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Biocontrol Potential Of Bacterial Isolate From River Environment Against Mosquito Larvae

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ABSTRACT

Mosquitoes serve as carriers for several illnesses, including malaria, which causes millions of deaths annually. Overcoming pesticide resistance remains a hurdle in achieving successful mosquito control. The ability of bacterial strains to impede the growth and development of mosquito larvae was assessed after they were isolated from samples of river water. The study isolated bacterial strains from water samples using serial dilution, analyzing their effects on mosquito larvae behavior and health. The mortality rate of larvae was measured and counted at each time interval. The study compared bacterial supernatants larvicidal efficiency with commercially available Bti formulations. The study aimed to determine the presence of anti-larval compounds. According to laboratory tests, four isolates considerably decreased larval survival rates, attaining over 85% mortality within 48 hours of exposure. The study promotes the shift from chemical-based strategies to more sustainable ones by incorporating these biological control techniques into already-existing frameworks, such integrated pest management (IPM). The study suggests that indigenous aquatic bacterial populations can serve as effective biocontrol agents against mosquito larvae, offering a promising alternative to chemical insecticides and reducing the transmission of mosquito-borne diseases.

Keywords: Mosquito larvae, Indigenous aquatic bacteria, Mosquito-borne diseases, Integrated Pest Management (IPM), Eco-friendly pest control, Larval survival rate.

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INTRODUCTION

Mosquitos are harmful vectors of infectious diseases like malaria, dengue, chikungunya, and zika, causing millions of deaths annually. In India, several vector-borne diseases pose significant risks to its population, including malaria, dengue, chikungunya, visceral leishmaniosis, Japanese encephalitis, and lymphatic filariasis. India contributes the highest number of malaria cases (79%) in the WHO's South East Asia region, with approximately 698 million people at risk [1]. India reported four Zika virus (ZIKV) cases in 2016-17, with widespread Aedes mosquito distribution and indigenous transmission indicating potential future large-scale outbreaks, potentially affecting 465 million people, including Gujarat and Tamil Nadu [2]. Uncontrolled urbanization is increasing mosquito-borne diseases, with Chikungunya experiencing a surge in cases. Kala-azar disease and lymphatic filariasis (LF) are endemic in Bihar, Uttar Pradesh, Jharkhand, and West Bengal, necessitating mosquito control to prevent further spread [3]. Mosquitoes pose a global threat to millions of people, posing a significant threat to human health and the environment. Traditional control methods, including organophosphates, insect growth regulators, and microbial agents, can negatively impact mosquito populations. Innovative tools, such as behavior-based strategies and plant-derived mosquitocidals, are being developed for eco-friendly control [4]. Despite the widespread use of chemical pesticides and personal avoidance, chemical resistance has resulted in a return of pests and detrimental impacts on nontarget creatures, the environment, and human health. The low toxicity of microbial pesticides to non-target animals and humans makes them an essential component of integrated pest control [5]. An important insect pathogen that is extremely harmful to mosquito larvae and similar dipterans is *Bacillus thuringiensis*[6][7]. *Bacillus thuringiensis* is selectively active against pests and unlikely to develop resistance, it is seen as advantageous for people, animals, and plants. In many nations, it is also a good substitute for conventional pesticides. Therefore, the prevalence of *Bacillus thuringiensis* in nature is evident. However, dirt is the organism's typical environment. The creature feeds on dead organic debris and develops spontaneously as a saprophyte. As a result, *Bacillus thuringiensis* spores remain in soil and develop vegetatively when nutrients are available. Additionally, *Bacillus thuringiensis* has just been discovered in maritime habitats [8]. The *Bacillus thuringiensis (Bt)* preparation is the most commonly used microbial pesticide in the world.

Research on bacterial species in river ecosystems is limited, but they offer a natural reservoir of microbial diversity, including bacteria with potential larvicidal properties. River ecosystems are home to microorganisms that play vital roles in nutrient cycling, organic matter decomposition, and ecological balance. They can yield bacterial species with insecticidal properties, shaped by ecological factors like nutrient availability and temperature. Studies have found bacterial strains from river environments with larvicidal activity against mosquito larvae, suggesting they are an underexplored source of novel biocontrol agents. These strains can target insect gut cells, disrupt physiological processes, or produce inhibitory compounds. Understanding these mechanisms is crucial for evaluating their potential as biocontrol agents, as they offer unique mechanisms and improved efficacy against resistant mosquito populations. Integrating bacterial biocontrol agents into mosquito management could provide a sustainable solution. This research work focused on summarizing available control strategies for mosquito vectors of public health importance. The study was based on the microorganisms present in the river environment other than *Bacillus thuringiensis israelensis (Bti)* that can act as a biocontrol agent against the mosquito larvae to prevent the hazardous diseases caused by them.

METHODOLOGY

Collection of Water Sample

Water samples were collected from pond and river environments in Pune. The water was collected in plastic bottles and transported to the laboratory [9].

Isolation of Bacterial Isolates

The serial dilution method was used to isolate bacterial strains from the water samples. 0.5 ml of each collected water sample was serially diluted in 4.5 ml of autoclaved saline water. to 10^{-5}. 0.1 ml from the last two dilutions was spread on Nutrient Agar (NA) plates and incubated at 37°C for 24 hours. Bacterial growth was observed on the plates after incubation. Four morphologically different isolates labelled C1, C2, C3, and C4, were initially selected and sub-cultured on NA plates and NA slants [10].

Identification and characterization of bacteria

The bacterial isolates were identified and characterised based on their colony, morphological, biochemical characters. Gram Staining, Catalase Test, Oxidase Test, Sugar fermentation Test, IMViC Tests, Nitrate Reduction Test, Gelatin Liquefaction Test, and Starch Hydrolysis Test were performed for the same [11].

Screening for larvicidal activity

Four bacterial isolates (C1, C2, C3, and C4) were inoculated into 100 ml sterile Luria Bertani (LB) Broth and incubated on a rotary shaker (100 rpm) for 48 hours. After incubation, cultures were centrifuged at 6000 rpm for 10 minutes at 4°C to obtain a cell free supernatant. This supernatant containing crude insecticidal toxins was collected for further analysis [12].

Qualitative assay

To observe changes in mosquito larvae behaviour and health after exposure to bacterial isolates, third instar mosquito larvae were used. Five larvae were placed in bumper tubes containing 10 ml distilled water, and 1 ml of each bacterial isolate supernatant was added to the respective tube. A negative control with only distilled water and larvae was maintained. Larval behaviour was continuously observed and recorded after 1, 4, and 8 hours for signs of erratic swimming, floating, curling, or colour changes.

Quantitative assay

To measure the mortality rate of mosquito larvae, the supernatants from isolates C1 to C4 were diluted in sterile saline to obtain 100%, 75%, 50%, and 25% concentrations in a total of 5 ml volume. Larvae were exposed to these dilutions to determine dose-dependent mortality. Third instar mosquito larvae were used for this bioassay. Five larvae were placed in bumper tubes containing 10 ml of distilled water. A negative control with only distilled water and larvae was maintained. 5 ml of bacterial supernatant at different concentrations (100%, 75%, 50%, and 25%) was added to each tube. The contents were gently mixed to ensure even distribution of the bacterial supernatant in the bumper tubes. The assay was performed in duplicates. The mortality of mosquito larvae was observed after 1 hour, 4 hours, and 8 hours of exposure to the bacterial supernatant. The number of dead larvae for each bacterial isolate (C1, C2, C3, and C4) was counted at each time interval, and the data were recorded.

Comparative analysis with the commercial larvicidal compound

The larvicidal efficiency of the bacterial supernatants was compared with that of commercially available *Bacillus thuringiensis israelensis* (Bti) formulation. Comparative analysis was performed using the supernatant from isolate C2. 1, 2, 3, 4 drops of *Bacillus thuringiensis israelensis* (Bti) formulation was added to the tubes containing larvae. The same protocol mentioned in the quantitative assay was followed this analysis [13]. To check the larvicidal efficacy of each dose response, the lethal concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}) was calculated by using the given formula:

Mortality calculation

$$\text{Mortality Rate (\%)} = \frac{\text{Number of dead larvae}}{\text{Total number of dead larvae}} \text{ X } 100$$

RESULTS

Isolation and Identification of Bacterial Isolates

After spreading water sample dilution on sterile nutrient agar plate growth was observed on few plates. Bacteria were isolated from river bank samples using serial dilution and spread plating. Four distinct colonies (C1, C2, C3, C4) were selected based on morphological differences (Figure 1). And were further streaked on sterile nutrient agar plates to obtained pure isolates.

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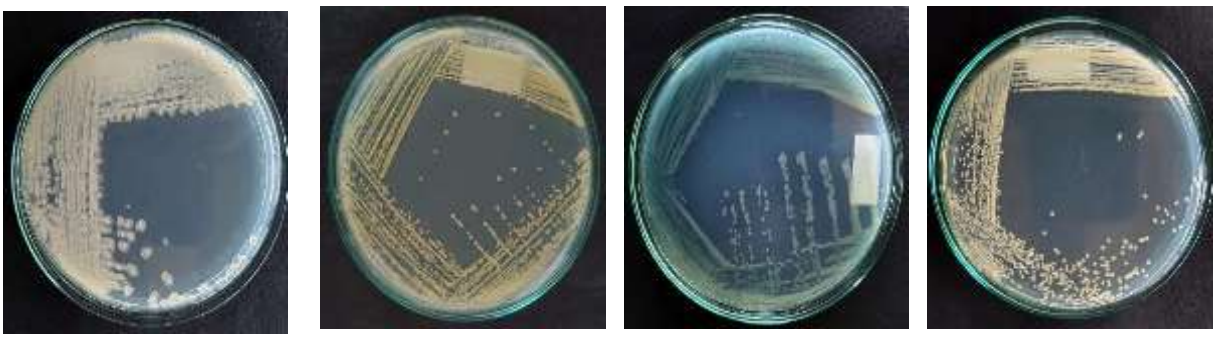

a. Colony 1 b.Colony 2 c. Colony 3 d. Colony 4

Figure 1: Bacterial colonies on Nutrient agar media of C1, C2, C3 and C4 isolates

Characterization and identification of bacteria isolates

The colonies of all four isolates were circular and small. C3 was green in color and showed Gram negative, rods shaped character. Rest all isolates were Gram positive. All colony characters along with their Gram nature and motility for all the four isolates are depicted in the Table 1

Table 1: Colony characters of all isolates

Colony	C1	C2	C3	C4
Size (mm)	2	1	1	1
Shape	Circular	Circular	Circular	Circular
Color	Creamy white	Yellow	Green	Creamy white
Margin	Entire	Regular	Entire	Regular
Elevation	Convex	Concave	Flat	Concave
Opacity	Opaque	Opaque	Translucent	Opaque
Consistency	Sticky	Sticky	Sticky	Sticky
Gram character	Gram positive rods	Gram positive cocci	Gram negative short rods	Gram positive cocci
Motility	Motile	Non-motile	Motile	Non-motile

*Note: (C1-Isolate 1, C2-Isolate 2, C3-Isolate 3, C4-Isolate 4)

Table 2: Catalase and oxidase test for all isolates

Colony	**Catalase**	**Oxidase**
C1	**+**	**+**
C2	**+**	**+**
C3	**+**	**+**
C4	**+**	**-**

*Note: [(+) = Positive, (-) = Negative]

As seen in Table 2, C1, C2, and C3 isolates was able to produce cytochrome oxidase enzyme while isolate C4 was not able to produce cytochrome oxidase. Hence oxidase test is positive for isolates C1, C2,

C3 and negative for C4 isolate. These organisms were able to produce catalase enzyme. Hence catalase test was positive for C1, C2, C3 and C4.

Fermentation test

Isolate C1 showed positive acid production for all tested sugars including mannitol, sucrose, maltose, glucose, galactose, and lactose, but no gas production was observed**.** Isolate C2 did not ferment glucose or lactose, as indicated by the absence of both acid and gas production as seen in Table 3. Isolate C3 fermented glucose, sucrose, and xylose with acid production but did not produce any gas. Isolate C4 fermented most of the tested sugars, including mannitol, lactose, maltose, sucrose, fructose, cellobiose, and xylose, showing acid production in all, but did not ferment galactose or arabinose, with no gas production.

Table 3: Sugar fermentation tests for C1, C2, C3 &C4

	Isolate C1		Isolate C2		Isolate C3		Isolate C4	
Sugars	**Acid**	**Gas**	**Acid**	**Gas**	**Acid**	**Gas**	**Acid**	**Gas**
Mannitol	+	-					+	-
Sucrose	+	-			+	-	+	-
Maltose	+	-					+	-
Glucose	+	-	-	-	+	-		
Lactose	+	-	-	-			+	-
Galactose							-	+
Fructose							+	-
Arabinose							-	+
Cellobiose							+	-
Xylose					+	-	+	-

*Note: (+) - Positive, (-) – Negative

Identification of the bacterial isolates

Bacterial isolates were characterized and identified depending on their colony characters, Gram characters and biochemical tests. Using Bergey's manual of determinative Bacteriology the genus were identified as C1 as *Bacillus,* C2 as *Micrococcus,* C3 as *Pseudomonas and* C4 as *Staphylococcus* (Table 4). The species of C2 isolate, which produced the most efficient and potent larvicidal compound was further identified using MALDI-TOF out sourced from Agharkar Research Institute as ***Micrococcus endophyticus.*** The results obtained are presented in the Table 5.

Table 4: Identification of isolates

Colony No.	Genus
C1	*Bacillus sp.*
C2	*Micrococcus sp.*
C3	*Pseudomonas sp.*
C4	*Staphylococcus sp.*

Table 5: MALDI Identification of C2

Sr. No.	Culture Code	Closest match	Score value
1.	C2	***Micrococcus endophyticus***	2.09

Four isolated bacterial colonies were inoculated in 100ml of Luria Bertani broth each respectively and incubated the culture with constant shaking (100 rpm) for 48 hours to promote growth and toxin production. After the incubation period, the culture was centrifuged at 6,000 rpm for 10 minutes at 4°C to pellet the bacterial cells.

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Screening for larvicidal activity

Qualitative Assay – Larval Mortality Test

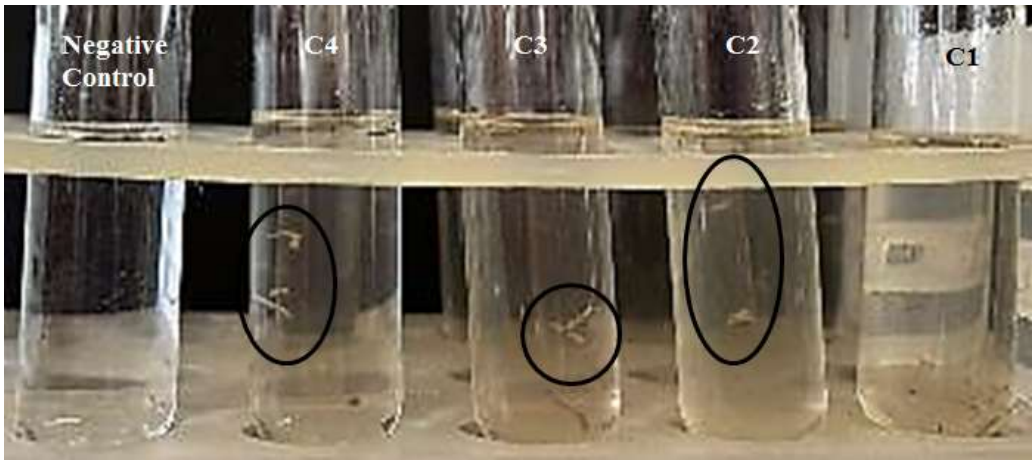

Figure 2: Bioassay setup for exposure of mosquito larvae to bacterial supernatant

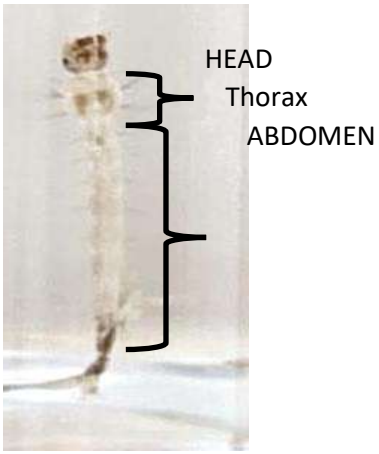

Figure 3: Mosquito larvae before exposure to bacterial supernatant

For screening of bacterial larvicidal activity 4 bumper tubes were taken, to each tube 5 mosquito larvae and 1ml of supernatant of C1, C2, C3 and C4 isolates was added (Figure 2). Changes in larval behavior due to the anti-larvicidal activity of the supernatant were observed after incubation of 1, 4 and 8 hours. Also, the number of dead larvae was recorded. After 1 hour supernatant of C2 could kill all the five larvae while other three supernatants could kill 2, 3, 1 respectively, as mentioned in Table 6 for each isolate.

Table 6: Larvicidal activity of all isolates against mosquito larvae

Isolates	Total number of larvae	1 hour		4 hours		8 hours	
		Number of live larvae	Number of dead larvae	Number of live larvae	Number of dead larvae	Number of live larvae	Number of dead larvae
C1	5	5	0	5	0	3	2
C2	5	0	5	0	5	0	5
C3	5	5	0	5	0	2	3
C4	5	5	0	5	0	4	1

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Larvae exposed to C2 isolate showed behavioral changes such as sluggish movement, erratic swimming visible distress, reduced activity, color change or mortality compared to the control group**.**

According to the data (Table 6), after exposure to bacterial supernatant C1 isolates is less effective as the time took was 8 hours to kill the mosquito larvae. C2 isolates is more effective as the time took was 1 hour to kill the mosquito larvae. C3 isolates is less effective as the time took was 4 hours to kill the mosquito larvae. C4 isolates is less effective as the time took was 8 hours to kill the mosquito larvae. Therefore, C2 isolates is effective so C2 isolates were processed further. Behavioural and physical changes in mosquito larvae were also noted after exposure to bacterial supernatant. Color change, neck / abdomen / appendages breakage settling down at the bottom was observed as the effect of the larvicidal activity of the bacterial supernatant. These changes are depicted in the Figure 4 and were compared with the unexposed larvae (Figure 3).

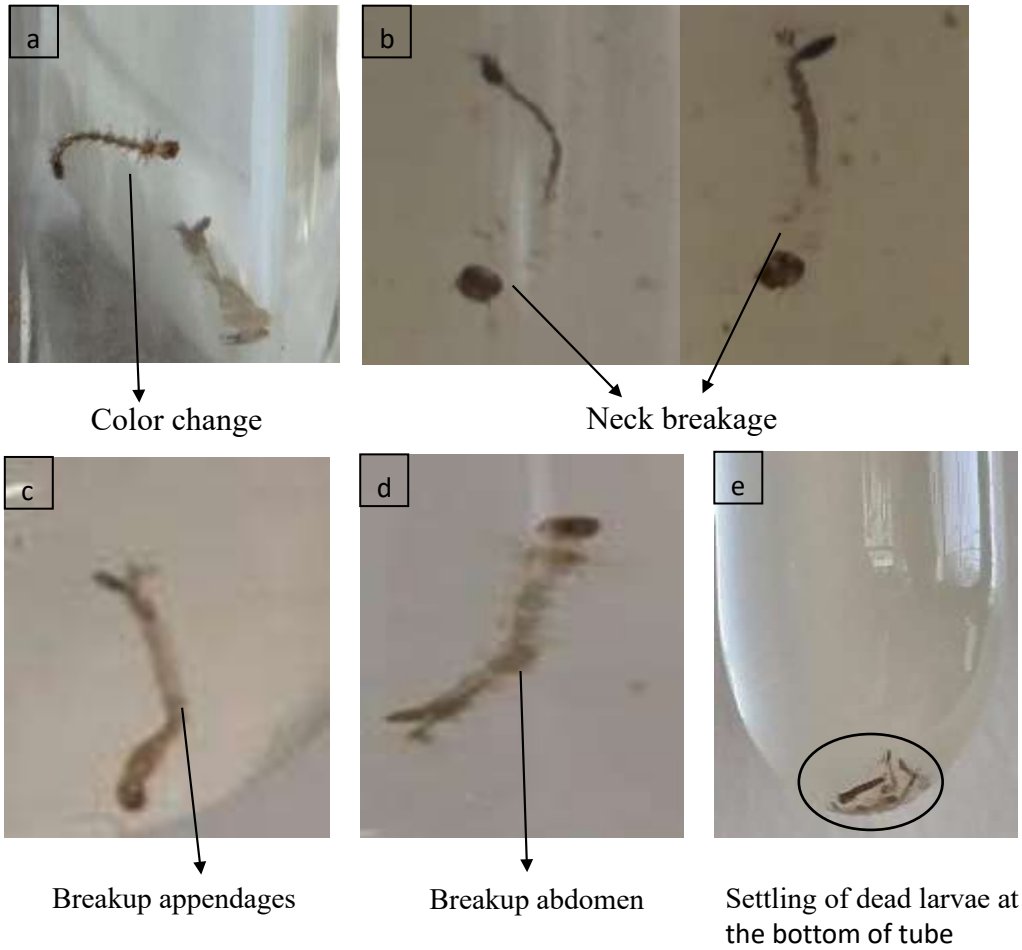

Figure 4: Mosquito larvae after exposure to C2 supernatant

Quantitative assay

The bacterial supernatant was diluted using sterile saline to obtain 25%, 50%, 75% and 100%. To check the larvicidal efficacy, mortality rate was calculated by using the formula given below.

Mortality Calculation

$$\text{Mortality rate (\%)} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

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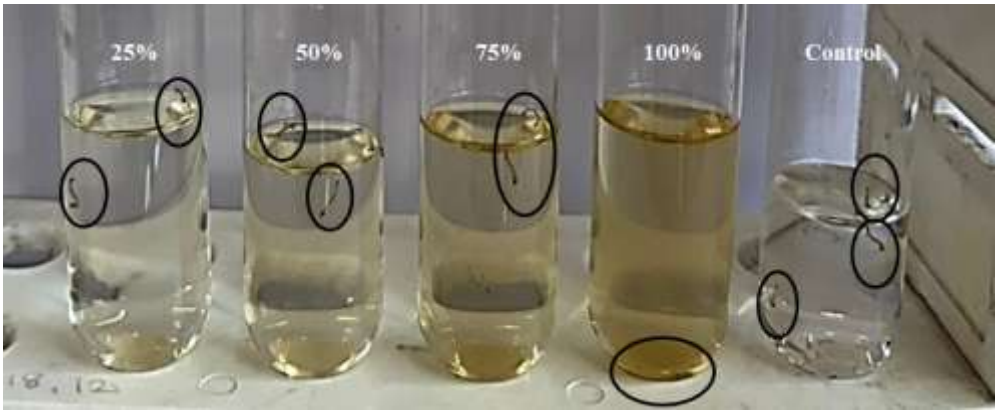

Figure 5: Exposure of larvae to different concentration of C2 bacterial supernatant

Table 7: Larvicidal activity of isolate C2 against mosquito larvae in 1 hours

Sr. No	Concentration	Initial Number of larvae	Number of dead larvae	Mortality Rate (%)
1.	100%	5	5	100
2.	75%	5	3	60
3.	50%	5	2	40
4.	25%	5	1	20

A positive correlation between the concentration of bacterial supernatant and the mortality rate was observed (Figure 5). 100% concentrated supernatant indicated a strong lethal effect with all five larval death (Table 7). This suggests that the bacterial supernatant has a dose dependent toxic effect, where increasing its concentration leads to a higher mortality rate.

Comparative analysis

The Comparative analysis of the larvicidal activity of *Bacillus thuringiensis israelensis* (Bti) formulation available in the market with the supernatant of the isolated bacteria against the mosquito larvae was performed by exposing five larvae to both solutions individually.

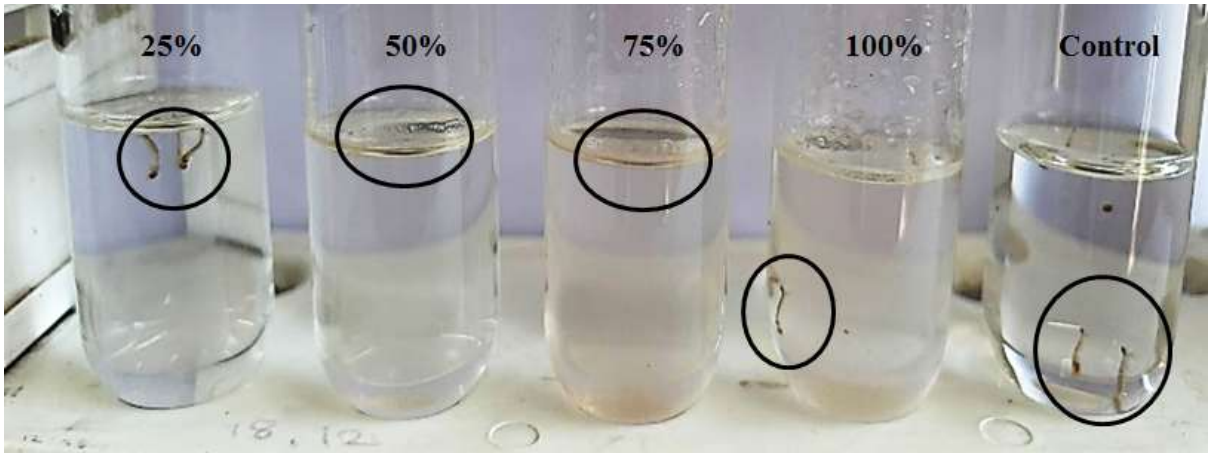

Figure 6: Exposure of larvae to different concentration of Bti

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Table 8: Larvicidal activity of Bti against mosquito larvae

Sr. No	Concentration (%)	Initial Number of Larvae	Number of Dead Larvae	Mortality Rate (%)
1	100	5	5	100
2	75	5	3	60
3	50	5	3	60
4	25	5	2	40

Bacillus thuringiensis Israelensis (Bti) formulation was effective at 100% concentration at 1 hour to kill all five larvae (Figure 6) but at lower concentrations of 50 and 25% it was less effective than the C2 supernatant. Bti could kill 2 and 3 at 50 and 25% (Table 8), whereas, C2 supernatant was potent to kill 3 and 4 larvae at the same concentration (Table 7). This suggests that the supernatant in its purified form can prove to be highly potent and efficient than the currently available formulations.

Identification of the nature of anti-larval compound by Thin Layer Chromatography

Thin Layer Chromatography (TLC) analysis of the samples labelled LB, C2, H, and Bti reveals distinct differences in compound separation, as observed from the varying intensities and R_f values of the developed spots (Figure 7). The samples on TLC plate developed with ninhydrine showed similar purple-pinkish spots as that of the Histidine, including the C2 supernatant, while the TLC plate developed with benzidine displayed brown colored spot only for xylose, confirming that the larvicidal compound present in the supernatant was proteineous in nature and not a carbohydrate.

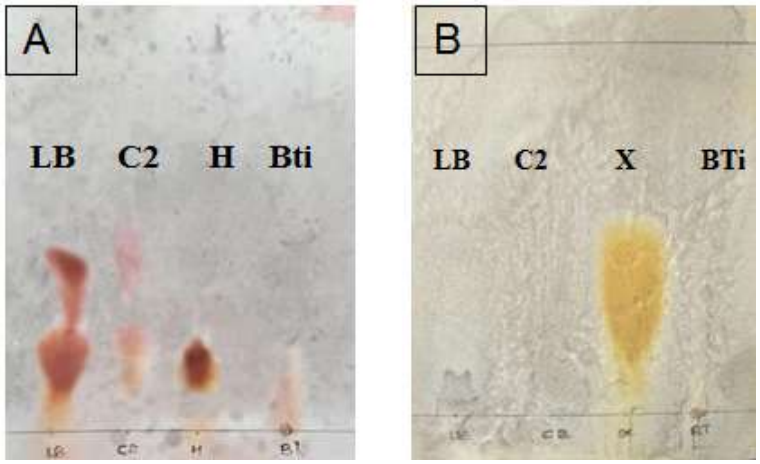

Figure 7: Thin layer chromatography A) TLC developed with Ninhydrine B) TLC developed with Benzidine. LB - Sterile uninoculated Luria broth media, C2 - Colony isolate 2 supernatant, H - Histidine (internal control), *Bti* - *Bacillus thuringiensis israelensis* formulation, X – Xylose sugar

DISCUSSION

The study evaluated the larvicidal potential of bacterial isolates from river water sources against mosquito larvae, finding that *Micrococcus endophyticus* (C2) demonstrated significant activity, achieving over 85% mortality within 1 hour.

Previous research, conducted by two groups [9] [12], demonstrated that Bt strains isolated from soil samples caused rapid and high larval mortality. In those studies, mortality rates of 100% were observed within 4 to 12 hours at higher concentrations. Similarly, [3] confirmed that soil-derived Bt isolates exhibited strong larvicidal activity, with complete mortality achieved within 12 hours across several dilutions. In contrast, in the present study, the isolates from river water required shorter exposure was exhibited to achieve comparable mortality rates; their overall effectiveness was evident and reinforces the potential of aquatic ecosystems as a source of potent biocontrol agents. This aligns with findings from [11] , who confirmed that Bt isolates produce endotoxins, particularly delta-endotoxins

(Cry and Cyt proteins), which become active in the alkaline environment of mosquito larvae midguts. Importantly, the study included a comparative assay with a commercially available *Bacillus thuringiensis israelensis* (Bti) formulation. While Bti induced high mortality, the C2 isolate from this study performed similarly under laboratory conditions. This observation reinforces the potential of using local, naturally occurring bacterial strains as biocontrol agents, offering a sustainable and cost-effective alternative to commercial products. Studies by [5], strongly advocate for transitioning to biological control methods, highlighting Bt as a preferred microbial pesticide due to its specificity, safety, and minimal ecological impact. Our findings directly contribute to this narrative, showcasing the viability of river-derived bacterial isolates as eco-friendly larvicides. Interestingly, while most existing studies have focused on isolating Bt from soil, compost, or agricultural environments, this study adds value by sourcing bacteria from river ecosystems— an underexplored microbial reservoir. Rivers and other freshwater bodies host diverse microbial communities influenced by seasonal changes, nutrient availability, and aquatic biodiversity. These factors may encourage the evolution of novel bacterial strains with unique insecticidal properties. The successful isolation and characterization of potent bacterial isolates from river water in this study highlight the untapped potential of such environments. Furthermore, the study aligns with global public health goals aiming to reduce the transmission of mosquito-borne diseases such as malaria, dengue, and chikungunya. As vector-borne diseases continue to rise, particularly in tropical regions like India and parts of Africa, the need for sustainable, community-level mosquito control strategies becomes more urgent. By demonstrating the effectiveness of locally sourced bacterial isolates, this research supports the development of region-specific biocontrol solutions that are both economically feasible and environmentally responsible. In terms of methodology, this study followed standard microbiological techniques for isolation, identification, and larvicidal testing, comparable to methods used in the reviewed research. The use of biochemical tests, colony morphology analysis, and comparative mortality assays lends credibility and reproducibility to the findings. While future work could benefit from molecular characterization and genome sequencing of the isolates to identify specific toxin genes, the present study provides a solid foundation for future research and potential field application.

CONCLUSION

Bacterial were isolated from pond and river environments to be used as biocontrol agents against mosquito larvae, offering a sustainable and environmentally friendly alternative to traditional chemical insecticides. The C2 isolate, identified as *Micrococcus endophyticus* produced the most potent larvicidal compound that would kill 5 larvae of *Ades aegypti*. This larvicidal compound was found to be proteinaceous in nature. When compared with the commercially available larvicidal formulation, *Bacillus thuringiensis israelensis* (Bti), supernatant of C2 was found to be more efficient at lower concentrations. In conclusion, further studies on optimization and purification of the larvicidal compound from the supernatant of the C2 isolate can lead to a better commercial formulation.

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