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STUDIES ON MOSQUITOCIDAL ACTIVITY OF METABOLITE FROM *PSEUDOMONAS* SPECIES

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ABSTRACT: Mosquitoes like *Aedes aegypti* and *Culex quinquefasciatus* act as a vectors in transmitting disease agents which are responsible for more than 500 million clinical cases estimated by the World Health Organization. The development of strategies to control mosquito population is currently being facing problems such as resistant varieties of mosquitoes, ineffectiveness of chemical insecticides and environmental issues. The alternative technique is biological control for minimizing mosquito population. Therefore, microbial insecticides can be considered as effective alternatives to chemical insecticides that lead to reduce the harmful effect of chemical insecticides on environment. In this research work the bacterial strains of *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094) were used to find their ability to produce the toxin which can inhibits the Mosquitoes growth. The *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094) has ability to produce extracellular exotoxin which has important mosquitocidal activity against the *Aedes aegypti*. The exotoxin produced by the *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094) was partially purified and then its mosquitocidal activity was confirmed. These biological control of mosquito population by exotoxins produced by *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094) might be helpful in designing the novel approaches in prevention of vector borne diseases by controlling the vector population.

KEYWORDS: *Pseudomonas Species*, Exotoxin, Mosquitocidal activity.

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1.INTRODUCTION

Over 2500 different species of mosquitoes are present throughout the World. The studies showed that in India three vector-borne diseases namely Malaria, Dengue, and Chikungunya are more prevalent [1]. The *Aedes aegypti* was found to be major vector for chikungunya and dengue transmission [2]. Reports suggests that about 151 districts of eight states/provinces of India have been affected by Chikungunya fever till 10 October 2006 [3] and thus it is important to control such mosquito vectors by control of their habitat control, use of insecticides, introduction of sterile male mosquitoes, reduction of the breeding rates and larvicides [4,6]. Presently use of chemical pesticides is more common to control mosquito population. However due to development of resistance and environmental concern it creates limitations for their use [5, 7, 8] as chemical pesticides adversely affect non-target populations also [9]. For example the insecticide, DDT was among the first used to control malaria and typhus. In 1947, it was first reported that resistance to DDT in mosquitoes *Anopheles taeniorhynchus has been* developed after four years in use [10]. Also *Aedes* mosquito has developed resistance to all major insecticide groups such as organophosphate, pyerthroids, organochlorine and carbamate. [11-16]. Therefore, to control the mosquito population biological means can be used as an alternative to chemical pesticides. This earlier research leads a platform to find new biological control of mosquito population. The present study revealed that there is a insecticidal activity among the metabolites of various bacteria like *Pseudomonas fluorescens, Pseudomonas pseudomallei and Pseudomonas aeruginosa* [17]. According to Vector Control Research Centre (VCRC), Pondicherry, larvae as well as pupae of vector mosquitoes can be killed by liquid formulation of *Pseudomonas fluroscens* metabolite and it is safe to mammalians [18]. For control of mosquitoes breeding in a variety of habitats two bacterial agents such as the *Bacillus thuringiensis* and *Bacillus sphaericus* are being widely used [19]. These bio-control agent acts against larval stages of mosquitoes when get ingested and act as stomach poison. However, some recent reports indicate development of resistance in mosquitoes against microbial agents too [20]. The studies showed that *Pseudomonas fluorescens* that contain delta endotoxin gene of *Bacillus thuringiensis*, produces 4 times more toxin protein and has the more potent in killing insect pests [21]. Lethal effects against vector mosquito have been showing by Bacteria such as *Pseudomonas fluorescens* [22, 23].In the present study the bacterial strains of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were used to check their ability to inhibit the growth of Mosquitoes. The *Aedes aegypti* larvae was grown in a pot in which extracellular broth of

Pseudomonas fluorescence (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) was added and then the mortality of those larvae was observed. These studies showed that the exotoxins produced by the *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) might be responsible for the killing of larvae. Hence these studies can be helpful to design new approaches to control the population of mosquitoes which might be helpful to prevent the transmission of various mosquito borne diseases.

2. MATERIALS AND METHODS

Bacterial culture maintenance

The bacterial strains of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were collected from the NCIM, National Chemical Laboratory, Pune, Maharashtra, India. These strains were maintained on nutrient agar slants as a working slant and master slant.

Inoculum Preparation

The inoculums of bacterial strains of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were prepared by using saline (0.75% NaCl). Approximately 1 ml of saline was taken in a test tube in which a loopful of the culture was added. Finally this inoculum was further used for the toxin production.

Toxin production

The bacterial exotoxin was produced from *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM-5094). The sterile Glucose Peptone Salt medium broth was taken in which these *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) inoculums was added. After that these broth was incubated on a rotary shaker for 150 rpm at 30°C for the 3 days**.** Finally after three days the produced toxins form *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were tested for its activity.

Extraction of toxin

The Glucose Peptone Salt medium broth inoculated with *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) was kept at 150 rpm at 30°C for the 3 days. After incubation the broth was centrifuged at 3000 rpm for 30 min. The cell debris was removed as a pellet and the supernatant in which the extracellular proteins and other components were taken for the further study.

Partial purification of toxin by ammonium sulfate precipitation

The extracellular broth of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) was having a toxin along with other components. Then the toxin was further partially purified by adding ammonium sulfate to the broth. The different concentrations of ammonium sulfate such as 20%, 40%, 60%, 80% and 100% were added to the broth and the activity after each concentration was checked. The concentration of ammonium sulfate showing highest activity was used for further studies.

Hatching of the eggs of *Aedes aegypti*

The hatching of *Aedes aegypti* eggs was carried out in a pot. Firstly the *Aedes aegypti* eggs sheet was taken. For hatching a clean and dry aluminium pot of capacity 5 liters was used. Then to that pot the sterile 2 liter of distilled water was added. The water in a pot was boiled to remove the excess amount of oxygen. Further the water was allowed to cool and then *Aedes aegypti* eggs sheet was added. After that pot was incubated at 30^0C and after 12 days the larvae's were harvested. Finally these larvae's was used for the further studies.

Mosquiotocidal activity of *pseudomonas species*

The mosquiotocidal activity of *pseudomonas species* was checked on *Aedes aegypti*. The extracellular broth of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM -5094) was used to check the mosquiotocidal activity on the *Aedes aegypti*. To check the mosquitocidal activity the clean and dry empty glass/plastic bottles of 250 ml capacity were taken. To which 50 ml of sterile distilled water was added. Then the 12 days old larvae of *Aedes aegypti* was added. Then each metabolite of *Pseudomonas* was added in different volumes such as 0.25 ml, 0.50 ml, 0.75 ml, 1 ml, 1.25 ml and 1.50 ml set respectively to make final concentration 0.5%, 1%, 1.5%, 2% and so on of metabolite containing broth. Further the bottles were incubation at room temperature for 72 hrs and larvicidal activity was monitered. The mortality of larvae was monitored on each day of incubation.

Protein estimation of partially purified exotoxin by Lowery method

After the ammonium sulfate precipitation the concentration of ammonium sulfate added which shows highest activity was further used for to check the protein content. The broth with highest activity was used and its protein content was determined by using Lowery method [24].

3.RESULTS AND DISCUSSION

Toxin production from *Pseudomonas fluorescence* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094)

The two species of Pseudomonas such as *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were used for the toxin production. These species were inoculated into the Glucose Peptone Salt medium broth and incubated for three days on rotary shaker at 30°C (Figure 1). After incubation the broth was centrifuged and cell debris was discarded and the supernatant was taken as a crude source of exotoxin. This procedure insured that good amount of crude exotoxin was produced by the *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) which can be partially purified by the ammonium sulfate precipitation.

Figure 1 Fermented broth of two species. A) *Pseudomonas fluroscens* B) *Pseudomonas caryophilly*.

Partial Purification Of Exotoxin

Ammonium sulfate precipitation was used for the partial purification of exotoxin. The cell free broths of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) were taken and in which different concentrations of ammonium sulfate was added such as 20%, 40%, 60%, 80% and 100%. This study revealed that good amount of proteins was precipitated at the different concentration of ammonium sulfate. The protein concentration at each different salt concentration can be estimated by Lowery method.

Hatching Of The Eggs Of *Aedes aegypti*

The *Aedes aegypti* eggs sheet (Figure 2) was used for to obtain the larvae. The *Aedes aegypti* eggs were hatched in aluminium pot of capacity 5 liters using *Aedes aegypti* eggs sheet (Figure 3). Then this pot was incubated at 30^0C for 12 days and after harvesting the larvae was used for further studies.

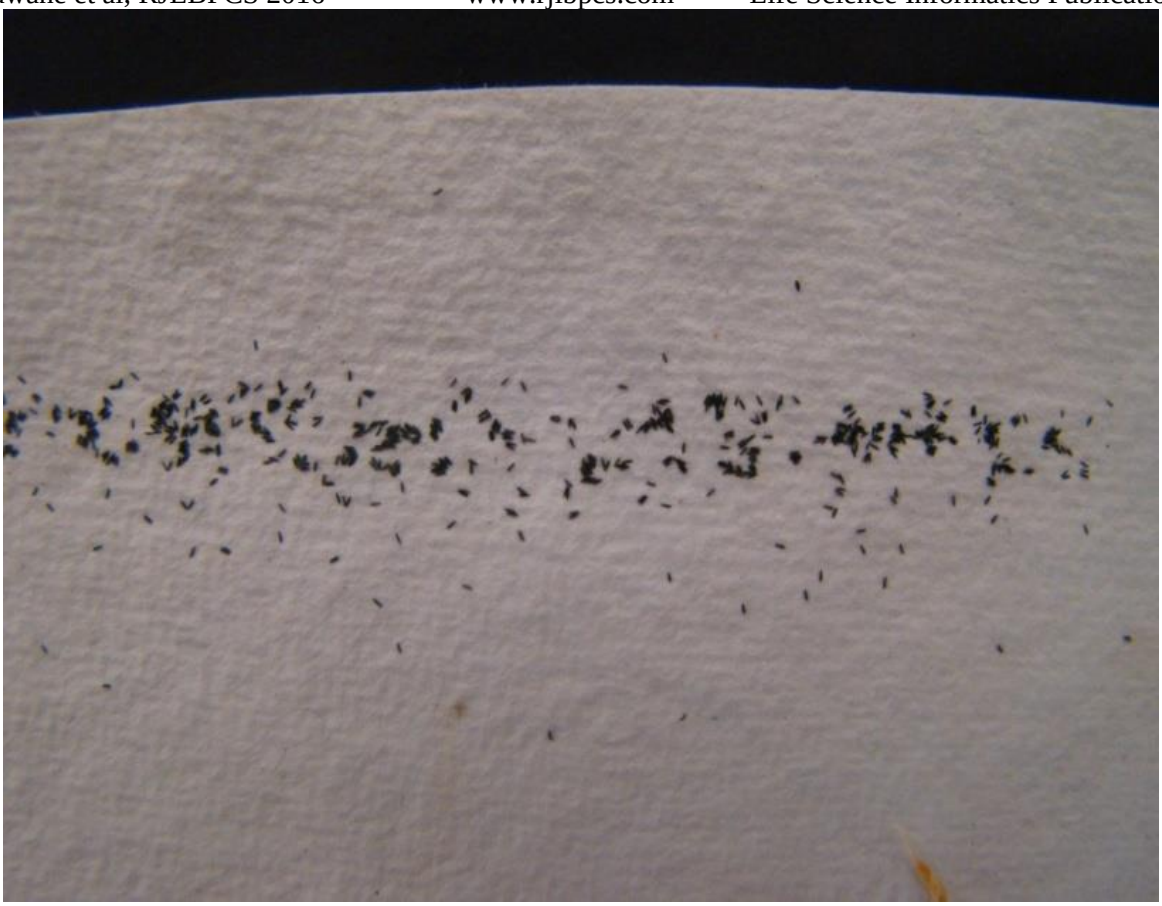

Figure 2 Egg sheat OF *Aedes aegypti.*

Figure 3 Hatching of *Aedes aegypti* egg in a pot.

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Mosquiotocidal Activity Of *Pseudomonas* Species

Mosquitocidal activity of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) was determined by using *Aedes aegypti* larvae. These larvae was incubated along with the different concentrations of cell free broths of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) saturated by the ammonium sulfate at different concentration. In this present study the cell free broth of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) at 60% saturation showed good results for the mosquitocidal activity. After the three days more number *Aedes aegypti* larvae were killed in cell free broth of *Pseudomonas fluorescence* (NCIM-2631) than *Pseudomonas caryophilly* (NCIM-5094) (Table 1 and 2). Hence the strain *Pseudomonas fluorescence* (NCIM-2631) found to be producing more amount of exotoxin than *Pseudomonas caryophilly* (NCIM-5094). Hence *Pseudomonas fluorescence* (NCIM-2631) has good Mosquiotocidal activity than *Pseudomonas caryophilly* (NCIM-5094) (Table 1 and 2).

Table 1 Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas fluorescens* (NCIM -2631).

Total mosquito larvae	Metabolite containing cell free broth of *p. fluroscens* (%)	Mosquito mortality				Percentage mortality
		First day	Second day	Third day	Total mortality	
10	0.5	0	0	0	0	0
10	1	0	0	0	0	0
10	1.5	0	0	0	0	0
10	2	0	0	0	0	0
10	2.5	1	0	0	1	10
10	3	2	0	0	2	20
10	3.5	2	1	0	3	30
10	4	3	0	1	4	40
10	CONTROL	0	0	0	0	0

Table 2 Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas caryophilly* (NCIM -5094).

Total mosquito larvae	Metabolite containig cell free broth of *p. caryophilly* (%)	Mosquito mortality				Percentage mortality
		First day	Second day	Third day	Total mortality	
10	0.5	0	0	0	0	0
10	1	0	0	0	0	0
10	1.5	0	0	0	0	0
10	2	0	0	0	0	0
10	2.5	0	1	0	1	10
10	3	0	1	0	1	10
10	3.5	0	1	1	2	20
10	4	0	1	1	2	20
10	CONTROL	0	0	0	0	0

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Mortality of *Aedes aegypti* larvae by metabolite of *Pseudomonas* Species

The Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas* Species was determined. The cell free broth of *Pseudomonas* Species in different volumes from 0.5 to 4 ml was added with the *Aedes aegypti* larvae. The studies revealed that as concentration of metabolite of *Pseudomonas* Species increases the mortality rate of *Aedes aegypti* increases. The metabolite of *Pseudomonas fluorescens* (NCIM-2631) has shown about 40% mortality of *Aedes aegypti* whereas the *Pseudomonas caryophilly* (NCIM-5094) showed 20% (Figure 4 and 5, Table 1 and 2). Hence this study revealed that the metabolite of *Pseudomonas fluorescens* (NCIM-2631) has greater mosquitocidal activity than metabolite of *Pseudomonas caryophilly* (NCIM-5094).

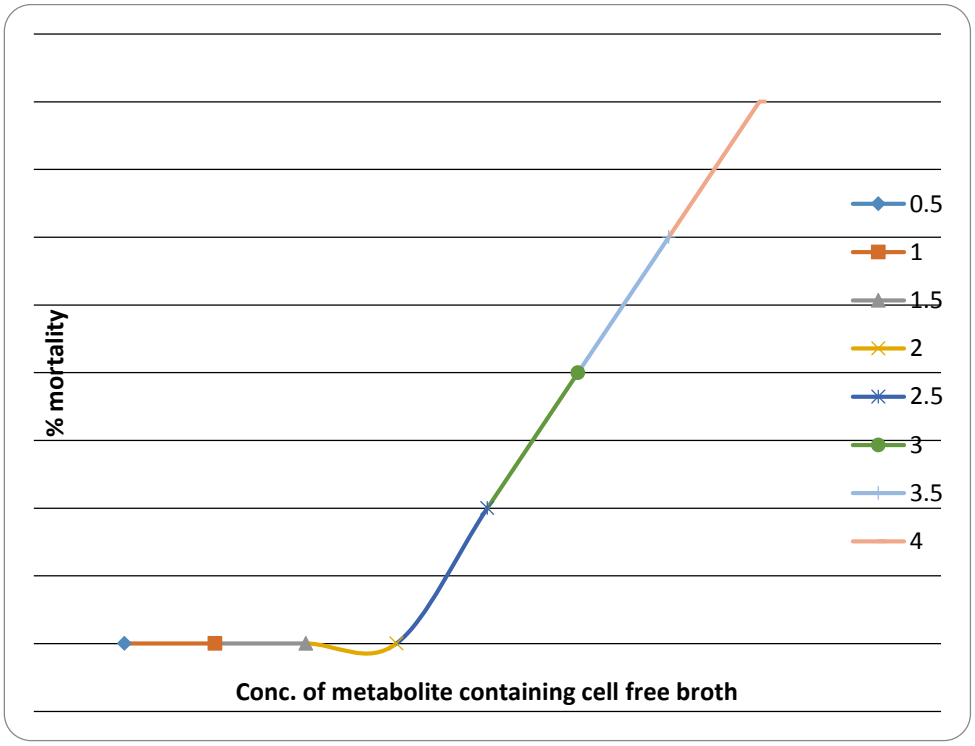

Figure 4 Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas fluorescens* (NCIM -2631).

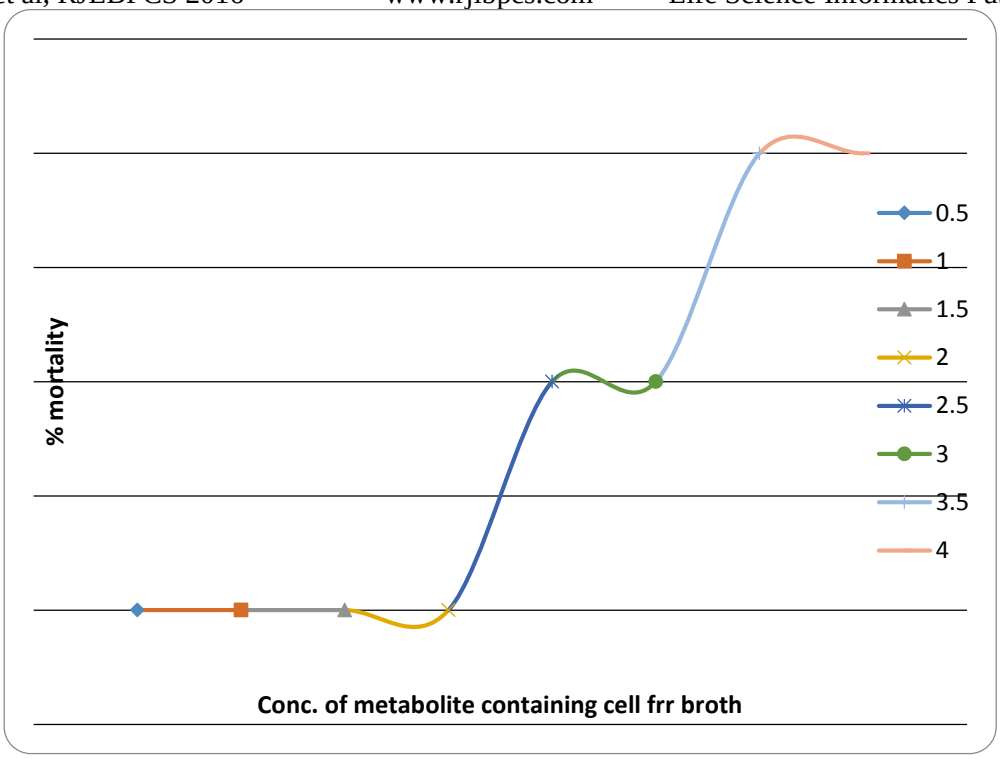

Figure 5 Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas caryophilly* (NCIM -5094).

Estimation of amount of partially purified exotoxin

The Mosquiotocidal activity was observed for the cell free broths of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094). Hence this study confirms that the cell free broth is having exotoxin which was responsible for the mosquitocidal activity. Hence to estimate the approximate amount of exotoxin produced by the cell free broth of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) Lowery method was used which showed that *Pseudomonas fluorescence* (NCIM-2631) producing 4 mg/ml protein whereas *Pseudomonas caryophilly* (NCIM-5094) producing 3 mg/ml protein.

4. CONCLUSION

Mosquito are the mainly responsible for the transmission of infectious diseases hence the increasing mosquito population is one of the threat to outbreak of infectious diseases. In the present study we demonstrated the mosquitocidal activity of *Pseudomonas fluorescence* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094). These *Pseudomonas* species are producing extracellular exotoxins which are mainly responsible for mosquitocidal activity. The Percentage of mortality of *Aedes aegypti* larvae for different concentrations of broth containing metabolite of *Pseudomonas* Species was carried. The metabolites of *Pseudomonas fluorescence* (NCIM-2631) have more larvicidal activity as it gives more percentage of mortality than *Pseudomonas caryophilly* (NCIM-5094). Hence these biological exotoxins produced by the *Pseudomonas* species might be important to control the population of mosquito and stop the disease transmission. Further work is needed to purify the exotoxin produced by *Pseudomonas* species using different purification techniques. Then this purified exotoxin can be specifically used for to control the mosquito population.

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