* for improved insecticidal activity, and bioinsecticide products that match the chemical counterpart.

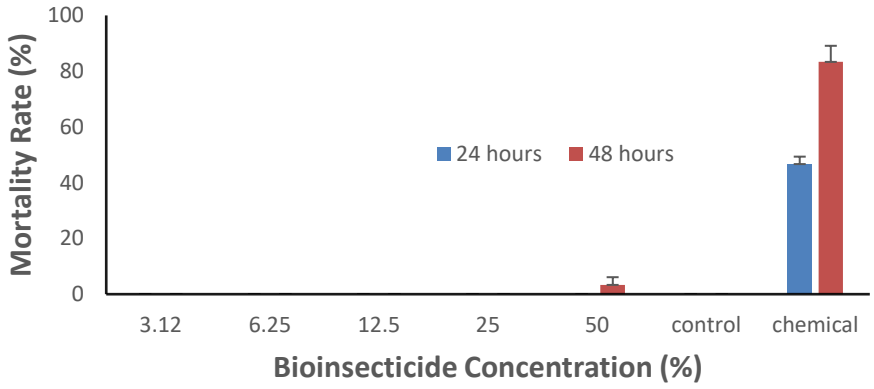

Figure 2. Mortality Rate of *Aedes aegypti* Larvae Exposed to *Bacillus thuringiensis* toxin

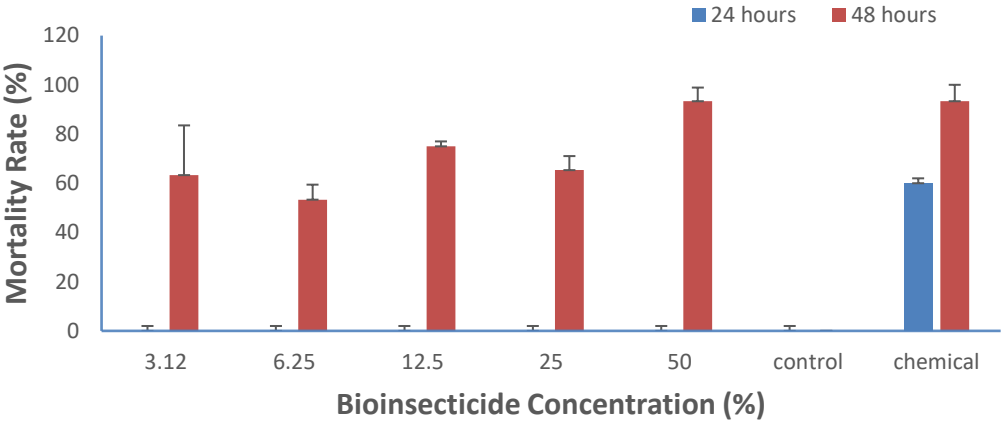

Figure 3. Mortality Rate of Adult *Musca domestica* Exposed to *Bacillus thuringiensis* toxin

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Conflict of interest

The Authors declare that there is no Conflict of Interest (COI) that may have affected their research.

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