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# Efficacy of *Bacillus thuringiensis as* Biocontrol Agent Against Mosquito Larvae

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**Abstract**: *A mosquito, deadliest arthropod vector in the world kills more than one million people every year according to the World Health Organization. It seems unbelievable that something so miniscule can kill so many people. The mission to accomplish a hit mosquito manage technique is by overcoming insecticide resistance. Mosquito borne diseases are a major life-threatening concern not only in India but across the world. The non-availability of vaccine or drugs against mosquito-borne diseases, and the limitations of traditional insecticide-based strategies have resulted in significant efforts to develop alternative eco-friendly methods. Bacillus thuringiensis (or Bt) is a Gram-positive, soil-dwelling, toxin producing bacteria. It is one of the best bio-larvicide for mosquitoes as there is less risk of developing a resistance towards it. The isolates of Bacillus thuringiensis confirmed an enormous degree of variant of their larvicidal interest. L-serine minimal agar media was used to isolate Bacillus thuringiensis as it inhibits the growth of positive strains of Bacillus spp. except B. thuringiensis. This phenomenon was utilized in isolation of B. thuringiensis strains from soil. This research work provides the evidence on alternative control of mosquito which is not only eco-friendly but also has negligible risk of mosquito developing resistance towards it.*

***Keywords:*** *mosquito-borne diseases, biocontrol, larvicidal, efficacy, Bacillus thuringiensis.*

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## Introduction:

Mosquitoes are the important vectors of various disease-causing agent that cause life threatening human disease than any other organisms. Diseases caused by mosquitoes include malaria, filariasis, dengue fever, chikungunya. Over one million people die from diseases caused by mosquitoes every year. WHO reported malaria to be the world's most important vector borne disease. Cases of these diseases have been reported in more than 100 countries, with

approximately more than 3 billion people living in endemic areas. More than 200 million cases of malaria and eight hundred thousand malaria related deaths are recorded every year. The increase in malaria cases is a result of detoriating health systems, increase in resistance of anopheline to insecticides, time taken to develop an effective vaccine and as well as *Plasmodium* to antimalarial drugs. Chemical insecticides have been used to control mosquitoes but overuse of chemical insecticides has resulted in damage to the environment which has caused adverse effect to our natural ecosystem. Keeping these negative effects in minds new methods were used to replace the chemical insecticides which included IPM (Integrated pest management). Their guidelines are based on public awareness, environmental planning and biological control that control the mosquitoes without causing any harm to environment thereby preserving it. Over reliance on insecticides to control mosquito has led to physiological resistance of mosquito vectors including *Anopheles gambiae* (Koekemoer et. al., 2011). Development of resistance to various types of insecticides such as organochlorides, organophosphates and carbamates (Bansal and Karam, 2007) possess various threat to the conventional control measures for vectors. In addition use of insecticides treated nets and spraying of insecticides in houses is highly effective but is also vulnerable to the development of resistance and behaviour change of vectors (Vulule et. al., 1994). In last decade there has been increased interest in biological control agents.

Organisms including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish have been investigated as potential agents for vector mosquito. In recent years several pathogenic strains of the *Bacillus thuringiensis* and *B. sphaericus* were reported to posses a high level of biological activity against mosquito larvae in the laboratory and field studies (Davidson et. al., 1981). As *Bacillus thuringiensis* are highly toxic to dipteran larvae it can be used as biolarvicides in mosquito eradication. The larvicidal substances of these are based on endotoxin proteins as parasporal crystals produced by bacterial cells. Toxicity is exerted by these proteins, causing a loss of osmoregulation and subsequent cell death in the midgut of affected organisms (Lacey, 2007). There are numbers of crystal-producing *Bt* strains in nature, though, not all are effective against insect pest (Roh et. al., 2007). First, distinctiveness of the *Bacillus thuringiensis* from its closely related species *B. cereus* because their biochemical and genomic composition is almost similar, except for the fact that *B. thuringiensis* produce a crystal inclusion endospore (Guo et. al., 2008). Single strains of *Bt* may contain more than one crystal protein genes (Letowski et. al., 2005). Research using *Bacillus thuringiensis* is often used to control mosquites either the formulation of *Bacillus thuringiensis* or culturing soil bacteria isolates. *Bacillus* sp is an important pathogen which is highly toxic to mosquito larvae and related dipterans hence it is considered beneficial to animals, humans and plants and is also a suitable replacement to chemical pesticides. Many western country researchers have reported biological activity of *Bt* to control the mosquito larvae both in field and laboratory as a safer insecticidal alternative (Charles et. al., 1996). *Bacillus thuringiensis* insecticides are divided into three groups. Due to the importance of *Bt* to control certain diseases such as malaria and dengue today it is the most successful commercial xenobiotics with its application when compared with chemical pesticides. *Bacillus thuringiensis* was first isolated by the Japanese scientist Ishiwata (1901) from

silkworm larvae, *Bombyx mori*. The purpose of our research is to provide an overview of *Bt* isolate and to summarize the current status and developmental trends of biological control based on published reports.

**Materials and Methods:**

**Sample collection:** Soil samples were collected aseptically from the soil at 5 cm depth of the various plants from Lakshmi dairy and organic farming Pvt. Ltd. Sampatchak, Patna. The water sample containing mosquito larva were collected from pond of Patna Women's College, Bailey Road, Patna.

**Isolation of *Bacillus thuringiensis*** : For isolation of microbes from soil, dilutions of normal saline were prepared. The $10^{-4}$,$10^{-5}$, $10^{-6}$dilutions were plated on NA media and L-Serine media and incubated for growth. The samples were sub-cultured and streaked on the nutrient agar media and L-Serine incubated at 37 degree Celsius for 24 hours.

**Screening of bacteria:** The morphological charecteristics of the colonies formed on NA and L-Serine media were studied taking their colour, texture, elevation and margin into account. Each suspected colony resembling to that of *Bacillus thuringiensis* were purified repeatedly subcultured on non-selective media i.e NA and preserved for further study. Each pure bacterial isolate was re-streasked on L-Serine agar media for further confirmation. Thereafter Gram staining and biochemical tests were performed.

**Microscopic study by Gram's staining method:** Gram's staining was performed to determine the differential staining reaction and shape of bacteria. Bacterial isolates which gave Gram positive, purple coloured with rod shaped appearance were suspected as *Bacillus thuringiensis.*

**Biochemical tests:** Bacterial isolate was further characterized on the basis of different biochemical tests like citrate utilization test to confirm the bacterial isolates were *Bacillus thuringiensis.*

**Preparation of bacterial culture for use in the bioassays :** Stock culture was taken by sterilized loop and serial dilution was done using distilled water which was autoclaved. Dilution was done upto $10^{-2}$.

**Test for Efficacy of *Bacillus thuringiensis:*** The test was based on the mortality rate of larva in the container containing the solution of the isolates and larvae.

**Results and Discussion:**

**Sample collection:** The five soil samples procured from the Lakshmi dairy and organic farming Pvt. Ltd. Sampatchak, Patna were from the rhizosphere of the various plants. The soil samples were of Basmati rice (*Oryza* sativa), Sponge gourd (*Luffa cylindrica*), Lady's finger (*Abelmoschus esculentus*) Bitter gourd *(Momordica charantia) and* Elephant foot yam (*Amorphophallus paeoniifolius*). They were obtained from a depth of 5 cm approximately maintaining aseptic condition. They were immediately brought in the laboratory of Patna Women's College.

The naming and classification of these soil samples based on their characteristics are showed in the Table 1.

**Table 1. Sampling and Characteristics of the soil samples**

| S. No. | Sample | Plant | Characteristics |
|---|---|---|---|
| 1. | A | Basmati rice (*Oryza* sativa) | Clayey, wet, light grey |
| 2. | B | Sponge gourd (*Luffa cylindrica*) | Silt like, dark brown to black, wet |
| 3. | C | Lady finger (*Abelmoschus esculentus*) | Light brown, semi-dry |
| 4. | D | Bitter gourd *(Momordica charantia)* | Light brown, silt like |
| 5. | E | Elephant foot yam *(Amorphophallus paeoniifolius*) | Light brown, dry |

**Isolation of *Bacillus thuringiensis* and study of its morphology and gram staining:** After being brought in the laboratory, the necessary testing and investigations of the 5 soil samples were done. Serial dilution of all the samples upto dilution of 10-6 were done. Dilutions of 10-4, 10-5 and 10- 6 of all the samples were inoculated on nutrient agar media and incubated for 24 hrs at 37 C.

Streaking on nutrient agar was done to obtain pure cultures and sub culturing was done. Gram staining was performed by taking the isolated colonies of the pure culture. Results from the study of morphology were recorded and are shown in Table 2.

**Table 2. Morphological Characteristics and Gram Staining**

| Sample | Margin | Colour | Elevation | Shape | Gram staining |
|---|---|---|---|---|---|
| A | Irregular and dry | Matte White | Flat | Bacilli | +ve |
| B | Irregular | Yellowish - white | Flat | Bacilli | +ve |
| C | Irregular or undulate | Pale white | Flat | Bacilli | -ve |
| D | Entire | Faint white | Convex | Cocci | +ve |
| E | Entire | Off white | Raised | Cocci | -ve |

The Table 2 shows that only sample A and sample B were gram positive and bacilli as per our requirement. Whereas Sample D and E were cocci in shape hence they were not proceeded with further investigation and experimentation. Samples A and B were inoculated on L-serine agar medium to confirm the presence of *Bacillus thuringiensis.*

**Inoculation on L-Serine agar:** It is observed that addition of L-serine to minimal synthetic media results in an inhibition in the growth of certain strains of *Bacillus* sp. but not *B. thuringiensis*. Thus L-serine-resistance phenomenon was used in isolation of *B. thuringiensis* strains from soil.

Sample A produced colonies on the minimal media whereas Sample B did not. Since Sample A was gram positive and had desired morphology and phenotypic characters it was suspected that Sample A was *Bacillus thuringiensis*.


**Fig. 1. Sample A colonies on L-serine agar**

**Biochemical tests:** Sample A isolate were subjected to biochemical test of citrate utilization for confirming the presence of *Bacillus thuringiensis*. All isolates of gram-positive rods gave positive test for citrate utilization test by producing blue colouration.

In Citrate utilization test, Simon citrate agar slant was prepared and it was streaked back and forth with a light inoculum picked from a well-isolated colony, incubated at 37°C and a colour change from green to blue was observed. Therefore, it tested positive for the citrate utilization test and it was confirmed that Sample A was *B. thuringiensis*.

**Fig. 2. Showing positive results of citrate utilization test**

**Bioassay :** The stock culture of *Bacillus thuringiensis* from slant was picked using sterile inoculating loop. It was diluted upto 2-fold dilution in sterilized distilled water. 1 ml of this solution was added in cup III and cup IV containing the mosquito larvae. Both the containers were incubated at 37°C for 24 hrs.

Soil sample A of Basmati rice (*Oryza* sativa) was used to isolate *Bacillus thuringiensis*. Colonies mostly appear matte white colour, flat, dry and with uneven borders. Colonies appeared purple under the microscope after Gram staining was performed showing its gram positive. Along with the growth on L- Serine minimal agar media confirmed the isolate was of *Bacillus thuringiensis.*

Hence, the experiment was proceeded using this isolate to test its efficacy as a bio-larvicide. Broth culture was added to the disposable cup containing live mosquito larvae. The cups were then incubated for 24 hrs at room temperature and the observation was recorded. A mortality rate of 66.6% was observed in the cup III and 70% in cup IV after 24 hrs. *Bacillus thuringiensis* showed promising results as biocontrol agent against mosquito larvae.

**Conclusion:**

Our study sheds light on the alternative control of mosquito which is not only eco-friendly and safe but also has negligible risk of mosquito developing a resistance towards it. *Bacillus thuringiensis* was isolated from basmati rice soil sample procured from Lakshmi dairy and organic farming Pvt. Ltd. Sampatchak, Patna. It was labelled as sample A, which produced matte white flat colonies with irregular, margin on nutrient agar media. The pure culture obtained after sub culturing and re streaking tested positive for Gram's staining. After growing on L-Serine minimal agar media it was confirmed that the isolate was *Bacillus thuringiensis*. The water

sample containing mosquito larva were collected from pond of Patna Women's College, Bailey Road, Patna. Hence, the experiment was proceeded using this isolate to test its efficacy as a bio-larvicide. A mortality rate of 66.6% was observed in the cup III and 70% in cup IV after 24 hrs.

In conclusion, *Bacillus thuringiensis* showed promising results as biocontrol agent against mosquito larvae. Biocontrol method is the future of insecticide and pesticide. Mosquito borne diseases being a global threat and mosquitoes having the ability of developing resistance towards chemical treatment biocontrol method emerges as the only hope.

**Future Prospect:**

Owing to the rise in mosquito-borne diseases, lack of vaccine or drug for these diseases and resistant species of mosquito found, more research pertaining to the field of eco-friendly method like biocontrol which has less risk of mosquito developing resistance needs to be encouraged.

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