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Evaluation of Larvicidal Efficacy of Actinomycetes, Isolated from the Soil, against Dengue Vector, *Aedes aegypti* L.

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Abstract

Soil samples were collected from various parts of Delhi and bacteria were isolated from collected soil samples through spread plate technique. The isolates were characterized based on their biochemical and morphological structures, and identified as *Actinomycetes* sp. The characterization of bacteria was confirmed according to Bergey's Manual of Systematic Bacteriology.The larvicidal potential of isolated bacteria was assessed against dengue vector, *Aedes aegypti* using standard WHO protocol. Preliminary and quantitative larvicidal activity was performed against the early fourth instars of *Aedes aegypti*, maintained in the laboratory under controlled conditions of 28 ± 1 °C and 80 ± 5 % RH with a photoperiod of 14h daylight/10h darkness. The investigations prove the significant larvicidal efficacy of the bacterial isolate; *Actinomycetes* exhibiting LC_{50} values of 4.9 mg/mL and LC_{90} values of 11.7 mg/mL. The present study concludes that non-pathogenic bacteria present in the natural environment can be used as potential bio-control agents against the larvae of *Ae. aegypti*. Further studies are needed to identify the bioactive secondary metabolite of bacteria effective against dengue vector larvae.

Keywords: Actinomycetes, Aedes, larvicidal, metabolite

Introduction

Mosquitoes are known vectors for many human diseases, the diseases prevalent in more than 100 countries across the world, infecting every year over 700,000,000 people globally and 40,000,000 of the Indian population (Ghosh *et al.* 2012). Driven by the increasing number of reported cases year after year; the dengue fever mosquito *Ae. aegypti*, continues to remain one of the most prevalent and serious disease vectors across the tropical and sub-tropical areas (WHO 2012). In India, Union health ministry reported a total of 50,222 dengue cases, which alarmingly increased to 75,454 cases causing 167 fatalities in 2013 (NVBDCP 2014). Synthetic chemical insecticides are popularly used as the first line of defence against mosquitoes owing to their quick action.

Over the last five decades the indiscriminate and frequent use of these synthetic insecticides has led to the multifarious problems *viz.*; insecticide resistance, permanent residual effect on the environment, destabilization of the ecosystem; and toxic hazards to human and non-target organisms (Warikoo and Kumar, 2013).This has augmented concerns considerably for public health and environmental security requiring detection of natural products that may be used against insect pests.

It is known that larvicides play a vital role in controlling mosquitoes in their breeding sites thus, making it is easy to deal with them in this habitat. This has stimulated a search for safer and effective alternative bioactive larvicidal material. Although various biocontrol measures are in vogue, their effective control of larval mosquitoes has not been hitherto highlighted. Microorganisms and microbial product with potential insecticidal activity can play an important role in controlling diseases by interrupting transmission mechanism by killing insect vectors at community level (Chaufaux et al, 1997).

Bacteria are probably best described as an opportunistic pathogen in insect habitats. *Bacillus sphaericus*and *B. thuringiensis*strains have been effectively used for the control of mosquitoes breeding in a variety of habitats (Merritt et al., 2005, Geetha and

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Manonmani 2010). These bio-control agents are targeted as stomach poisons against larval stages of mosquitoes. However, there are some reports indicating development of resistance in mosquitoes against microbial agents too (Mir et al., 2003). These reports have prompted many workers to look for new microorganisms and/or their metabolites with mosquito control potential. Soil is one of the most diverse habitats on earth and contains one of the most diverse assemblages of organisms. *Pseudomonas fluorescens* Migula has been reported to be effective against larvae and pupae of mosquito vectors, though it showed more pronounced effects on pupal stages (Prabakaran et al, 2003).

Limited reports are available regarding the soil bacteria as novel agents of mosquito control. Keeping this in view and need for an environment friendly new microorganism for mosquito control, present investigations involve the isolation of non-pathogenic bacteria from soil collected from Delhi, India; their identification and characterization. The larvicidal efficacy of microbial isolates was assessed against Indian strain of dengue vector, *Aedes aegypti* L. This could help in formulating strategy for mosquito management programmes.

Materials and Methods

Collection of Soil Samples

The soil samples were collected from a depth of 5 cm, at random from different locations of New Delhi, India. The selected locations include; Banks of Yamuna River (S1), Garden of University of Delhi (S2), Garden of IGNOU (S3) and Premises of Acharya Narendra Dev College (S4). The samples collected were brought to the laboratory in sterile polythene bags to avoid any external contamination. The soil samples were dried and stored at 4°C for further study.

Isolation of Bacteria

The soil samples were diluted up to 10^{-6} and 0.1 mL aliquots of each sample were spread over Nutrient agar medium, sterilized at 121°C in 15 lbs pressure for 15 min incubated at 28 °C for 48h. Five replicates were maintained for each dilution. Loopful of individual colonies of bacterial isolates were picked up and purified by streaking on starch casein agar (SCA); sterilized at 121°C in 15 lbs pressure for 15 min and supplemented with amphotericin B 50 µg/ml to prevent the growth of bacterial and fungal contaminants. The inoculated plates were incubated at 28°C for seven days. After incubation the bacterial isolates were purified and kept at 4°C for further investigations.

Identification of *Actinomycetes*

Isolated bacterial strains were identified based on their morphological, microscopic, biochemical and staining characteristics. The identification was confirmed according to Palleroni (1986).

Microscopic study by Gram's staining method

Gram's staining was performed to determine the size, shape and arrangement of bacteria.

Motility test by hanging drop technique

The motility test was performed by hanging drop technique as described by Cowan (1985). Hanging drop slide was prepared by broth culture and examined under 100X power objective.

Biochemical Characterization of Bacteria

The tests were carried out as described by Cheesbrough (1985).

(a) TSI agar slant

The test organisms were cultured into TSI agar slant (comprise of agar, phenol red, 1% lactose, 1% sucrose, 0.1% glucose, sodium thiosulphate and ferrous sulphate) by stab or streak method. Fermentation of any of the three sugars in the medium will produce acid by-products which will change the colour of the phenol red to a yellow colour.

(b) Carbohydrate fermentation test

The 0.2 ml of nutrient broth culture of the isolated organisms was inoculated into the tubes containing three sugars; dextrose, maltose and mannitol. The tubes were incubated for 24 h at 37ºC. The production of acid was indicated by the colour change from red to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube.

(c) Catalase test

The 3 mL of catalase reagent (3% H_2O_2) was taken in a test tube. The isolated colony was taken with a glass rod and merged in the reagent. The formation of bubble indicated positive test.

(d) Methyl Red test

Single colony from the pure culture of the test organism was inoculated in 5 ml of sterile MR-VP broth. After 5 days incubation at 37ºC, 5 drops of methyl red solution was added and observed for colour formation. Development of red or yellow colour indicated positive or negative result, respectively.

(e) Voges -Proskauer (V-P) test

The test organisms were grown in 3 ml of sterile MR-VP broth at 37ºC for 48 h. Thereafter, 0.6 mL of 5% alpha-naphthol and 0.2 mL of 40% potassium hydroxide containing 0.3% creatine was added per mL of broth culture. The broth was shaken well and allowed to stand for 5-10 minutes to observe the colour formation. Development of pink-red colour indicated positive result.

(f) Indole test

The test organisms were cultured in 3 ml of peptone water containing tryptophan at 37ºC for 48 h. One ml of diethyl ether was added, shaken well and allowed to stand until the ether rises to the top. Then 0.5 ml Kovac's reagent was gently run down the side of the test tube to form a ring in between the medium and the ether. Development of brilliant red coloured ring indicated positive test (Cheesbrough, 1985).

(g) Citrate test

A tube containing citrate medium was inoculated with a small amount of bacteria and incubated at 30-37ºC for 24-48 h. The positive test result shows growth in citrate medium with colour change to blue.

(h) Hydrogen sulphide production

A deep inoculation was performed in the H_2S medium with bacteria and the tube was incubated at 30-37ºC for 24-48 h. The positive test results ina black precipitate in the medium.

(i) Urease test

The bacteria were grown in urease medium containing a pH indicator. The presence of urease will change the colour of medium to red.

(j) Oxidase test

Oxidase reagent dropper was held upright between thumb and forefinger. The tube was squeezed to break a glass ampule inside the plastic tube. The dropper was turned upside down and a few drops of reagent were applied to a strip of filter paper. With a sterile needle, the colony of bacteria was picked and streaked on the reagent-soaked filter paper. If oxidase is present, the reagent will undergo a chemical reaction resulting in a violet or purple colour change.

Extraction of Extracellular compounds of Bacteria

The bacterial isolate was inoculated into 500 mL conical flask containing 200 mL of starch casein liquid medium and incubated at 28± 2°C aerobically on a rotary shaker at 200 rpm for seven days. The cell-free culture filtrates were separated by centrifugation at 10,000 rpm for 15 min for assessment of larvicidal activity. The bacterial cell pellet was collected and washed two times with sterile distilled water and re-suspended in sterile distilled water. The bacterial concentration was determined by dry cell weight.

Rearing of Mosquitoes

The present investigations employ the dengue fever mosquito, *A. aegypti*, originated from fields of Delhi and surrounding areas. The colony was maintained in an insectary at 28±1°C, 80±5% RH and 14:10 L/D photoperiod (Warikoo*et al.* 2013). Wet cotton was kept on the top of each cage to provide water for the mosquitoes. Water-soaked split raisins were kept in the cage, mainly as a source of the food for the male mosquitoes. Periodic blood meals were provided to female mosquitoes for egg maturation by keeping restrained albino rats in the cages. The eggs were collected in an enamel bowl lined with Whatman filter paper on all the sides and half-filled with de-chlorinated tap water. The eggs were allowed to hatch in trays filled with de-chlorinated water. The newly hatched larvae were reared in enamel trays (25 x 30 x 5 cm) containing de-chlorinated water. The larvae were provided daily with food consisting of finely ground dog biscuits and yeast in the ratio of 3:2 by weight. Care was taken to prevent formation of any scum on the surface of water. Pupae formed thereafter were collected into an enamel bowl filled with water and kept in the cage for adult emergence.

Larvicidal Bioassay of Bacteria against *Aedes aegypti*

Laboratory bioassay of the formulations was conducted against IV instar larvae of laboratory reared mosquito species, *Ae. aegypti.* Different doses of the formulation (in terms of mg protein mL^{-1}) were prepared by diluting the concentrated formulation with the required quantity of sterile distilled water and mixing in a vortex mixer. The early fourth instars of mosquitoes were taken, in batches of 25, in plastic bowls containing 89 mL of distilled water and transferred to glass jar containing 110 mL of distilled water and 1 mL of bacterial formulation. Four replicates were carried out simultaneously for each extract making a total of 80 larvae for each test. Controls were exposed to the distilled water alone. During the treatment period, the larvae were not provided with any food. The dead and moribund larvae were recorded after 24 hours as larval mortality.

Statistical analysis of data

The larvicidal tests with more than 20% mortality in controls and pupae formed were discarded and repeated again. If the control mortality ranged between 5-20%, it was corrected using Abbott's formula (Abbott 1925).

Corrected Mortality =

$$\frac{\text{\% Test Mortality - \% Control Mortality X 100}}{\text{100- \% Control Mortality}}$$

The data were subjected to regression analysis using computerized SPSS 16.0 Programme. The LC_{50} and LC_{90} values with 95% fiducial limits were calculated in each bioassay to measure difference between the test samples.

Results and Discussion

Biological control with entomopathogenic bacteria has been increasingly used as a larvicide to control populations of various medically important dipterans of the genera*Culex* and *Aedes*. Like chemical larvicides, these agents can cause drastic density-dependent mortality, killing all larvae within 24–48 h, after breeding site treatment. Moreover, they are selective to insects and are consequently considered soft to non-target fauna commercial products. Natural soil sample is an excellent residence for plentiful microbes which have ability to produce secondary metabolites applied in bio-control activities. Several varieties of microorganisms, including fungi, bacteria and viruses, have been reported to be entomopathogenic. Studies have proved *B. thuringiensisisraelensis*(*Bti*) and *B. sphaericus*(*Bsp*) as entomopathogenic bacteria that have abilityto control the larvae of *Ae. aegypti*(Merritt et al., 2005, Geetha and Manonmani 2010).

Actinomycetes are very well known and successfully exploited as a source of secondary metabolites. There are over 23,000 known microbial secondary metabolites, 42% of which are produced by Actinobacteria, 42% by fungi and 16% by other bacteria (Kekuda et al. 2010). They are effectively used against *Culexquinquefasciatus*by producing bioactive compounds (Sundarapandin et al., 2002). Keeping this in view, investigations were conducted to isolate natural harmless bacteria from the soil samples. The studies resulted in the isolation of, *Actinomycetes* sp., from different soil samples. The bacteria were identified on the basis of their staining, morphological and biochemical characteristics, the results of which are presented in Table 1. The isolate colony was dark grey in colour, non-motile and spiral-shaped (Table 1; Fig. 1, 2). The bacteria was proved to be Gram positive, non-acid fast and caused sugar fermentation. Kekuda et al. (2010) reported isolation of two strains of Gram positive Actinomycetes, identified as Streptomyces, causing hydrolysis of starch and casein. Vijayakumar et al (2010) isolated a total of thirty different marine actinomycetes from marine soil and sediment samples collected from different locations of Muthupet mangrove, Tamil Nadu, by serial dilution plate technique on starch casein agar medium. Recently, Anwar et al. (2014) isolated 51 species of *Actinomycetes* from salt range, Pakistan.

Our study reports that the larvicidal efficacy of isolated *Actinomycetes sp.* against IV instars of*Ae. aegypti*exhibiting dose-mortality relationship. The larvicidal bioassay revealed the significant LC_{50} values of 4.9 mg/mL and LC_{90} values of 11.7 mg/mL, respectively resulting in 100% mortality at 12 mg/mL. These results are in conformity with Kekuda et al (2010) who isolated two species of *Actinomycetes* from the soil and reported higher larvicidal efficacy of isolate 2 as compared to isolate 1 against *Ae. aegypti* larvae, with 100% larval mortality at 5mg/mL. Thirty actinomycetes isolated from marine soils were investigated for their larvicidal activity against *Anopheles* mosquitoes, out of which, 23 isolates were reported to exhibit larvicidal activity in preliminary screening (Vijayakumar et al., 2010).

Table 1: **Morphological and Biochemical characteristics of *Actinomycete***

S. No.	Identification Tests	*Actinomycetesp.*	Observation
Staining Characteristics			
1.	Gram Stain	Purple colour	Positive
Morphological Characteristics			
1.	Colour	Dark grey	-
2.	Shape	Spiral	-
3.	Motility	Non-motile	Negative
Biochemical Characteristics			
1.	Indole production test	No red coloured ring formed	Negative
2.	Methyl Red test	Yellow colour	Negative
3.	Voges-Proskauer test	No pink red colour	Negative
4.	Citrate utilization test	Blue colour	Positive
5.	H_2S Production test	Absence of black precipitate	Negative
6.	Urease test	Red colour	Positive
7.	Catalase test	Formation of bubbles	Positive
8.	Oxidase test	No purple colour	Negative
9.	Triple sugar iron test	Red colour	Alkaline
10.	Dextrose fermentation test	Yellow colour withproduction of gas	Positive
11.	Maltose fermentation test	Yellow colour with production of gas	Positive
12.	Mannitol fermentation test	Yellow colour withproduction of gas	Positive

Table 2:Larvicidal activity of *Actinomycetes* against early fourth instars of *A. aegypti*

Time of exposure	Larvicidal activity	S.E	χ2(*df*)	Regression Coefficient	
	LC_{50} (mg/mL)	LC_{90} (mg/mL)			
24 hours	4.913 (2.760#-8.296*)	11.757 (27.335#-92.217*)	0.506	11.234 (4)	3.382
48 hours	3.942 (1.910#- 5.928*)	10.429 (7.493#- 12.386*)	0.56	2.426(4) 3.721	

Lower fiducial limit; * upper fiducial limit

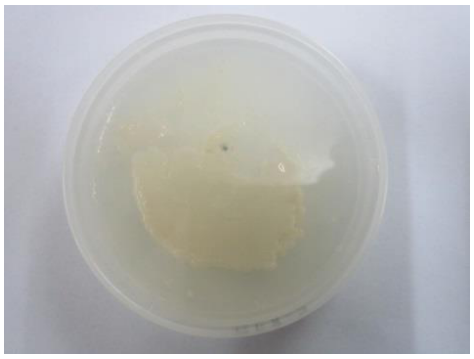

Fig1: *Actinomycetes* colony isolated on agar plate

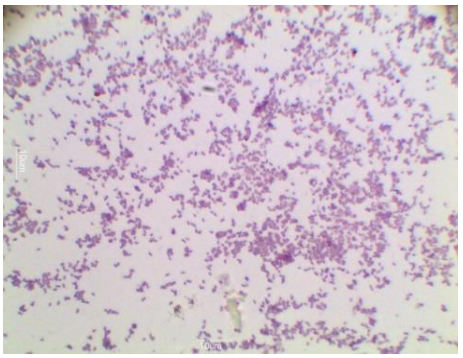

Fig2: Gram's staining of *Actinomycetes* bacteria

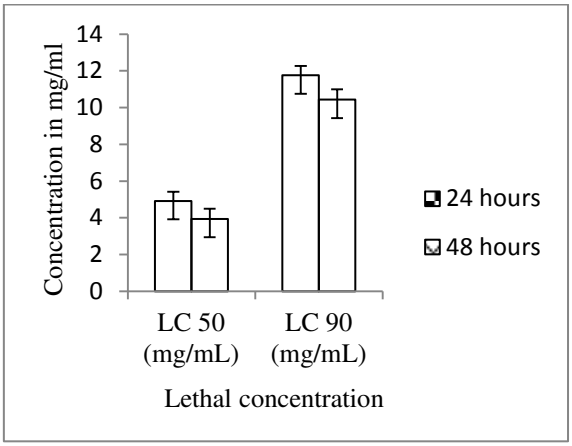

Fig3: Lethal concentration in 24h and 48h

Prabhakaran et al (2003) developed a formulation from the metabolite(s) of a novel *Pseudomonas fluorescens* Migula strain (VCRC B426) and tested against IV instars and pupae of three species of vector mosquitoes, *An. stephensi*Liston, *Cx. quinquefasciatus* Say and *Ae. aegypti*L. They reported the larvae and pupae of *An. stephensi* being the most susceptible to the formulation, followed by those of *Cx. quinquefasciatus* and *Ae. aegypti*. The LC_{50} values calculated were 70.4, 511.5 and 757.3 µg protein/mL respectively, for the larvae of three species, and 2.0, 9.4 and 19.2µg protein/mL, respectively, for the pupae. Dhanasekaran et al.(2010) reported the strong larvicidal activity of *Actinomycete* isolates againstAnopheles larvae, thoughonly 4 isolates exhibited the potential of inhibiting (100%) the growth of larvae.

An attempt has been made to assess the potential role of *Actinomycetes* to control mosquitoes. Keeping in view the resistance developed by the mosquito larvae against chemical insecticides, bio-control agents are generally preferred in vector control measures due to their less deleterious effect on non-target organisms. Thus, it is worthwhile to identify new active compounds from these agents against mosquitoes. Further studies of the active principles involved and their mode of action, formulated preparations for enhancing potency and stability, toxicity and effects on non-target organisms and the environment, and field trials are needed to recommend *Actinomycetes*as an anti-mosquito product used to combat and protect from mosquitoes in a control program.

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