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Optimization of *Bacillus thuringiensis* Growth Conditions for Enhanced Larvicidal Activity against Anopheles Mosquitoes Larvae

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Abstract

One of the conventional ways of tackling malaria is the application of insecticides to mosquito breeding places. The study's potential contributions to knowledge include providing a comprehensive understanding of how to maximize the effectiveness of Bti extracts, which can be applied in various ecological contexts and potentially lead to more effective mosquito control strategies. This study bridges the gap in identifying the optimal growth conditions for Bacillus thuringiensis to enhance its larvicidal activity against Anopheles mosquitoes larvae. This study aimed at enhancing the potential of Bacillus thuringiensis in the control of mosquito larvae. Bacillus thuringensis was isolated from soil samples in Katsina metropolis. Optimization of the culture conditions for the growth of the bacterium was carried out using selected parameters (pH, temperature, salinity and inoculum volume) based on single factor at a time analyses. Mosquito larvae were obtained from Kofar Marusa water outlet, Katsina and characterized based on morphological and larvicidal stages (instar). The larvae mortality test was carried out using the prepared inocula of B. thuringensis, and the mortality rate among the larvae was recorded at intervals. The results obtained indicate that the population range of Bacillus thuringiensis ranged from 2.01 × 10^3 ± 0.03 CFU/mL to 3.62 × 10^3 ± 0.02 CFU/mL. The highest and lowest percentage mortality obtained were 85% and 50% respectively. The initial culture conditions used involved a temperature of 29 ^{0}C, a pH of 7.5, a salinity of 2% and inoculum volume of 1% v/v. The optimum conditions achieved involved a temperature of 30 ^{0}C, pH value of 7.5, salinity of 0% w/v and inoculum volume of 20% v/v. This indicates that B. thuringiensis represents an effective alternative to chemical insecticides in the control of mosquito larvae. Considering the fact that Bt toxins are safe for non-target species and human health, and so far, no resistance among the target has been detected, coupled with their specificity and eco-friendly nature, this study recommends using integrated pest management campaigns involving the use of bioinsecticides to serve as a better alternative to chemical insecticides which are harmful to the ecosystem.

Keywords: Bacillus thuringiensis, Biocontrol, Malaria, Mosquito larvae, Optimization.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is bacterial insect pathogens that rely on insecticidal pore-producing proteins known as Cry and Cyt toxins to destroy its insect larval hosts. The organism differs from other members of the *Bacillus* group due to its entomopathogenic properties, mainly the elaboration of parasporal crystals/insecticidal proteins (Cry and Cyt toxins) during the sporulation phase, which are mainly comprised of delta endotoxins. The toxins are harmless to humans, animals, plants and vertebrates, but are extremely specific to their target insects. The organism resides in different ecosystems across the world environment (Kabir *et al.*, 2014; Hamedo, 2016). Bt subspecies *israelensis* operates through a well-known protein mechanism that causes toxicity in insects due to the crystal protein it possesses.

Some strains synthesize proteins that are usually secreted into the medium during the vegetative growth phase of *B.thuringiensis* and have been found to have insecticidal properties against a number of insects, thus extending the overall host range of this bacterium (Milne *et al.*, 2008). The insecticidal proteins encompass two classes of proteins designated as: vegetative insecticidal protein (Vip) and secreted insecticidal protein (Sip).

Numerous *Bt* strains that exhibit actions towards dipteran, coleopteran or lepidopteran insects have been studied (Bravo *et al.*, 2011). Recently, *Bt* strains that are active against the insect orders Hymenoptera, Homoptera, Orthoptera and Mallophaga, as well as other

other non-insect species including nematodes and protozoa, have been identified (Wei *et al.*, 2003).

A number of *B. thuringiensis* strains have the capacity to produce nonproteinous, thermostable and secretable secondary metabolites, which exhibit non-specific toxic activity against a wide range of insects in addition to mammals (Liu *et al.*, 2014). This category of secondary metabolites, which have been generally termed as ß-exotoxins (example: thuringiensin), are low molecular weight (700-Da) analogues of the nucleotide adenine. The production of ß-exotoxin is more common in some serovars than others (Hermandez *et al.*, 2000); as such, it becomes mandatory that ß-exotoxin should be absent in any *B. thuringiensis* formulations in the US, Europe, Canadian and African formulations (Glare *et al.*, 2000; Gomos-Cebolla *et al.*, 2020).

From the foregoing, *Bti* toxins have a great role to play towards eradicating mosquito larvae; however, to maximize the effectiveness of *Bti* in controlling mosquito populations, optimizing the production of these toxins is crucial. This involves adjusting various growth conditions, such as temperature, pH, salinity, and inoculum volume, to enhance toxin yield. Recent studies have demonstrated that by fine-tuning these parameters, researchers can significantly increase the potency of *Bti* extracts, achieving higher mortality rates in mosquito larvae. For instance, optimizing conditions led to a remarkable increase in toxicity, with some studies reporting very high mortality rates under ideal conditions (Ma *et al.*, 2023).

Even though there had been some studies exploring the optimization of larvicidal toxins from *Bti* before, these studies have not systematically explored the combined effects of temperature, pH, salinity, and inoculum volume on the toxicity of *Bti* extracts, leading to suboptimal conditions being used in larvicidal applications. Moreover, there is insufficient research on how varying environmental conditions, such as salinity and temperature, influence the efficacy of *Bti* against different mosquito species, particularly in natural settings. Additionally, previous studies often focused on single concentrations of *Bti* extracts without assessing the full range of concentrations that could optimize larvicidal activity. To address these gaps, this research employs a systematic approach to optimize multiple parameters (Temperature, pH, salinity, and inoculum volume) simultaneously, rather than in isolation, which is a common limitation in earlier research. Similarly, this study aims to achieve a very high mortality in the mosquito larvae, which may even surpass the mortality rates reported in previous studies (Bahrami *et al.*, 2024). The study's potential contributions to knowledge include providing a comprehensive understanding of how to maximize the effectiveness of *Bti* extracts, which can be applied in various ecological contexts and potentially lead to more effective mosquito control strategies. Moreover, the optimization of the conditions for toxin production can lead to complete mortality of mosquito larvae, thus supporting the potential of *Bti* as a viable alternative to chemical insecticides, which are often harmful to non-target organisms and the environment (Land *et al.*, 2023). Finally, if this study can achieve higher mortality rates under optimized conditions, it can provide a benchmark for future research into the effects of environmental factors on *Bti* efficacy, encouraging more nuanced studies that could lead to improved formulations and application strategies in the mosquito control program.

Therefore, this study was carried out to optimize *Bt* culture conditions from soil samples and determine its toxicity against mosquito larvae.

MATERIALS AND METHODS

Soil sampling

The study was carried out within Katsina State, located between latitudes 11.1303 °N and 13.3825 °N and longitudes 6.8675 °E and 9.1506 °E. Specific areas within Batagarawa and Katsina local government areas were selected for the study. Soil samples were obtained from UMYU Biological Garden, Kofar Durbi Garage, UMYU Agricultural farm and Saulawa quarters, all within Batagarawa and Katsina LGAs, using clean polythene bags. The criterion used for the selection of sampling points was high mosquito infestation due to the presence of stagnant water, thus providing a good environment for the isolation of *Bt* and mosquito larvae. All samples were processed at the Microbiology, Biology and UMYU Central laboratories using standard techniques.

Isolation and screening of *Bacillus thuringiensis (Bt)*

Isolation of *Bt* was done using the heat-shock technique by boiling mixture containing 9 mL of purified water in a test tube and 1gram of soil sample to eliminate all vegetative cells and stimulate *Bacillus* spores (Andrzejczak *et al.*, 2008). Subsequently, 0.1 milliliter of the soil suspension in each tube of the soil was serially diluted up to 10^{-7}, and 0.1 mL was inoculated in plates containing prepared Luria-Bertani agar. The plates were incubated for 24 hours at room temperature where colonies with large, shiny, white, round, irregular margins which spread over the plates were selected as presumptive *B. thuringiensis* (Amin *et al.*, 2015).

Bacteria Identification The screened bacteria were identified using microscopic morphology, relevant biochemical tests (catalase, motility, haemolysis, parasporal, and gelatin hydrolysis) and 16S rRNA molecular analysis (Amin *et al.*, 2015). The extraction of genomic DNA was conducted using the phenol-chloroform method (Mahuku, 2004). Initially, the isolate was sub-cultured on nutrient broth at 37 °C for 18 hrs. After that, a 200 µL content was centrifuged for 5 minutes at 10,000 rpm, the supernatant was disposed and an extraction buffer and 10 µL of lysozyme were added and kept at 37 °C for one hour. Upon that, 20 µL was added to the cell solution along with proteinase K, and it was then incubated at 65°C for one hour to facilitate cell lysis. Subsequently, the phenol-chloroform extraction process was initiated by adding 200 µL of the mixture with a 25; 24; 1 solution of phenol, chloroform and isoamyl alcohol. The mixture was centrifuged for ten minutes at 12,000 rpm, leading to the separation of organic and aqueous phases. The uppermost watery phase, which contained DNA, was then meticulously collected using a pipette and moved to a fresh 1.5 µLmicrocentrifuge tube. Following the addition of 100 µL of cold 70% ethanol to precipitate the DNA, a DNA pellet was formed by centrifuging the mixture for 10 minutes at 12,000 rpm.

This pellet was washed with ethanol to eliminate impurities. After air-drying to remove any remaining alcohol (ethanol)×1, TEdistilled water was used to dissolve the DNA pellet. The extracted DNA Finally, DNA was finally stored at -20°C for preservation. The quality control of the extracted DNA was confirmed using purity assessment through spectrophotometry (Viljoen *et al.*, 2022), and the extracted DNA was checked by running it in an electrophoresis gel. Appearance of DNA on gel with minimal or no protein contamination was observed (Mahuku, 2004). Using the Taq Mix (2×) (Master Mix) and universal primers 27F (forward) and 1426R (reverse), the 16S rRNA gene was amplified by PCR. The Polymerase Chain Reactions were set up individually for each sample, using the following parameters the Annealing at 58 °C for 30 seconds, Denaturation at 95°C for 30 seconds and Extension at 72°C for 30 seconds, repeating steps 2 to 4 for 30 to 35 cycles and completing the last extension at 72°C for five minutes. To verify successful PCR amplification, agarose gel electrophoresis was used to check for the presence of particular amplicons. When agarose powder was dissolved in 1× TAE (Tris-Acetate-EDTA) buffer and heated in a microwave oven, a 1.5% agarose gel was created. To help visualize the molten agarose gel under UV light, 2 µL ethidium bromide was used as a stain. After being poured into a gel casting tray, the gel was left to solidify. For well creation, comb slots were placed at one end of the gel (Mahuku, 2004).

Optimizing *Bacillus thuringiensis* growth conditions

In order to enhance the growth performance of *B. thuringensis* isolates, relevant parameters were selected and optimized. The parameters optimized were temperature, pH, salinity and inoculum volume. The following were the initial isolation cultivation conditions used: temperature: 29 °C, pH: 7.5, salinity: 2% and inoculum volume: 0.1 mL. Each of these conditions was subjected to the Single Factor Optimization (SFO) analyses which allowed the selection of much significant variables that might enhance bacteria performance. For each SFO analysis, the best-performing factor was used to replace the value previously utilized for the ensuing experiments. The individual parameter values used were: Temperature (25°C, 30°C, 35°C, and 40°C), pH (6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0), salinity (0%, 2%, 4%, 6% and 8% w/v), and inoculum volume (1%, 5%, 10%, 15%, and 20% v/v).

Larvicidal mortality assay against Anopheles mosquito using *Bt*

Bacterial (*Bt*) inocula were prepared and quantified using haemocytomeric measurements. The seeds thus prepared were incubated in an orbital shaker at 250 rpm and 30 ^{0}C, for 48 hours (Poopathi, 2012). The effect of *Bt* against Anopheles mosquitoes was determined at the larval third instar stage. The larvae were transferred using a Pasteur pipette into 100 mL capacity beakers containing 40 mL of distilled water. Different concentrations of the bacterial preparations were added to the beakers and mixed to obtain a uniform suspension (Shankar *et al.*, 2016). The control experiment consists of 40 mL of distilled water in a beaker inoculated using the mosquito larvae without *B. thuringensis*. The number of larvae that remained in relation to the original number of larvae was used to calculate the mortality rate among the larvae after 24hours (Monnerat *et al.*, 2005).

Statistical Analysis

The obtained data were analysed statistically using one-way Analysis of Variance (ANOVA) to detect significant differences in the larvicidal activity across the various inoculum sizes. Similarly, regression analysis was done to show the linear relationship between inoculum size and larvicidal activity.

RESULTS

Bacteria isolation, screening and identification

A total of 40 different isolates were screened from the initial isolation process, designated as A1 to D10 (Figure 1).

The pre-screened assays include Gram staining, catalase tests, and colonial morphology, which limited the entire isolates into *B. megaterium, B. pumilis, B. coagulans* and *B cereus* strains. The final screening revealed only 4 isolates to be *Bt* which were further processed. The isolates underwent additional screening by gelatinase, haemolysis on blood agar, motility, Gram's staining, and parasporal body detection. Furthermore, inoculation of mosquito larvae in the enriched nutrient broth culture of the potential isolates so as to obtain possible *B. thuringensis* isolates, based on their morphological growth pattern, and biochemical test results (including streaking on chrome medium, to differentiate *B. thuringiensis* from other species based on blue colour formation, as described in the media manual), Gram's stain and parasporal bodies detection (Table 1). The 16S rRNA results showed 98.08% similarity of the screened isolate to be *B. thuringensis*.

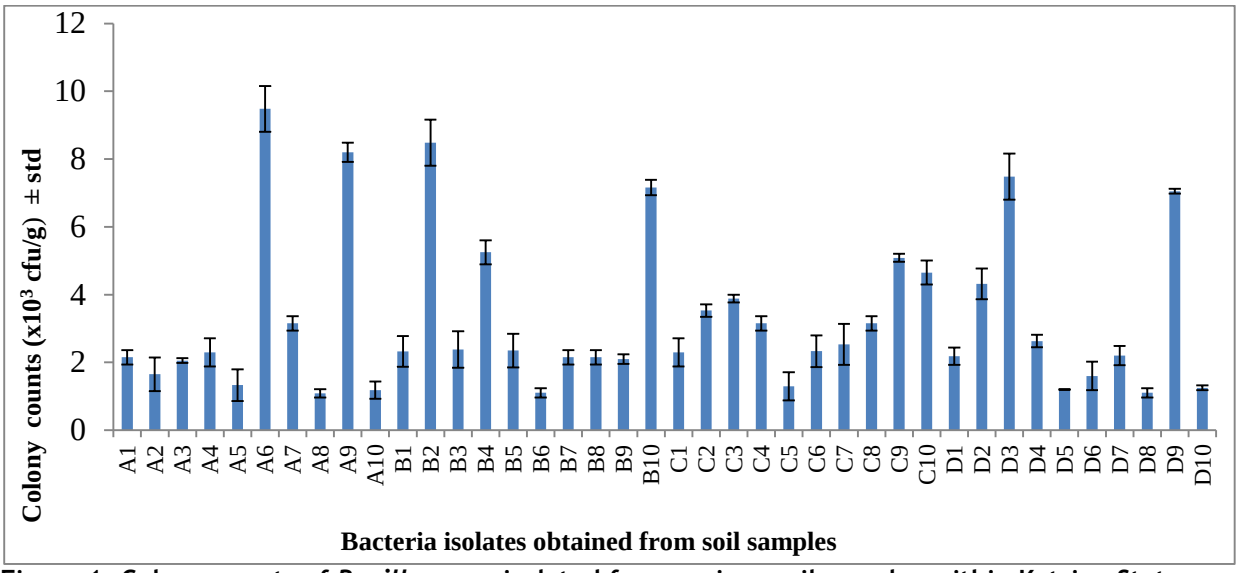

Figure 1: Colony counts of *Bacillus* spp. isolated from various soil samples within Katsina State.

Table 1: Morphology and Biochemical characteristics of the screened *Bacillus* species

Isolate	Colony Morphology	Gram reaction	Biochemical characteristics				Presumed Organisms
			C	M	H	GH	
C2	Tenacious, yellow colored colonies with irregular shape	+ve rods	+	-	-	-	*Bacillus megaterium*
A2	Tenacious, yellow colored colonies with irregular shape	+ve rods	+	-	-	-	*Bacillus megaterium*
A7	Opaque, off white with light filamentous appearance	+ve rods	+	+	-	-	*Bacillus pumilis*
B8	Opaque, off white with light filamentous appearance	+ve rods	+	+	-	-	*Bacillus pumilis*
B9	Opaque, off white with light filamentous appearance	+ve rods	+	+	-	-	*Bacillus pumilis*
B1	Light brown to creamy white colonies	+ve rods	+	-	-	-	*Bacillus coagulans*
A4	Pink or purple flat colonies surrounded by precipitate zone	+ve rods	+	-	+	-	*Bacillus cereus*
A10	Pink or purple flat colonies surrounded by precipitate zone	+ve rods	+	-	+	-	*Bacillus cereus*
A1	Off white and slightly curved colonies with rounded ends	+ve rods	+	+	+	+	*Bacillus thuringiensis*
C1	Off white and slightly curved colonies with rounded ends	+ve rods	+	+	+	+	*Bacillus thuringiensis*

Key: C = Catalase test, M = Motility, G=Gelatin hydrolysis, H=Hemolysis.

Sequencing of 16 rRNA genes

Figure 2 shows the gel electrophoresis results of the sequencing of the PCR products, while the 16S rRNA sequencing results were shown in Table 2.

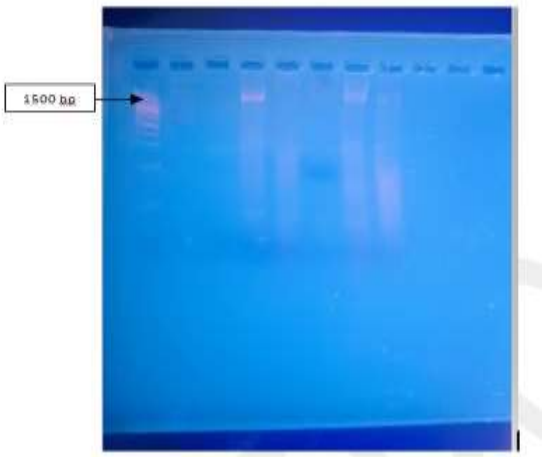

Figure 2: An image of Agarose gel electrophoresis of 16S rRNA gene sequencing that has been amplified for *Bacillus thuringiensis*

Table 2: Summary of the identified *Bacillus thuringiensis*

Sample ID	Prediction (%)	GenBank Accession No.	BLAST Predicted Organism
A1	98.08%	PP724433.1	*Bacillus thuringiensis* strain DKV_Bt_SAk-12

Physicochemical Parameters optimization

During temperature optimization, there was no colony development at 20 ^{0}C. However, colony development at 25 $^\circ$C reached 2.88 × 10^3 ± 0.07 CFU/mL, while 30 ^{0}C recorded the highest counts of 3.62 × 10^3 ± 0.02 CFU/mL. This was observed to start decreasing to 2.79 × 10^3 ± 0.01 CFU/mL due to increasing heating effects at 35 ^{0}C, while the least count of 1.10 × 10^3 ± 0.15 CFU/mL was recorded at 40 ^{0}C. Therefore, the temperature value of 30 ^{0}C was selected as the optimum (Figure 1). Results of pH optimization showed that at pH 6, the colony count was 2.05 × 10^3 ± 0.07 CFU/mL. This gradually increased to a maximum value of 2.60 × 10^3 ± 0.03 CFU/mL at pH 7.5, before decreasing to the lowest value of 0.99 × 10^3 ± 0.09 CFU/mL at pH 8.5. No growth was obtained at pH 9. Therefore, pH 7.5 was selected as the optimum. During salinity optimization, the highest colony count of 2.01 × 10^3 ± 0.01 CFU/mL was obtained at 0% w/v. The colony counts thereafter decreased with increasing salinity, up to 1.02 × 10^3 ± 0.03 CFU/mL at 4% w/v. By the time the salinity concentration reached 6% w/v, the isolates failed to growth. During the inoculum volume optimization, there was a uniform increase in bacterial colony count as the inoculum volume increased. At 1% v/v, the colony counts were 0.19 × 10^4 ± 0.002 CFU/mL, which increased to 0.53 × 10^4 ± 0.01 CFU/mL when the inoculum volume was increased to 10% v/v and the highest colony count of 2.52 × 10^4 ± 0.04 CFU/mL was obtained at an inoculums volume of 20% v/v. Therefore, the optimum conditions achieved involved a temperature of 30 ^{0}C, pH value of 7.5, Salinity of 0% w/v and inoculum volume of 20% v/v.

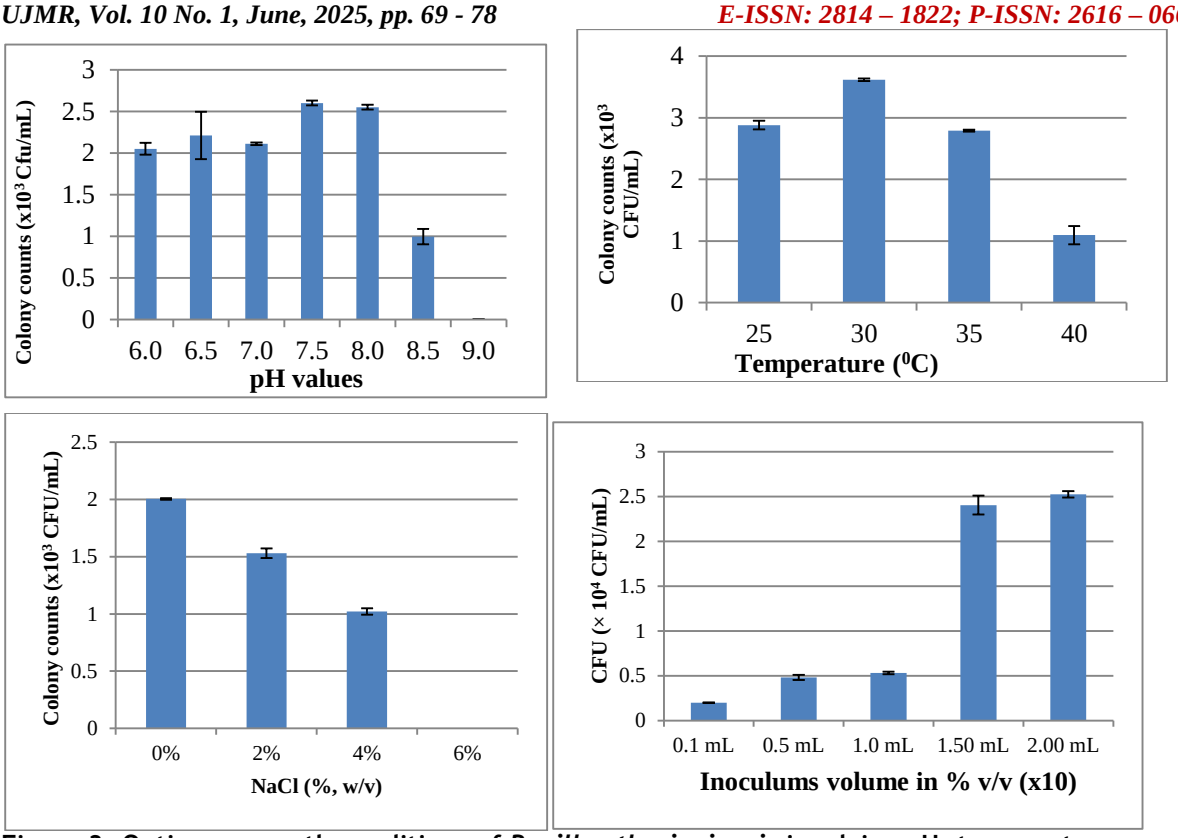

Figure 3: Optimum growth conditions of *Bacillus thuringiensis* involving pH, temperature, salinity and inoculum volume

Larvicidal activity against the *Anopheles* mosquito using the optimized *Bacillus thuringiensis*

After the larvicidal test was conducted, the highest percentage mortality was obtained when the inoculum volume was 2 mL, leading to 85% and the lowest was obtained from the inoculum volume of 0.125 mL, leading to 50% mortality (Table 3).

Table 3: Mosquito Larvae test using Optimized *Bt*

Inoculums Volume (%, v/v)	% Mortality	P-value
1	48.33 ± 7.64	0.0002
5	65 ± 8.66	
10	78.33 ± 2.89	
20	88.33 ± 2.87	

NB: $\%\ Mortality = \frac{number\ of\ dead\ larvae}{number\ of\ initial\ larvae} \times 100$

Similarly, the results of the regression analysis showed a linear relationship between the amount of inoculum and percentage mortality, as governed by the equation: y = 1.9966x + 52.028, with an R^2 value of 0.8948.

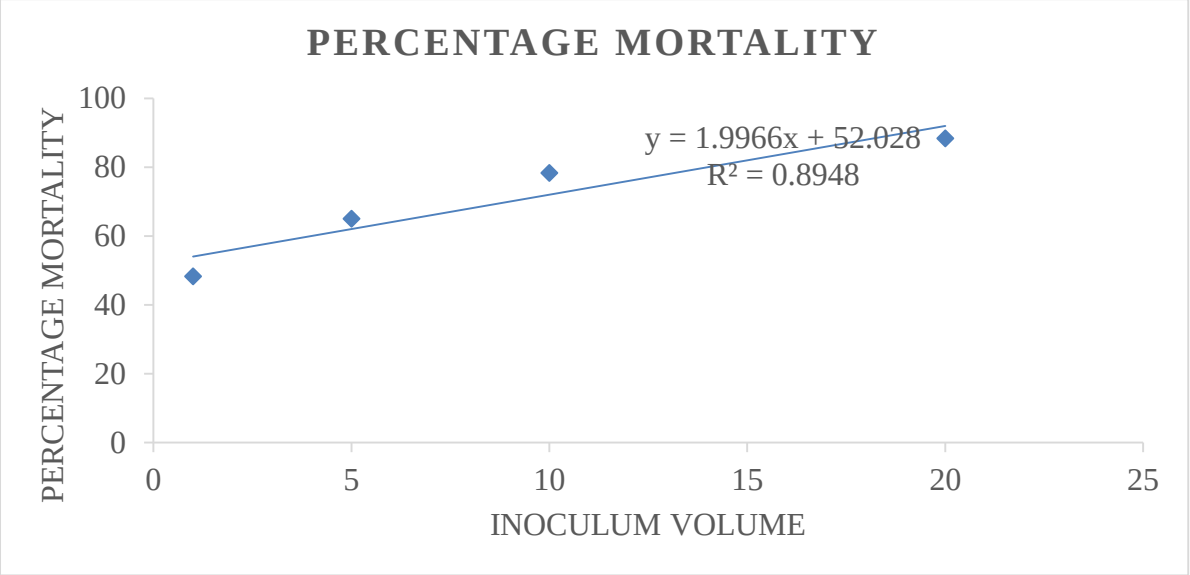

Figure 4: Regression analysis of the relationship between mortality and inoculum volume

DISCUSSION

Previous research had identified these isolates from garden soil (Nazina *et al.*, 2001), garage (Toledo *et al.*, 2006), and gutter (Hassan *et al.*, 2021). Applying various assays, such as parasporal body detection, has been employed by previous researchers to isolate *Bacillus thuringiensis* from the soil (Hassan *et al.*, 2021). The isolation of these bacteria from the places mentioned can be linked to their spore formation ability, which aids their survival in various forms of environmental stress.

In oil-contaminated, rhizosphere and garden soils, Bacilli are known to exist in a number of beneficial interactions with other microbes. On the whole, genetic factors and transfer of mobile genetic elements facilitate the adaptability, survival and colonization of Bacilli in these soil ecosystems (Bishop *et al.*, 2014).This indicates its ability to withstand oxidative stress due to H_2O_2 (catalase), move around the environment to search for nutrients, colonise or escape unfavorable conditions (motility), and secrete a variety of lytic enzymes that can degrade substrates, which may be used for the nutrition of the isolates (haemolysis and gelatinase).

The survival of *Bacillus thuringiensis* was optimized at 30 °C. This is associated with the optimal range for enzymatic activity and protein functioning, above or beyond which cell growth may be slowed or even terminated. A previous research had also indicated that the best growing temperature is between 30-35 °C for *Bacillus thuringiensis* (Ibrahim *et al.*, 2010). Similarly, the optimum pH of 7 is linked to the fact that *Bacillus* is a neutrophile. Around this pH, various enzymes and transport systems function optimally; however, abnormally acidic/alkaline conditions can compromise living cells or even kill the bacteria totally (Bravo *et al.*, 2011; Haruna *et al.*, 2014).

Moreover, *Bacillus thuringiensis* is not a halophile; therefore, it survives best at a salinity of 0%. However, it may tolerate low (up to 4%) salinity concentrations. This observation is linked to the fact that the growth and sporulation of this bacterium are better at lower salinity, since higher salinity is associated with more energy expenditure to maintain cellular homeostatic (da Costa *et al.*, 2020). Finally, the fact that higher inoculum size (2 mL) is associated with better growth and larvicidal activity in these bacteria is due to the introduction of a higher initial population of bacteria, leading to faster establishment and activity, in the presence of enough nutrients and low competition (Trisyono *et al.*, 2023).

Bacillus thuringiensis is known to produce toxins to target insect pests, including Cry and Cyt toxins. This property facilitates the virulence of the bacterium, and helps it invade, perforate and colonise the insect host. This property is enabled by its possession of genetic mechanisms for elaborating toxins (Patel *et al.*, 2019). The high killing effect exerted by *Bacillus thuringiensis* against the larvae in this study points to its capacity to generate endotoxins, which can be employed to manage insect pests, giving it enormous potential as a biological control agent. The molecular mechanism of toxicity and the variables influencing the high specificity of the toxins produced by this bacterium have been the subject of numerous investigations (Duarte Neto *et al.*, 2020).

The mortality rates obtained in the current study showed a trend of higher inoculum volumes leading to increased mortality aligns with

findings from various global studies. A study conducted in Thailand evaluated the effects of different inoculum volumes of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) on *Anopheles minimus*. Researchers observed that an inoculum volume of 1 mL resulted in 50% mortality, while 10 mL achieved 80% mortality. These results are consistent with the current study, indicating that larger inoculum volumes enhance mosquito mortality. Similarly, in India, researchers assessed the impact of varying inoculum volumes of *Bti* on *Anopheles stephensi*. They reported mortality rates of 45% with a 1 mL inoculum and 85% with a 20 mL inoculum. This study corroborates the current findings, demonstrating a positive correlation between inoculum volume and mosquito mortality (Poopathi & Archana, 2012).

A Brazilian study investigated the relationship between inoculum volume and mortality in *Anopheles darlingi*. The researchers found that a 5 mL inoculum resulted in 60% mortality, while a 15 mL inoculum led to 90% mortality. These findings further support the trend observed in the current study. Moreover, an Indonesian study focused on *Anopheles sundaicus* and reported mortality rates of 50% with a 1 mL inoculum and 87% with a 20 mL inoculum. These results are in agreement with the current study, highlighting the efficacy of increased inoculum volumes (Masri *et al.*, 2020).

Moreover, optimizing physicochemical parameters is crucial for maximizing the production of Cry and Cyt toxins by *Bti*. In the current research, optimal conditions were identified as a temperature of 30°C, pH 7.5, 0% w/v salinity, and an inoculum volume of 20% v/v. These conditions likely facilitated enhanced bacterial growth and toxin production. Studies have shown that factors such as carbon and nitrogen sources, mineral salts, and pH significantly influence δ-endotoxin synthesis. For instance, the presence of starch and soybean meal as carbon and nitrogen sources, respectively, along with specific concentrations of FeSO and MgSO, has been found to increase δ-endotoxin production. Additionally, maintaining optimal pH and temperature conditions ensures the stability and activity of the toxins produced (Ennouri *et al.*, 2015). Statistically, the results showed that inoculum volume significantly affects the % mortality ($p = 0.0002$, $F_{cal} = 24$, $F_{crit} = 4.07$).

Eco-friendly pest control, particularly in vector management, is increasingly recognized as a sustainable alternative to chemical pesticides, with *Bacillus thuringiensis israelensis* (*Bti*) emerging as a key biological agent for mosquito control. In Nigeria, regulatory policies support integrated vector management (IVM) that emphasizes the use of biological control methods, with the National Malaria Elimination Programme promoting environmentally friendly strategies to combat malaria transmission. The regulatory framework mandates rigorous safety and efficacy evaluations for new vector control products, ensuring they do not pose risks to human health or the environment. Internationally, organizations like the World Health Organization (WHO) and the Environmental Protection Agency (EPA) have established guidelines that endorse the use of *Bti*, highlighting its safety for non-target organisms and humans. Real-world applications of *Bti* have demonstrated its effectiveness in various settings, including urban areas where aerial spraying has successfully reduced mosquito populations without harming beneficial species. Additionally, *Bti* is utilized in agricultural practices to manage mosquito larvae in irrigation systems, contributing to sustainable farming. Public health campaigns, such as those in Puerto Rico, have integrated *Bti* into their strategies to combat mosquito-borne diseases like Zika and dengue, showcasing its potential to significantly reduce disease transmission. Overall, the combination of supportive regulatory policies and successful real-world applications positions *Bti* as a vital component of eco-friendly pest control strategies (Land *et al.*, 2023; McKie *et al.*, 2023).

CONCLUSION

The molecularly identified *B. thuringiensis* strains from gutter, oil-contaminated, garden and rhizosphere soils were found to grow best at the optimised conditions of: pH of 7.5, temperature of 30°C, salinity of 0% w/v and inoculum volume of 20% v/v, which were able to record 85% mosquito larvicidal effect within 24 hours of inoculation, a significant improvement upon the initial results of 50% larvicidal effect. The study is, however, limited because laboratory conditions may show variation compared to field conditions; similarly, variation in bacterial strains may lead to changes.

It is recommended that the timing and frequency of *Bti* applications should be linked to mosquito breeding sites to ensure effectiveness, likewise the application of *Bti* in appropriate formulations (liquid, briquette, powder or granular) should be based on the specific breeding sites identified, and upon the application of the *Bti* treatments, the treated sites should be regularly monitored to evaluate the effectiveness of *Bti* in reducing mosquito larvae populations.

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