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Bio-control of mosquito larvae *Culex quinquefasciatus* by using *Bacillus sphaericus*

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Abstract
Mosquitoes acts as a vector for various kinds of diseases to public health worldwide. In India 99% of infection occurs when the mosquito *Culex quinquefasciatus* spreads a worm *Wuchereria bancrofti* that causes lymphatic filariasis through a mosquito bite. For many years' mosquitoes were controlled based on spraying chemical insecticides to kill larvae and adults. The alternate, more effective ways of controlling them are environment friendly bio-insecticides. In the present study the bio insecticidal property of *Bacillus sphaericus* 3910 were tested against *Culex quinquefasciatus* at different larval stages with different concentration of the bacterial species. It was observed that 0.4 and 0.5g/100mL of the bacterial concentration was more effective against all 4 stages of the larvae. After the bioassay the bacterial protein was isolated and its molecular weight was identified. To determine the bio insecticidal property the bacterial protein was also isolated from the dead larvae treated with bio insecticide and the molecular weight was identified. Moreover, the plasmid DNA of Bacillus sphaericus was transformed to E. coli for broad spectrum of pathogenicity. Hence *Bacillus sphaericus* can be used as a bio control agent against the *Culex quinquefasciatus* larval population.

Keywords: *Lymphatic filariasis, Wuchereria bancrofti* Bio-insecticides, *Culex quinquefasciatus* and *Bacillus sphaericus,* bioassay, transformation

1. Introduction
Mosquitoes are found in damp, marshy places near stagnant water, all over the world. They are especially abundant in the tropical and sub-tropical countries. The current study focuses on the southern house mosquito *Culex quinquefasciatus* transmits zoonotic diseases that affects humans and animals such as lymphatic filariasis, avian malaria, encephalitis and West Nile fever. In India it is the primary vector of *Wuchereria bancrofti* a nematode that causes lymphatic filariasis. *Bacillus sphaericus* is a gram-positive, rod shaped, aerobic bacteria and synthesizes a parallelepiped parasporal crystal, which contains toxic protein lethal to *C. quinquefasciatus* larvae. It is very effective in the presence of high levels of organic matter and the capacity to recycle itself in dead mosquito larvae, resulting in greater persistence and larvicidal effect by the product. This is the major advantage for the biocontrol of the mosquito larvae in environmental conditions. The toxin of *Bacillus sphaericus* consists of two polypeptides with apparent molecular Sizes of 51and 42 kDa (BinB and BinA) are encoded by highly conserved chromosomal genes. When *the Culex* larva ingests the *Bacillus sphaericus* present in the water the crystal toxin gets solubilized resulting in the release of protein in the larval gut. The protease present in the mid-gut region breaks down the crystal protein to 43 and 39 kDa proteins results in major increase of toxicity. Hyun-woo park, Clare M. Mangum, He Zhong and Sabrina R. Hayes John A. Mulrennan, Sr., studied the isolation of *Bacillus sphaericus* with improved efficacy against *Culex quinquefasciatus*, [5] the toxicity of the isolated bacteria from sediment samples were compared with strains 2297 and 2362 of *Bacillus sphaericus* proven the greater efficacy towards the 4th instar larvae of *culex quinquefasciatus*. In the current study, *Bacillus sphaericus* strain 3910 was tested on all 4 stages of the larva and proved to be effective against all stages of the larva by the bioassay technique. Alkaline extraction of toxin from spores of the mosquito pathogen, *Bacillus sphaericus* strain 1593 [4] by Elizabeth west Davidson reported that NaOH extraction of toxin from spores can lead to decrease in the toxicity of bacteria against the *C. quinquefasciatus* as the spores are present in the cell wall.

The purification and fractionation of protein is inactivated by enzymatic activity and spore viability is decreased by 10-50%. This drawback was overcome in the current study by using the natural sporulating bacteria to control the *Culex* larval population. Transfer of the Toxin Protein Genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and Their Expression [6] by Catherine *et al* suggested that the genetic engineering helps to couple the toxic activity of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* by transformation to obtain transformants toxic to *Culex, Anopheles* and *Aedes* larval population. Similarly, in the present study the *B. sphaericus* plasmid DNA is transformed in to *E. coli* to achieve broad spectrum of pathogenicity against *Culex quinquefasciatus.* The current study focused mainly on the growth and sporulation of *Bacillus sphaericus* 3910, collection of all stages of *C. quinquefasciatus* to perform bioassay, pathogenicity of the bacteria against *C. quinquefasciatus* larvae, protein analysis, transformation of plasmid DNA of *B. sphaericus* to wild type *E. coli* cells, antibiotic resistance activity of transformants and determination of transformants by restriction digestion of DNA using HindIII restriction enzyme to achieve broad spectrum pathogenicity.

2. Materials and Methods

Bacterial cultures

The lyophilized sample of the bacteria *Bacillus sphaericus* was obtained from the Microbial Type Culture Collection (MTCC) and Microbial Institute of Technology, Chandigarh, India. The Lyophilized bacterial sample was stored under refrigerated condition. The bacterial cultures were prepared for the experiment by inoculating a loop full of lyophilized sample in Nutrient Yeast Extract Mineral salt medium (NYSM) and Soybean Hi-Veg medium. The inoculated broth cultures were maintained in orbital shaker at 37 degrees C, 100 RPM for 24 to 48 Hrs. The bacterial characteristics were identified using motility test, hanging drop method, gram staining and endospore staining. The biochemical tests were carried out on the bacteria like catalase test, oxidase test, carbohydrate fermentation test, methyl red and voges proskauer test, citrate utilization test, urease test, indole test and nitrate reduction test.

2.1 Mosquito Larval cultures

The mosquito larvae of *Culex quinquefasciatus* were collected in and around the Chennai city, India. The National Institute of Malarial Research (ICMR) guided in the culture and identification of larvae and mosquito of *Culex quinquefasciatus.* The University of Madras, Department of Zoology guided in the collection of larval samples from the mosquito breeding places necessary for the project. The *Culex quinquefasciatus* larvae were grown in the collected water rich with organic matter. Each stage of the larvae was maintained separately. The water was changed once in 2days. It was fed once in a day with dog biscuit and yeast in the ratio of 5:1. The culture tank was stirred thrice in a day to maintain the larvae in active condition. The culture tank was covered with muslin cloth to prevent the settling of dust on the water and to protect the larval respiration. The larvae are maintained under room temperature and provided with 20hours of light and 4-6hours of dark. They were aliquoted as 10 larvae /50ml of water containing organic matter. The larvae were transferred using aspirator. The morphology of the larva was studied by isolating and visualizing under the dissection microscope.

2.2 Bioassay and Protein analysis

The *Culex quinquefasciatus* larvae were grown, cultured and tested under laboratory conditions in the organic matter containing water. The *Bacillus sphaericus* cultures were grown for 48 hours and selected for the bioassay. The larvicide was obtained by centrifuging the culture broth and the wet weight of the pellet was taken in different concentration as (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.l, 0.2, 0.3, 0.4, 0.5) dissolved in 100 ml of distilled water and slowly mixed to make it a diluted larvicide. The 4th instar larvae of *culex quinquefasciatus* of 100 numbers was added to 90 ml of distilled water. The volume was completed to 100ml by adding 10 ml of the diluted larvicide. The bioassay was performed using 14different concentration (0.01/100 ml, 0.02/100 ml, 0.03/100 ml, 0.04/100 ml, 0.05/100 ml, 0.06/100 ml, 0.07/100ml, 0.08/100 ml, 0.09/100 ml, 0.l/100 ml, 0.2/100 ml, 0.3/100 ml, 0.4/100 ml, 0.5/100ml). The larval mortality rates were measured after 48 hours with all the containers were supplied with feed until the target period. The larval mortality rate was calculated by counting the dead larvae in each different concentration beakers. A separate study of the 1st, 2nd and 3rd instar larvae was tested with different concentration (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.l) of bacterial larvicides. 10 larvae from each stage were transferred to 45 ml of distilled water separately and 5 ml of bio insecticide of different concentration was added to each stage of the larva. The larval mortality rate was measured after 48 hours. The bioassay was evaluated separately for the 1st, 2nd, 3rd instar larva 50mL of solution containing 10 numbers of larva in 45mL of distilled water with 5mL of larvicide and for the 4th instar larva 100mL of solution containing 100 numbers of larva in 90mL of distilled water with 10mL of larvicide and the culture sample without the bio larvicide was taken as control respectively. The protein was isolated from *Bacillus sphaericus* and bioassay sample was estimated by Bradford method calorimetrically at 595 nm. The Protein separation and molecular weight was determined by Ammonium sulphate precipitation, dialysis and SDS PAGE. The culture broth of *Bacillus sphaericus* was centrifuged at 10,000 RPM for 5 minutes. The pellet was transferred to pre-chilled pestle and mortar and small amount of sodium acetate buffer (0.05M) pH 5.2 was added to grind the pellet. The solution was made up to 30ml using sodium acetate buffer and centrifuged at 10,000 RPM at 4ºC for 10 minutes. The supernatant was collected, estimated and used for ammonium sulphate precipitation. To the 30 ml of filtrate powdered ammonium sulphate was added to 80% saturation (0.561 g/ml) under stirred condition using magnetic stirrer. The protein was separated from the solution by centrifugation at 8000 RPM for 10 min at 4ºC, the pellet was dissolved in known volume of sodium acetate buffer. Followed by dialysis the dialysis membrane (6cm) was cut off and activated by incubating the required length at 60ºC for 15 min followed by rinsing in distilled water. The dialysis bag containing the protein sample was immersed in cool sterile sodium acetate buffer and subjected to dialysis at 4ºC. The dialysate was changed every 4 hours with dilution rate of 2:1, 2:2, 1:2 (Buffer: water) for the efficiency of the process. The proteins present in the dialysis bag were collected and used for SDS PAGE.

Different samples were loaded to the polyacrylamide gel such as bacterial protein sample, mosquito protein sample, dialyzed bacterial protein and larval protein sample. Each sample were mixed with equal volume of sample buffer and boiled for 2 minutes and cooled at room temperature. The staining gel was carefully separated from the plates and incubated in the staining solution overnight in shaker. De-staining the gel in the de-staining solution until the bands were intensely observed.

2.3 Bacterial Transformation

In the current study the larvicide property of *Bacillus sphaericus* is transferred to other bacteria lacking the property i.e. *E. coli* to increase the pathogenicity against *Culex quinquefasciatus* larvae. Here in this study *E. coli* is selected as a competent cell because of its habitat in the dirty water and sewage water drains. The plasmid DNA was isolated from *Bacillus sphaericus*. The *Escherichia coli* was isolated from its environmental inhabitants. The *E. coli* sample was gram-stained and cultured in eosin methylene blue agar, incubated at 37ºC for 48 Hours. *E. Coli* culture grown on LB broth was placed on ice for 10 – 20 minutes were centrifuged at 6000 RPM for 3 minutes and 15 mL of cold 0.1M calcium chloride solution is added to the cell pellet and mixed well. The tubes were stored at 4ºC for 30 minutes. The same procedure was repeated again. The pellet was suspended gently in 0.6µL of cold 0.1M calcium chloride solution to obtain the competent cells. To the electroporation cuvette 100µL of competent cells (*E. coli*) and 5 µL of plasmid DNA of *Bacillus sphaericus* was added and placed in the electroporator for the transformation process. The presence of plasmid DNA in the competent cells were identified by the growth of transformed bacterial colonies on the LB media containing penicillin G. Restriction Digestion of DNA is performed on the transformants for further analysis and confirmation. Aliquot of 10µL of plasmid DNA was taken from each sample (*Bacillus* sample, recombinant sample, *E. coli* sample), separately and labeled as 1,2,3,4 to this 10µL of 2x assay buffer, 2 µL of Hind III restriction enzyme and made up to 50 µL by adding 28 µl of nuclease free water. The mixture was mixed gently and tubes were incubated at 37ºC for 1 hour. Four different samples were prepared for agarose gel electrophoresis to observe the restriction pattern of DNA i.e. plasmid DNA of *Bacillus sphaericus*, wild type *E. Coli*, recombinant *E. Coli* and 1 KB DNA ladder digested with restriction enzyme as control.

3. Results

In this study *Bacillus sphaericus* cultured on nutrient yeast extract mineral salt medium (NYSM) appears creamy white colonies with mat formation and on soybean medium it appears dense creamy white colonies with waxy growth. *Bacillus sphaericus* was observed to be a motile organism by motility test, found to be a gram-positive bacterium with rod shaped cells in chains. Endospore staining reveals the spore is spherical and located at the terminal position of the bacteria. The spores were stained in green and the vegetative cells were stained in red colour. Using biochemical analysis *Bacillus sphaericus* was observed to be catalase positive, as the presence of catalase enzyme that cleaves the hydrogen peroxide into hydrogen and molecular oxygen with the release of air bubbles. In the oxidase test *Bacillus sphaericus* oxidizes the reagent NN tetra methyl paraphenylene diamine dihydrochloride to a coloured product indophenol. The colourless disc changes to blue colour was observed shows oxidase positive. In the carbohydrate fermentation test *Bacillus sphaericus* did not involve in the fermentation of lactose and sucrose. And acid fermentation of dextrose was observed. In the Methyl red and voges proskauer test absence of colour change observed on addition of the MR/VP reagents to the bacterial broth. *Bacillus sphaericus* did not utilize citrate as carbon source and thus no production of acetate and alkaline carbonates, no colour change was observed in the citrate utilization test. The bacterium hydrolyzes urea to release ammonia and carbon dioxide as it contains the urease enzyme, addition of phenol red to the broth changes the colour from yellow to red was observed. In the Indole test *Bacillus sphaericus* grown on peptone rich medium on addition of Ehrlich's reagent to the culture broth no colour change was observed as the bacterium lacks tryptophanase to utilize tryptophan, thus absence of indole production. The difference in the concentration of the larvicide and the mortality rate is due to the activeness of the larva. This shows that the sensitivity varies at each stages of the larvae. But the toxicity of the bio-larvicide remain the same even for large population of mosquito larvae. Complete mortality rate of the 1st, 2nd, 3rd, 4th instar larvae was observed at the concentration of 0.04, 0.06, 0.09, 0.4 g/100mL refer to table 1,2,3,4. The *Bacillus sphaericus* larvicidal activity was confirmed and the mortality at different stages of the larvae was observed by bioassay. Also, the persistence and toxicity of the bacterium in polluted water was determined.

The protein was isolated from *Bacillus sphaericus* using Bradford's method is estimated to be 0.34mg/mL; the protein isolated from bioassay was estimated to be 0.32mg/mL; To separate the fractional proteins the samples are further analyzed using ammonium sulphate precipitation and dialyzed and the bacterial protein was estimated to be 0.32mg/mL and the bioassay sample was estimated to be 0.30mg/mL. The molecular weight of the protein was determined by SDS PAGE as 132kDa with 35 and 54 kDa respectively. The plasmid DNA of *Bacillus sphaericus* was successfully transformed to the competent cells of wild type *E. coli* using electroporation. The *E. coli* cells obtained the recombinant character of *B. sphaericus* was observed as the colonies are formed by the catalyzation of penicillin G to 6 amino-penicillianic acids. The normal *E. coli* cells lacks resistance towards the antibiotic penicillin G. Further the plasmid DNA transformation was confirmed using restriction digestion of DNA. Hind III restriction enzyme was used to fragment the DNA. It was observed that the Hind III digested samples - plasmid DNA of *Bacillus sphaericus* and the plasmid DNA of transformed *E. coli* cells were identified on the same lane. This shows that the plasmid DNA was restricted on the same site by the restriction enzyme Hind III in both the samples and it is absent in the other samples i.e. in the digested sample of wild type *E.coli*, undigested sample of plasmid DNA of *B.sphaericus* and undigested sample of plasmid DNA of transformed *E.coli*. Thus, the LB media containing penicillin G and restriction digestion of DNA confirmed the recombinant *E. coli* cells. The transformed *E. coli* cells were also tested against the *Culex quinquefasciatus* larvae in laboratory conditions and larval mortality was observed. So, the broad spectrum of pathogenicity against the *Culex* larvae has been achieved.

Table 1: 1ST Instar Stage

Concentration of *Bacillus sphaericus*	Dead larvae	Live larvae
0.01g/100ml	8/10	2/10
0.02g/100ml	9/10	1/10
0.03g/100ml	9/10	1/10
0.04g/100ml	10/10	1/10
0.05g/100ml	10/10	0/10
0.06g/100ml	10/10	0/10
0.07g/100ml	10/10	0/10
0.08g/100ml	10/10	0/10
0.09g/100ml	10/10	0/10
0.1g/100ml	10/10	0/10
0.2g/100ml	10/10	0/10
0.3g/100ml	10/10	0/10
0.4g/100ml	10/10	0/10
0.5g/100ml	10/10	0/10

Table 2: 2nd Instar stage

Concentration of *Bacillus sphaericus*	Dead larvae	Live larvae
0.01g/100ml	7/10	3/10
0.02g/100ml	7/10	3/10
0.03g/100ml	8/10	2/10
0.04g/100ml	8/10	2/10
0.05g/100ml	9/10	1/10
0.06g/100ml	10/10	0/10
0.07g/100ml	10/10	0/10
0.08g/100ml	10/10	0/10
0.09g/100ml	10/10	0/10
0.1g/100ml	10/10	0/10
0.2g/100ml	10/10	0/10
0.3g/100ml	10/10	0/10
0.4g/100ml	10/10	0/10
0.5g/100ml	10/10	0/10

Table 3: 3rd Instar Stage

Concentration of *Bacillus sphaericus*	Dead larvae	Live larvae
0.01g/100ml	6/10	4/10
0.02g/100ml	6/10	4/10
0.03g/100ml	7/10	3/10
0.04g/100ml	8/10	2/10
0.05g/100ml	8/10	2/10
0.06g0100ml	8/10	2/10
0.07g/100ml	9/10	1/10
0.08g/100ml	9/10	1/10
0.09g/100ml	10/10	0/10
0.1g/100ml	10/10	0/10
0.2g/100ml	10/10	0/10
0.3g/100ml	10/10	0/10
0.4g/100ml	10/10	0/10
0.5g/100ml	10/10	0/10

Table 4: 4th Instar Larvae

Concentration of *Bacillus sphaericus*	Dead Larvae	Live Larvae
0.01g/100ml	80/100	20 /100
0.02g/100ml	81/100	19/100
0.03g/100ml	85/100	15/100
0.04g/100ml	86/100	14/100
0.05g/100ml	90/100	10/100
0.06g/100ml	90/100	10/100
0.07g/100ml	90/100	10/100
0.08g/100ml	93/100	7/100
0.09g/100ml	94/100	6/100
0.1g/100ml	96/100	6/100
0.2g/100ml	99/100	4/100
0.3g/100ml	99/100	4/100
0.4g/100ml	100/100	1/100
0.5g/100ml	100/100	1/100

4. Discussions

Environmentally friendly bioinsecticides have shown controlling the mosquito population. Evaluation of *Bacillus sphaericus* bio insecticide produced with white soybean meal as culture medium for the control of *Culex quinquefasciatus* [1]. This study reported that using white soybean as culture medium for growing *Bacillus sphaericus* can yield increased sporulation and thereby high level of toxicity to the mosquito larvae. Similarly, in the present study high soybean medium was used to culture the bacterium and thereby high number of spores was observed to be produced at 36hours of bacterial culture and at 48hours the bacteria culture was highly toxic to the *Culex quinquefasciatus* was observed. Also, this soybean medium is selective medium for the bacteria where the bacteria utilizes the medium well and mature culture is found at 36-38hours of incubation period. The study by Andre *et al* [1] with Five different dilutions of the bioinsecticides were used 10mg/L, 4mg/L, 2mg/L, 1mg/L, and 0.5mg/L to the recipients containing the L4 larvae, and the larval mortality rate of *C. quinquefasciatus* was highly susceptible to the product prepared with white soybean meal produced an LD50 of 0.90mg/L and an LD90 of 2.26mg/L. Another study revealed that the elimination of Mn^{2+} from the complex growth medium of *Bacillus sphaericus* leads to decrease in the sporulation i.e. cells grown either in complex or defined media the toxicity remains the same even at low spore density. The toxin was observed to be unstable and the activity was destroyed due to heat and freeze-thaw cycle or both lead to cell wall damage by Paula Myers and Alan a Yousten [3]. The current study used Nutrient yeast mineral salt media which contains Mn^{2+} and Hi soybean media for the growth and culture of the bacillus sphaericus. For bioassay 48hours bacterial cultures grown on soybean media is used. The 4th instar larvae reached 100% mortality at 0.4g/100mL and 90% mortality at 0.05g/100mL. This shows the growth and sporulation of the bacteria is enhanced by the Soybean Hi-veg media and thereby increasing the toxicity. when compared to the NYSM media the growth and sporulation of *Bacillus sphaericus* was better in Soybean Hi veg media. Transfer of the Toxin Protein Genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and Their Expression [2]. This study suggests that *B. sphaericus toxin genes* can be expressed at high level in *B. thuringiensis* subsp. *israelensis* and capable of forming crystalline structures, their expression of the *B. sphaericus* toxin genes does not interfere with the synthesis of the endogenous *B. thuringiensis* subsp. *israelensis* crystal proteins. As *B. sphaericus* toxin is toxic mainly to the larvae of *Culex* and *Anopheles* species, while the B. thuringiensis subsp. israelensis inclusions are more active against *Culex* and *Aedes* species and *B. sphaericus* also has the benefit of longer persistence in polluted aquatic ponds. The transformants produced crystal proteins of both *B. thuringiensis subsp. Israelensis* and *B. sphaericus*, was highly active against the three mosquito species and showed wide spectrum of toxic activity by Catherine *et al* [2]. In the same way the current study focused on the transformation of *B. sphaericus* plasmid DNA to *E. coli* cells. As like *B. sphaericus*, *E. coli* cells also persist in organic rich polluted waters which are the mosquito breeding places. So, *E. coli* is chosen as a recipient for the expression of *B. sphaericus* plasmid DNA by electroporation transformation. The transformants have gained the penicillin G antibiotic resistance activity from *B. sphaericus* and have grown on the

culture medium containing it. Further determined by restriction digestion of the DNA using Hind III and found that the transformants contain the plasmid DNA of *B. sphaericus*. The transformed *E. coli* cells were tested under laboratory conditions against the *Culex quinquefasciatus* larvae and larval mortality was observed. So, the broad spectrum of pathogenicity against the *Culex* larvae has been achieved.

5. Conclusions

Hence *Bacillus sphaericus* can be used as a bio-larvicide specific for the mosquito larvae *Culex quinquefasciatus* and the toxicity is effective against all 4 stages of the larvae is proved by bioassay refer to figure 1.1,2.1,3.1,4.1. The bacterial and bioassay sample proteins are isolated, estimated to achieve protein purification and separation and molecular weight of the protein was determined. Successful transformation of *Bacillus sphaericus* plasmid DNA to wild type *E. coli* cells and proven that the transformants have obtained the recombinant character. *Bacillus sphaericus* can be used as effective bio-control agent against *Culex quinquefasciatus* to prevent the spread of the vector borne disease lymphatic filariasis.

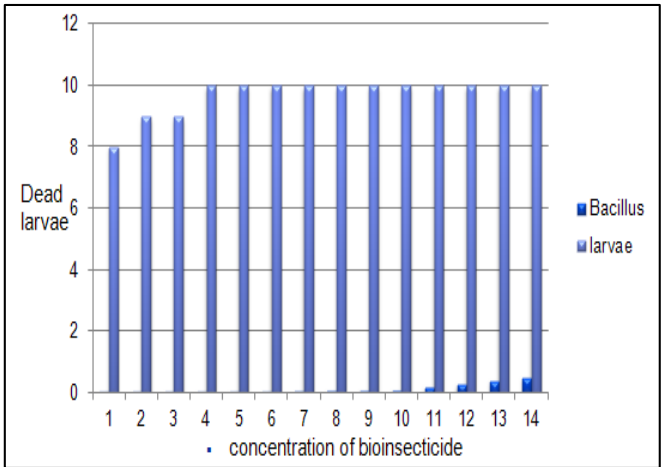

Fig 1: 1st Instar Larva mortality

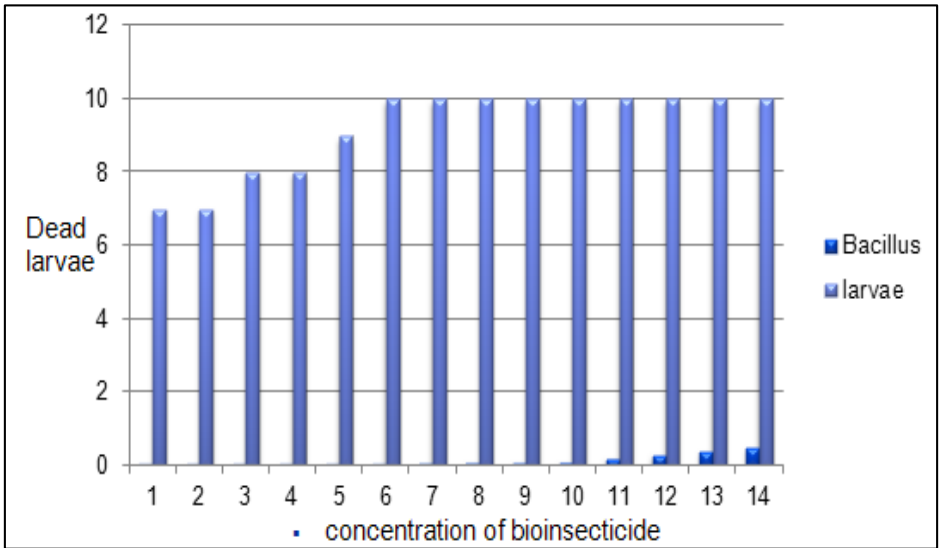

Fig 2: 2nd Instar Larva mortality

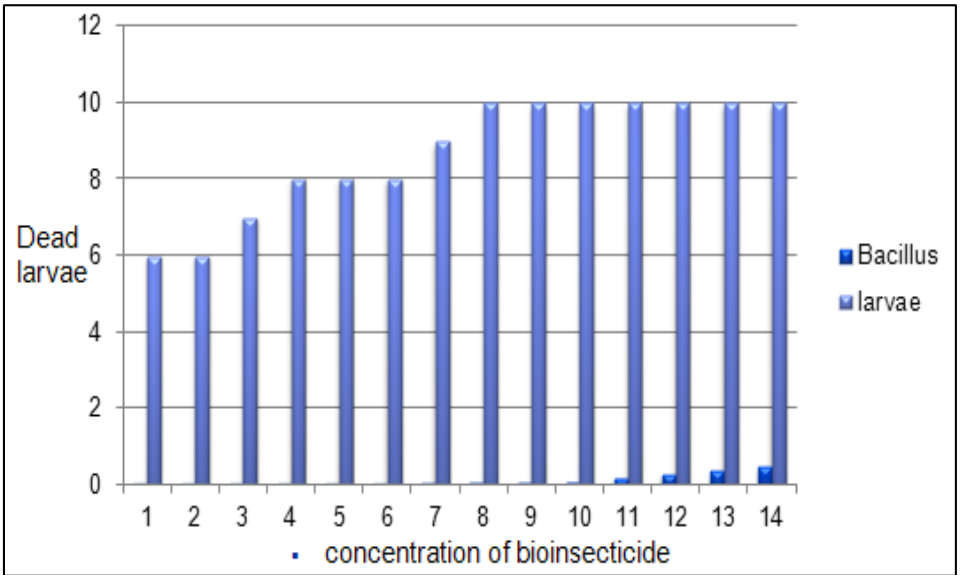

Fig 3: 3rd Instar Larva mortality

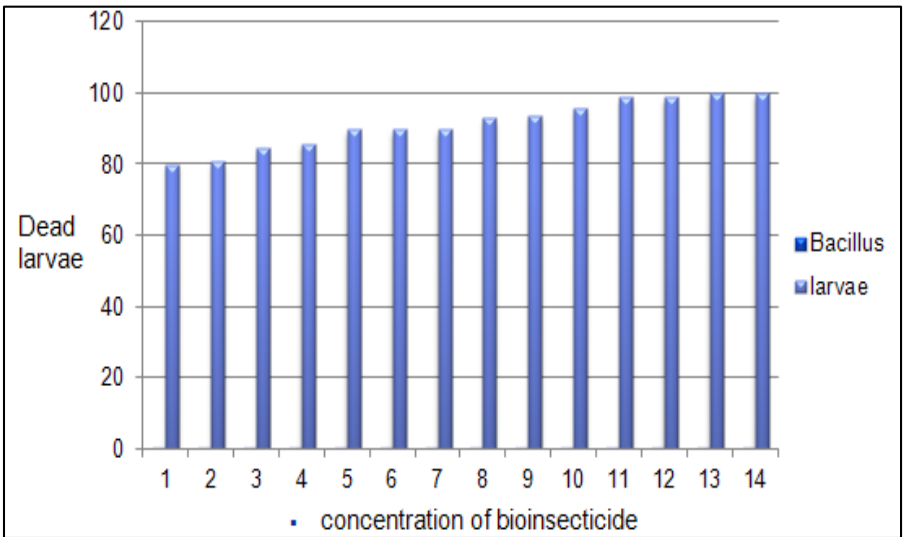

Fig 4: 4th Instar Larva mortality

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