International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 16-08-2023, Accepted: 02-09-2023, Published: 18-09-2023
Volume 8, Issue 9, 2023, Page No. 73-77

Immobilization of *Pseudomonas aeruginosa* in alginate beads for the sustained production of mosquito larvicides against dengue vector *Aedes aegypti* (Diptera Culicidae)

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Abstract
Mosquitoes are at the center of entomological research because of their importance as vectors of various viral and parasitic diseases affecting humans. They carry the causative agents of diseases such as malaria, dengue, chikungunya, west nile fever, japanese encephalitis, and zika. The measures to control vector mosquitoes include physical, chemical, and biological methods. Chemical methods are hazardous to the environment as the mosquitoes are developing resistance to the initial dosage. Biological methods such as using Bt toxin and gambusia fishes in the breeding field are generally used to support/complement the chemical methods. Although several species of *Pseudomonas* can produce mosquito larvicide as exotoxin, its survivability is very limited in the field due to various environmental factors. The sodium alginate immobilization techniques have been proposed for a long to increase the field sustainability of *Pseudomonas* but with limited success. The advantage of these alginate immobilization spheres is that the Pseudomonas being trapped inside the beads, they release the exotoxin which comes out of the permeable membrane of the beads. At the same time, the permeable membrane allows the nutrients to pass in. In this study, we have immobilized *Pseudomonas aeruginosa* in calcium alginate beads and then it is allowed to produce the larvicidal formulations with the continuous input of the medium and the simultaneous output of the formulations at regular intervals. The larvicidal formulations are bio-assayed against the *Aedes aegypti* larvae. The results of the bioassay showed that the larvicides collected were efficient enough to kill the larval mosquitoes.

Keywords: Immobilization, sodium alginate beads, mosquito larvicide, *pseudomonas*, fermenter, dengue

Introduction

The public health burden of mosquito-borne diseases such as dengue, malaria, chickungunya, Japanese encephalitis and zikaare continuously increasing (Gould *et al*., 2008; Golding *et al*., 2015). Most of the vector-borne diseases have complex zoonotic cycles which include a primary vertebrate reservoir or host such as human and other animals and an arthropod which is the vector (Ramalho-Ortigao *et al*., 2020).Vector control is the only available option to control these diseases (Beier *et al*., 2008). However, being hazardous to the environment and remaining for long periods, chemical insecticides are still continuously used in the control operations of vector mosquitoes. Although, chemical insecticides have been used for several decades to control mosquitoes, development of resistance in mosquitoes often leads to increase the dosage and frequency of application. However, the concentration of the chemical insecticides cannot be increased beyond a certain level as it is very hazardous to the environment (Brown *et al*., 1988; Brattsten *et al*., 1986; Hemingway *et al*., 2000). Therefore, an alternative, more effective, and environment-friendly control agents are the need of the hour. Hence developing an effective, environmentally non-hazardous, eco-friendly bioinsecticide in an economically cheaper way is the dream of many scientists in this field. Microbial larvicides such as *Bacillus thuringiensis* and *B. sphaericus* are advantageous and used widely as larvicide for mosquito control (Balaraman 1995; Lee and Zairi 2005; Medeiros *et al*. 2005; Armengol *et al*.2006) [1, 21]. However, environmental instability of most of the microbial larvicides and development of resistance in vector mosquitoes to endotoxins of *B. sphaericus* (Nielsen-Leroux *et al*. 1995; Poopathi *et al*. 1999; Su and Mulla 2004), warrants novel, eco-friendly approaches in mosquito control operations. In the present study, we have developed a method for the sustained release of mosquito larvicide by encapsulating the *Pseudomonas* species in polymeric matrix of alginate beads. The metabolites of certain Pseudomonas species are acting as potent mosquito larvicide. Direct field application of *Pseudomonas* species is very limited as they would be inactivated by various environmental factors such as temperature, sunlight etc., and their interaction with the other organisms and competition among other bacteria for available nutrients. In our study, *Pseudomonas aeruginosa* was immobilized in alginate beads to release the metabolites into the environment. In addition to field usage, the immobilized bacteria can be used to produce a large quantity of larvicide in the laboratory using a bioreactor. Although the continuous fermentation in the bioreactor is highly advantageous, removal of the microbial cells in the spent broth is highly challenging (Singh *et al*., 2017) [37] and we have overcome this problem by means of immobilizing Pseudomonas in sodium alginate in the form of beads, where the Pseudomonas species still can maintain its viability for a relatively longer time with limited nutrient availability and produce mosquito larvicide continuously. The spent broth was collected continuously for seven days at regular intervals of every 24 hours. The larvicidal potential of the collected metabolite was tested as per the standard WHO protocols against *Aedes aegypti*.

Material and method

Rearing of the mosquito larvae

Mosquito larvae were collected from fields and morphologically identified according to the classification keys provided in the photographic manual of mosquito identification (Cutwa MM, O'Meara GF 1999). The mosquito larvae were reared in an enamel tray with a volume of one liter / 300 larvae and were kept at 27 °C and humidity of 85. The larvae were fed with the larval food of yeast and dog biscuit (3:1).

Pseudomonas aeruginosa

The *Pseudomonas aeruginosa* MTCC 4713 was obtained from Madurai Kamaraj University. The strains were sub-cultured on nutrient agar plates incubated at 37 C for 24 hours and maintained in slants till further use (Murty *et al*., 1994 and Roy *et al*., 2010).

Culture and harvesting of *P.aeruginosa*

A loop of pure culture of *P.aeruginosa* taken from the nutrient agar slant was inoculated in 1000ml nutrient broth and kept under 37ºC incubation for 24 hours. Then the cultures were centrifuged at 2000rpm for 20 minutes. The supernatant was discarded, and the pellets were collected.

Immobilization in sodium alginate

Immobilization of *P.aeruginosa* in alginate beads was done based on the method of Bettman and Rehm (1984). The sodium alginate (3%) was dissolved in distilled water and autoclaved at 121 C for 15 min. The fresh bacterial pellet of *P.aeruginosa* (3g) was mixed in 100mL sterilized sodium alginate solution. This mixture was extruded drop by drop into a sterile 0.2M Calcium chloride solution using a sterile syringe. To improve the structural integrity of the beads, the mixture was stirred for about 15 minutes. Finally, these beads were washed with sterile distilled water and were then harvested using a strainer.

Fig 1&2: *Pseudomonas* immobilized in sodium alginate

Fermentation process ofmosquitolarvicide

The *Pseudomonas aeruginosa* immobilized in sodium alginate was placed in a beaker with 700 ml of sterilized tap water and kept undisturbed in room temperature. Thespent broth of 100 ml was collected at regular intervals of 24 hours for seven days and the sterilized tap water was replaced to the beaker. The larvicidal efficacy of the spent broth was determined by bioassay, based on WHO protocol.

Bioassay

The spent broth was subjected to bioassay to check the mortality of the vector *Aedes aegypti* mosquito. Twenty-five third instar larvae of the mosquito species were transferred to 100 ml tap water containing various concentrations (100%, 50%, 25%, 12.5%) of spent broth. Larval controls were maintained throughout the bioassay. The bioassay was carried out in three batches and triplicates were done in each batch for all the concentrations. After exposure of the larvae, it was kept undisturbed overnight. After 24 hours and 48 hours, the larval mortality was recorded. The percentage of larval mortality and LC 50 of the spent broth was calculated.

Calculation of percentage mortality

To check the Percentage mortality of larvae is the ratio of the number of larvae killed to the total number of larvae used.

$$\% \text{ mortality} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae used}} \times 100$$

LC50 determination

The LC50 of the spent broth was determined based on WHO guidelines 2005. The minimum concentration inhibits 50% of the larval population was considered as LC50. The mortalities of treated groups were calculated according to Abbott's formula and statistically analyzed by probit analysis (Finney DJ 1971) by the SPSS statistical software Ver 15.0 (SPSS Inc, USA)

$$\text{Mortality (\%)} = [(X - Y)/X]\ 100,$$

WhereX=%survivalincontrolandY =%survival intreatedmosquitoes.

Results

The *Pseudomonas* immobilized in alginate beads was able to produce larvicide for up to 7 days and the larval mortality was recordedThe percentage mortality was calculated by

counting the number of dead larvae at each concentration. The larval mortality was higher on exposure to 100% concentration and the lowest at 25% concentration and are graphically represented (Fig 3 & Fig 4). The LC 50 values of the 24 hours and 48 hours were calculated for seven days and tabulated in Tables 3 and 4.

Table 1: Percentage mortality of *Ae.aegypti* at 24 hours vs. the spent broth

Hours	Days	100%	50%	25%	Larval Control	Alginate Beads control
24	1	76	60	52	0	0
	2	68	60	40	0	0
	3	60	40	36	0	0
	4	44	24	12	0	0
	5	20	12	4	0	0
	6	12	4	0	0	0
	7	4	0	0	0	0

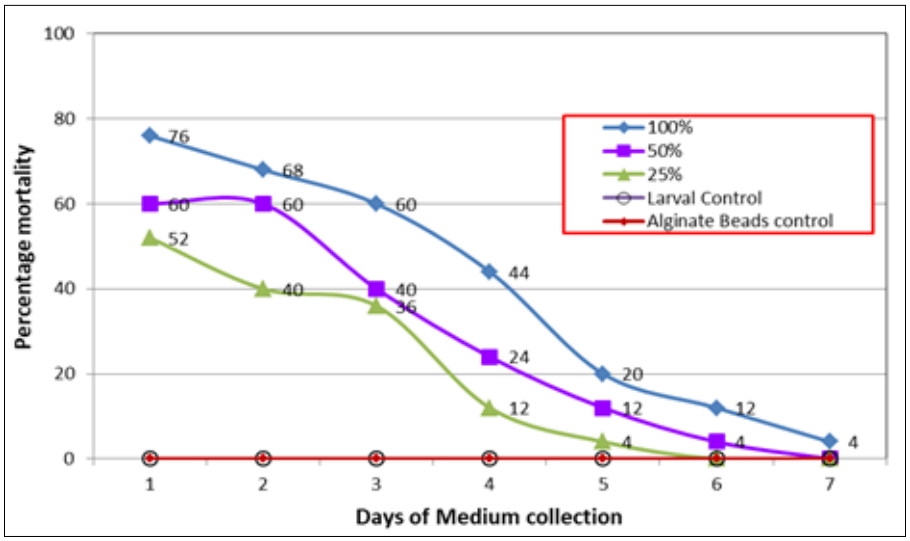

Fig 3: Percentage mortality of *Ae.aegypti* at 24 hours vs. the spent broth

Table 2: Percentage mortality of *Ae.aegypti*at 48 hours vs. the spent broth

Hours	Days	100%	50%	25%	Larval Control	Alginate Beads Control
48	1	92	76	56	0	0
	2	84	64	48	0	0
	3	72	52	44	0	0
	4	52	40	24	0	0
	5	24	16	12	0	0
	6	12	4	0	0	0
	7	4	0	0	0	0

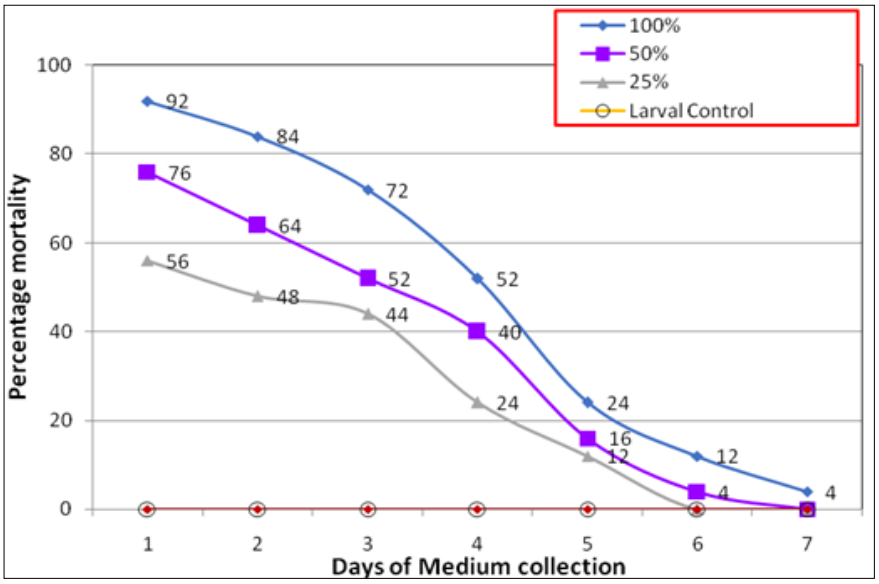

Fig 4: Percentage mortality of *Ae.aegypti*at 48 hours vs. the spent broth

Table 3: LC 50 value of 24 hours and 48 hours of larval exposure

Days	LC_{50}/24 hours	Fiducial limits	
		Lower	Upper
1	24.443	9.215	35.033
2	37.018	23.385	48.832
3	66.615	47.322	118.088
4	124.422	93.185	220.015
5	366.438	182.114	4185.091
6	411.527	200.846	7522.977
7	640.084	--	--

Days	LC_{50}/48 hours	Fiducial limits	
		Lower	Upper
1	21.538	14.17	27.327
2	28.008	19.048	35.154
3	46.98	33.75	63.723
4	87.263	65.198	160.079
5	282.92	157.467	1673.141
6	411.527	200.846	7522.977
7	640.084	-	

Conclusion

In the micro bioreactor, the larvicidal agent was collected for five days which prolonged the production of mosquito larvicides which showed the mortality rate even after 120 hours. The larvicidal agent is efficient in the control of *Ae. aegypti* larvae which is responsible for the spread of dengue and chikungunya viruses. Thus the metabolites can be a potential substitute for the insecticides against mosquito vectors. The present novel approach has proved that the micro bioreactor can effectively be utilized to produce larvicide or any metabolites from the alginate bead encapsulated bacteria. The present study showed that the *Pseudomonas* sp. immobilized in alginate bead can release mosquito larvicides. Controlled release formulation based upon entrapment in alginate matrices is finding wide applicability for several reasons, e.g., decrease in environmental pollution and toxicity with much more effective control compared with conventional chemical formulations (Kuppusamy M., 1987) [20]. These bead matrices can be used for the controlled release of insecticides at rates sufficiently effective to kill mosquitoes in the field. The metabolites from *Pseudomonas* species have immense potential to counter the threat associated with human health from mosquito vectors. The present study showed a promising approach to producing mosquito larvicide which can be scaled up to large quantities of larvicide in the future.

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