# BIOCONTROL POTENTIAL OF NOVEL *BACILLUS SPP.* ISOLATED FROM SOIL SAMPLES AGAINST MOSQUITO LARVAE

[1]Priya S. Patel, [2]Dr. Reena G. Desai

[1]PG research scholar, [2]Head of Department,
[1] Department of Microbiology
[1,2]Dolat-Usha Institute of Applied Sciences & Dhiru-Sarla Institute of Management & Commerce, Valsad, India.

***Abstract:*** Bio-pesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria and certain minerals. The perfect pesticide is one, which is easily applied, reasonably inexpensive, non-toxic to non-target organisms, and eliminates the pest quickly before it becomes a threat. Certain types of bacteria have been developed to use as pesticides that are very close to perfect pesticide model and *Bacillus* was one of the most crucial and important genus (Rishikesh *et al*., 1983) in this connection, And also as a suitable replacement to chemical pesticides. 11 potent *Bacillus spp.* were isolated from different soil samples like garden soil, agricultural soil, animal dung contaminated soil etc. as a control strategy of mosquitoes larvae. This 11 potent isolates (B.t 1, B.t 2, B.t 3, B.t 4, B.t 5, B.t 6, B.t 7, B.t 8, B.t 9, B.t 10, B.t 11) were confirmed as *Bacillus* based on microscopic observation and biochemical characterization. Further parasporal crystal was observed by phase contrast microscopy and larvicidal protein was extracted, and it was quantified by Folin Lowery's method. Isolate coded as B.t 1 showed highest protein concentration so it was use to check larvicidal activity against 2 types of larvae which was *Aedes* and *Culex*. The larvicidal activity (Which were measured by mortality rate and change in morphology of the larvae) was observed at $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ & $10^{-6}$ dilution Factors and at interval of every 2 hours. B.t 1 isolate which was confirmed as *Bacillus flexus* by 16S rRNA sequencing showed 100% mortality of both larvae at the highest concentration of $10^{-1}$ and $10^{-2}$ at 24 hours while 100% mortality was recorded in other dilution factors at 48 hours which was observed by change in morphology in phase contrast microscope. In control system (Distilled water) no mortality was recorded also after 72 hours. From this study, it is concluded that *Bacillus flexus* is a very potent biolarvicide that brings about mortality of mosquito larvae at short duration of time. The present study proved that the mosquitocidal properties of the *Bacillus flexus* isolated from soil was evaluated as target species of mosquito vectors. This is an ideal eco-friendly approach for the vector control programs.

***IndexTerms*** **- Bio-pesticide, *Bacillus spp*., Mosquito, Larvicidal.**

## I. INTRODUCTION

Mosquitoes are the important vectors of various diseases causing agents, responsible for transmission of pathogens causing more life threatening human disease than any other organisms. Over one million people worldwide die from mosquito borne disease, which include Malaria, Filariasis, Dengue fever, yellow fever, Chikungunya causing serious health problems to world human populations. Chemical insecticides have been used in the last century to successfully control mosquitoes; However, uncontrolled use of chemical insecticide has resulted in irreparable damage to environment, disrupt natural ecosystems leading to re-emergence and increase in mosquitoes population. Globally there are continues efforts to overcome these problems, and great emphasis is placed recently on eco-friendly and economically viable methodologies for insect control. An alternative approach for mosquitoes control is the use of natural products such as plant and microorganisms [1]. Interestingly, *Bacillus spp.* is an important insect pathogen which is highly toxic to mosquito larvae and related dipterans, hence it is considered beneficial to human, animals and plants and also as a suitable replacement to chemical pesticides in many countries. *Bacilli* are present in an extremely large area of environments ranging from sea water to soil, and are even found in extreme environments. This bacterium could be one of the major sources of potential microbial bio pesticides because it retains several valuable traits [11].

## II. MEDIA

1. Sterile nutrient broth (Hi-Media Composition)
2. Sterile M9 medium (Andrzejczak *et al.*)
3. Sterile nutrient agar medium (Hi-Media Composition)

## III. RESEARCH METHODOLOGY

### 3.1 Sampling

7 different soil samples like garden soil, agricultural soil, animal feces, cattle dung contaminated soil, soil and leaf of night blooming jasmine and soil near pond were collected in sterile plastic container from valsad city and nearby areas to isolate *bacillus spp.* [13]. The soil samples were taken at a depth of 10cm below the soil surface after scrapping off the surface material with sterile spatula and samples were placed in sterile plastic bag for further processing.

### 3.2 Sample processing

Before samples were processed; physiochemical characteristics of soil sample was analysed like temperature of the soil, pH of the soil, colour of the soil etc. After that 1 gm of sample was taken from the collected sample and added into sterile 250 ml conical flasks containing 99 ml of sterile distilled water. The conical flasks were then covered with cotton plug and placed on an

orbital shaker at 250 rpm for 4 hours. This procedure ensures the microbe attached to the soil particle dispensed into the distilled water and to ensure the samples are homogenised. After 4 hours, 5 ml of the soil-water samples were collected using a sterile pipette and added into test tube, immediately tubes were then subjected to heat shock treatment in a water bath set at 80ºC for 10 minutes. This step was to ensure all microbes in vegetative form are killed, leaving behind only the microbial spores. After heat shock treatment, the samples were vortexed (Lobo *et al*, 2018).

**3.3 Enrichment**

For the enrichment 1ml of treated sample was added into sterile 9 ml nutrient broth .The inoculated nutrient broth was kept at 37ºC for 24.

**3.4 Isolation of novel *Bacillus spp*.**

The enriched bacterial cells were sedimented by centrifugation and cells were resuspended in 0.9% NaCl solution. The suspensions were serially diluted and 0.1 ml of aliquot was taken and spread on sterile M9 media supplemented with 0.2 mM of L- serine [2]. The plates were incubated at 37ºC for 48 h. After 48 hrs all plates of M9 were studied for different colonial characteristic.

**3.5 Purification and Preservation of novel *Bacillus* isolates**

The colonies which showed *Bacillus* like colony morphology, like medium/large, round/irregular, smooth/ glossy/less glossy /rough, convex, white/creamy and spread out over the plate were selected and streaked again on sterile nutrient agar plates. Then the inoculated plates were incubated for 24 h at 37ºC to obtain pure culture. Pure isolates suspected to be *bacillus spp.* were kept in nutrient broth or on nutrient agar slant at 4ºC for further characterization.

**3.6 Identification of isolates**

All selected isolates showing colony characteristics like medium/large, round/irregular, smooth/glossy/less glossy/rough, convex, white/creamy and spread out over the plate were studied by gram staining and motility these isolates also further studied with conventional identified and conventional biochemical test like catalase test, oxidase test, Indole production test, Methyl red (M-R) test, Voges proskauer (V-P) test, Citrate utilization test, Urea hydrolysis test, Triple sugar iron (TSI) agar test, Nitrate reduction test, Sugar (Glucose, Sucrose, Maltose, Mannitol) utilization test, Starch hydrolysis test, Casein hydrolysis test, Gelatine hydrolysis test, Blood hemolysis test was performed as described by procedure according to (Claus and Berkeley, 1986).

**3.7 Phase Contrast Microscopy**

Gram positive colonies were inoculated into sterilized 250 ml conical flasks containing 50 ml sterile nutrient broth and flask was placed on orbital shaker at 250 rpm for 4 days. The purpose was to induce sporulation of the bacterial cells. Wet mount slides were prepare from inoculated nutrient broth after 2 days of inoculation in order to visualize the vegetative cell and endospore. Wet mount slide was also prepared at $4^{th}$ day from inoculated nutrient broth to visualize the endospore and parasporal inclusion bodies which were important in *Bacillus spp.* identification.

**3.8 Protein extraction and estimation**

For protein extraction and estimation sterile 10 ml nutrient broth was prepared and inoculated with test isolates and incubate the inoculated nutrient broth at 37 ºC for 4 days to induce autolysis. After complete autolysis (after 4 day) 10 ml of the inoculated nutrient broth containing lysed cells were pipetted into centrifuge tube. Crude protein was obtained by centrifugation at 10,000 rpm at 4°C for 15 minutes. The supernatant was discarded and the pellets were resuspended in 2 ml of sterilized distilled water. The protein concentrations in the crude sample were determined by using the folin lowry's method (Lowry O H *et al*., 1951) with bovine serum albumin (BSA) as a standard.

**3.9 Bioassay and activity of novel *Bacillus* isolate against mosquito larvae**

**3.9.1 Breeding of mosquito larvae**

Water containers were left to stand in open space at ambient temperature of about 30°C for approximately 7 days to facilitate lying of eggs by the mosquito. It was depend on the climatic condition because in summer it takes more time for larvae emergence. The water container was monitored daily to observe the emergence of the larvae. The larvae of mosquitoes were harvested using sieve. Then the larvae of the mosquitoes were identified based on their general characteristics and separated into different groups (*Aedes, Anopheles*, and *culex*) for the further larvicidal experiment.

**3.9.2 Bioassay**

In primary screening, *Bacillus spp.* isolates having highest amount of protein and having different crystal characteristic were selected and tested against larvae of mosquitoes.

**3.9.2.1 Inoculum preparation**

The prime objective of inoculum preparation was to achieve a high level of viable biomass in a suitable physiological state for use as inoculum. For that 9 ml sterile nutrient broth was prepared and inoculated with 1 ml suspension of selected *bacillus* isolate (B.t 1) for the larvicidal experiment and incubated at 37 C for 24 hrs.

**3.9.2.2 Larvicidal experiment**

The selected *Bacillus spp*. (B.t 1) from inoculated nutrient broth were diluted $10^{-1}$, $10^{-2}$, $10^{-3}$ up to $10^{-6}$ with sterile distilled water by adding 1ml of inoculum in 9ml of sterile distilled water. Then 6 larvae were transferred into each plate with labelled dilution factor of *Bacillus spp*. The test plates were kept at room temperature and the mortality rate was checked for each dilution factor. Larval mortality was recorded at every 2 hours interval. The control test was also carried out using distilled water.

## IV. RESULTS AND DISCUSSION

### 4.1 Screening and isolation of *bacillus spp*.

11 bacterial strains were isolated from the 7 different soil samples using sterile M9 medium with L-serine supplement agar plate. All putative isolates with typical colony morphology (i.e. large, round/irregular, smooth/glossy/less glossy/rough, convex, white/creamy and spread out over the plate) were determine as *bacillus spp*. Motility test was performed, and only motile isolates were further selected for morphological and biochemical characterization. The 11 bacterial isolates (B.t 1, B.t 2, B.t 3, B.t 4, B.t 5, B.t 6, B.t 7, B.t 8, B.t 9, B.t 10, and B.t 11) are gram-positive, rod shaped, and spore formers.

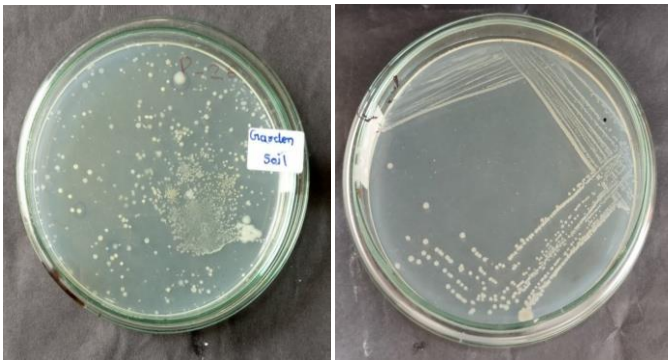


Figure 4.1: Creamy white colonies indicating a *Bacillus* colony (left). Single colonies obtained after sub-culturing (right).

### 4.2 Identification and characterization of isolates

All 11 isolates were examined under bright field microscope to observe their microscopic features. All 11 isolates were gram positive, rod shaped and motile.

TABLE 1: Results of Gram staining, Spore staining, Catalase and Oxidase test

| Isolates | Gram staining | Formation of spore | Catalase | Oxidase |
|---|---|---|---|---|
| B.t 1 | Gram +ve | +ve | +ve | -ve |
| B.t 2 | Gram +ve | +ve | +ve | -ve |
| B.t 3 | Gram +ve | +ve | +ve | -ve |
| B.t 4 | Gram +ve | +ve | +ve | +ve |
| B.t 5 | Gram +ve | +ve | +ve | +ve |
| B.t 6 | Gram +ve | +ve | +ve | +ve |
| B.t 7 | Gram +ve | +ve | +ve | +ve |
| B.t 8 | Gram +ve | +ve | -ve | +ve |
| B.t 9 | Gram +ve | +ve | -ve | +ve |
| B.t 10 | Gram +ve | +ve | -ve | +ve |
| B.t 11 | Gram +ve | +ve | -ve | +ve |

+ve = Positive

-ve = Negative

TABLE 1 showed that all 11 isolates were gram positive rod shaped, motile and spore former. Out of 11 isolates B.t 1 to B.t 7 isolate showed positive reaction with regard to catalase test, while other isolate B.t 8 to B.t 11 showed negative reaction to catalase test. Positive reaction represents the presence of catalase enzyme in the isolates. For oxidase test B.t 1, B.t 2, and B.t 3 showed negative reaction to oxidase test while other isolates give positive reaction to oxidase test.

TABLE 2: Results of biochemical tests

| Isolates | IN | MR | VP | CIT | URE | TSI | NR | GL | SU | MAL | MAN | STR | CAS | GL | HL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B.t 1 | -ve | +ve | +ve | -ve | -ve | +ve | +ve | ⊕ | ⊕ | ⊕ | ⊕ | +ve | +ve | +ve | β |
| B.t 2 | -ve | +ve | +ve | +ve | -ve | +ve | +ve | ⊕ | ⊕ | ⊕ | ⊕ | +ve | +ve | +ve | γ |
| B.t 3 | -ve | +ve | +ve | -ve | -ve | -ve | +ve | ⊕ | ⊕ | ⊕ | ⊕ | +ve | +ve | +ve | γ |
| B.t 4 | -ve | +ve | +ve | -ve | ve | +ve | +ve | - | + | + | + | +ve | +ve | -ve | β |
| B.t 5 | -ve | +ve | +ve | +ve | -ve | -ve | +ve | - | + | + | + | +ve | +ve | +ve | γ |
| B.t 6 | -ve | +ve | -ve | +ve | -ve | +ve | +ve | + | - | ⊕ | + | +ve | +ve | +ve | γ |
| B.t 7 | -ve | +ve | -ve | +ve | -ve | +ve | +ve | - | - | - | - | +ve | +ve | -ve | γ |
| B.t 8 | -ve | +ve | -ve | +ve | -ve | -ve | -ve | + | - | + | +ve | +ve | -ve | +ve | β |
| B.t 9 | -ve | +ve | +ve | +ve | -ve | -ve | -ve | - | - | - | - | +ve | +ve | +ve | γ |
| B.t 10 | -ve | +ve | +ve | -ve | -ve | +ve | +ve | + | + | + | ⊕ | +ve | +ve | +ve | γ |
| B.t 11 | -ve | +ve | +ve | +ve | -ve | +ve | +ve | + | + | + | - | +ve | +ve | -ve | β |

+ve = Positive, -ve = Negative, + = acid, ⊕=acid + gas, - = absence of acid and gas, β hemolysis = Complete hemolysis, γ hemolysis =No zone around colony

IN:Indole production test, MR: Methyl-red test, VP: Voges - proskaur's test, CIT:Citrate utilization test, URE:Urea hydrolysis test, TSI:Triple Sugar iron (TSI) agar test, NR:Nitrate reduction test, GL:Glucose utilization test, SU:Sucrose utilization test, MAL:Maltose utilization test, MAN:Mannitol utilization test, STR:Starch hydrolysis test, CAS:Casein hydrolysis test, GL:Gelatin hydrolysis test, HL:Blood hemolysis test.

The isolates showed negative reactions with regard to indole production test. They showed positive reactions with regard to MR test. The summary of the test results is shown in TABLE 2.

**4.3 Phase contrast microscopy**

Phase contrast microscopy was carried out after determined the colonies were gram positive through gram staining. This procedure was important to confirm the isolates are *Bacillus* by viewing the endospore and parasporal bodies. Besides that, phase contrast microscopy was also done for vegetative phase cells to confirm the isolates were rod shape. Wet mount slides were prepared after 4 days of incubation for each isolate. Isolates that showed presence of both endospore and parasopral bodies were selected for further characterization. All 11 isolates possessed presence of endospore and parasporal bodies.

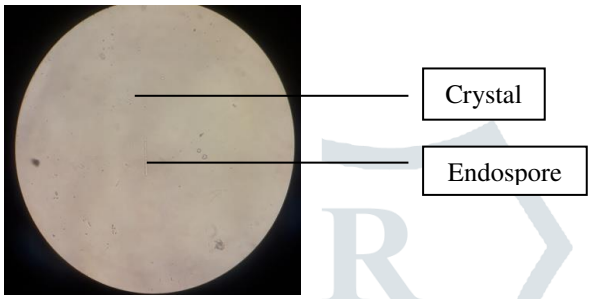


Figure 4.2: Sporulated form of *Bacillus* at 100X magnification

Parasporal body is a dark oval region and the endospore is bright rod shape. All 11 sporulated samples showed dark coloured crystal proteins and unstained endospore under phase contrast microscope. Based on this observation, all 11 isolates were confirmed to be *Bacillus*.

**4.4 Protein Estimation**

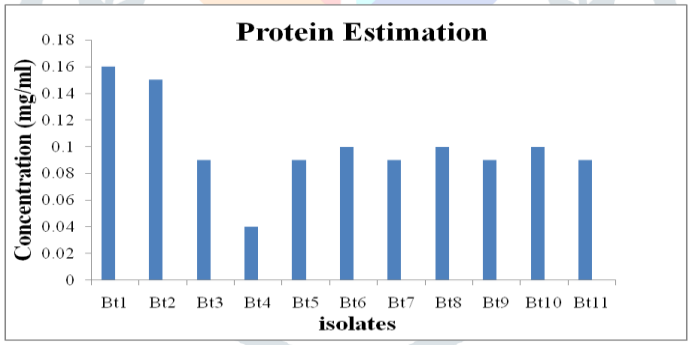


Figure 4.3: Total protein contain

In this study, total protein concentration was determined by Folin Lowry's method. All 11 isolates possessed protein as their parasporal crystal which is made up of proteins. That was the important part of the insecticidal *bacillus spp.* because it is toxic for the insect. Among them, B.t 1, and B.t 2 isolate gave the highest protein content of 0.16 and 0.15 mg/ml respectively whereas B.t 4 isolate gave least protein content of 0.02 mg/ml (Figure 4.3).

**4.5 Results of Larvicidal Activity**

TABLE 3: Bioactivity of novel *Bacillus isolate* isolate (B.t 1) against *aedes* and *culex* mosquito larvae at different hour's interval).

| | **4 hours** | | | | **12 hours** | | | | **24 hours** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Aedes*** | | ***Culex*** | | ***Aedes*** | | ***Culex*** | | ***Aedes*** | | ***Culex*** | |
| Dilution factor | No. of live larvae | No. of dead larvae | No. of live larvae | No.of dead larvae | No. of live larvae | No. of dead larvae | No. of live larvae | No. of dead larvae | No. of live larvae | No. of dead larvae | No. of live larvae | No. of dead larvae |
| Control | 6 | 0 | 6 | 0 | 6 | 0 | 6 | 0 | 6 | 0 | 6 | 0 |
| $10^{-1}$ | 5 | 1 | 5 | 1 | 3 | 3 | 3 | 3 | 0 | 6 | 0 | 6 |
| $10^{-2}$ | 5 | 1 | 5 | 1 | 4 | 2 | 4 | 2 | 0 | 6 | 0 | 6 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $10^{-3}$ | 6 | 0 | 6 | 0 | 4 | 2 | 4 | 2 | 1 | 5 | 1 | 5 |
| $10^{-4}$ | 6 | 0 | 6 | 0 | 4 | 2 | 4 | 2 | 1 | 5 | 1 | 5 |
| $10^{-5}$ | 6 | 0 | 6 | 0 | 5 | 1 | 5 | 1 | 2 | 4 | 2 | 4 |
| $10^{-6}$ | 6 | 0 | 6 | 0 | 5 | 1 | 5 | 1 | 3 | 3 | 3 | 3 |

In this study toxicity was demonstrated by death at specified period of time and change in morphology of the larvae (Wei *et al*., 2003). 100% mortality of both mosquito larvae (*Aedes* & *culex)* was recorded at 24 hours at highest concentration $10^{-1}$ and $10^{-2}$ dilution factor while 100% mortality was recorded in other dilution at 48 hour. The control tubes which contain sterile distilled water; no mortality was recorded also after 72 hours.

Figure 4.4: Before (left) and after (right) ingestion of *bacillus* isolate (B.t 1) (*Aedes* mosquito larvae)

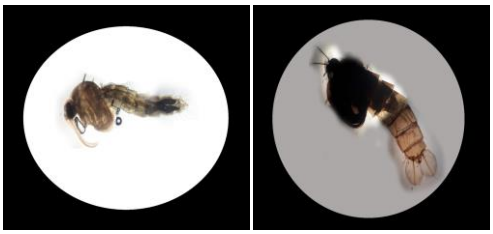

Figure 4.5: Before (left) and after (right) ingestion of *bacillus* isolate (B.t 1) (*Culex* mosquito larvae)

The dead larvae were examined under phase contrast microscope, it was observed that the larvae were swollen and the general morphology of the dead larvae which include change in coloration, gut shape, general body shape (as a result of feeding on the *bacillus*) was different from the normal one not fed with the *bacillus*.

## V. CONCLUSION

In the perspective of biological control, a spore-forming soil bacterial strain has been isolated during the study period. *Bacillus flexus* strain NBRC 15715 is a gram-positive, rod-shaped, spore forming bacterium. Physiological, biochemical and molecular characterization revealed its identity. The strain is virulent against *Aedes* larvae and also efficient against *culex larvae*. Information on its patogenicity against mosquito has not been reported till now. In the present study, special attention was devoted to the search for new isolates of *Bacillus* from soil sample. Such studies were intended to provide novel *bacilli* culture that were more toxic for endogenous mosquito species than the existing strains and could be used in the future as safe biological control agents under the local environment condition.

## VI. ACKNOWLEDGMENT

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