Article

Biolarvicidal activity of *Peanibacillus macerans* and *Bacillus subtilis* isolated from the dead larvae against *Aedes aegypti* – Vector for Chikungunya

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Abstract
Two bacterial species were isolated from dead mosquito larvae. They were identified as *Peanibacillus macerans* and *Bacillus Subtilis*. They were examined for their mosquito larvicidal activity against chikunguya vector *Aedes aegypti* (Diptera: Culucidae). The LC_{50} values of *P. macerans* and *B. subtilis* were recorded 70.99, 50 $\times 10^6$ cells /ml and 58.97, 49 $\times 10^6$ cells /ml for 24h and 48h, respectively. The LC_{50} value of the procured culture *Bacillus thuringiensis* subsp *israelensis* also detected. It was noted as 152.02 and 50 $\times 10^6$ cells /ml for 24hrs and 48hrs. *A. aegypti* was the most susceptible to *B. subtilis*. It has the highest relative susceptibility (RS) value.

Keywords biocontrol; Chikunguya; *Aedes aegypti*; *Peanibacillus macerans*; *Bacillus subtilis*; *Bacillus thuringiensis* subsp *israelensis.*

1 Introduction

Mosquitoes are some of the most adaptable and successful insects on earth. Mosquito-borne diseases are a major problem in almost all tropical and subtropical countries and currently there are no successful vaccines against the most mosquito borne diseases (CDC, 2008; Milam et al., 2000). It transmits some of the world's most serious vector borne diseases, such as malaria, encephalitis, filariasis, yellow fever, dengue and chikungunya (Rozendaal, 1997; Reinert et al., 2004; Parthiban and David, 2007; Radhika et al., 2011). Vector control is primordial and very essential means for controlling transmission of filariasis, malaria, Japanese encephalitis and dengue in human society (Ohkuma et al., 2003; Kaushik and Saini, 2009). Biological control is a method which uses biotic agents that are toxic or lethal to target insects (Rodrigues et al., 1999; Bellows, 2001; Headrick and Goeden, 2001). In view of the increasing resistance of mosquitoes to chemical insecticides and the lack of new alternative methods to control mosquitoes, biocontrol method using microbes is being considered as a possible control measure. Biological control agents can adapt to mosquito breeding habitats and pose no danger to people (Das, 2003; Kaushik and Saini, 2008). Hence the present work, the microbial larvicidal activity against *A. aegypti* was carried out by using two bacterial strains isolated from dead mosquito larvae.

2 Material and Methods

2.1 Rearing of mosquito larvae of *A. aegypti*

The larvae of *A. aegypti* were collected from Indian Council of Medical Research (ICMR), Madurai, Tamil Nadu, India. The collected larvae were reared in laboratory rear cages (17″ ×12″ × 8″). Chick blood meal and glucose water were given as feed for female and male adult mosquitoes, respectively. 5 % of Dog biscuits and yeast extract were given as a feed for larvae. They were allowed to ovulate (Murugesan et al., 2009). After emerging from the eggs, the larvae were transferred to enamel pans which contained water and larval feed. The water in the enamel pans was changed every two days, until the larvae reached the appropriate (third instar) larval development stage to be used for the bioassay. The insectary was maintained at a temperature of 26 ± 2º C with relative humidity of 80% to 90%.

2.2 Isolation and identification of microorganisms from dead mosquito larvae

Naturally dead mosquito larvae were collected from natural breeding habitats. They were washed with sterile distilled water and macerated by adding 1 ml of sterile distilled water with glass rod. The suspension was then serially diluted up to 10^{-7}. Samples from various dilutions were plated on nutrient agar medium (g/l Peptone-5, meat extract-1, yeast extract-2, NaCl-5, Agar-15, pH- 7.0 ± 0.2) and incubated at 37 ºC ± 2ºC for 24 h. The same procedure was also followed to isolate the microorganisms from the control live larvae. After 24 h, the morphological characters were observed. The isolated microorganisms from the dead larvae were identified by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, India.

2.3 Suspension formulated for bioassays

Isolated bacterial species were cultured in nutrient broth for 24h at 37ºC ± 2ºC. The culture was centrifuged at 2000g for 10 minutes to collect the cell pellet. This bacterial biomass was used for the bioassay. *Bacillus thuringiensis subsp israelensis* (*Bti*) was procured as reference culture from MTCC and cultured in Luria Bertani broth (g/l Tryptone-10, Yeast extract- 5, Sodium chloride-10).

2.4 Bioassay for evaluating the larvicidal activity against *A. aegypti*

Third instar larvae of *A. aegypti* (25 numbers) were taken in 250ml container containing 200ml of tap water (Khyami-Horani et al., 1999; Cavados Fonseca et al., 2001). Bioassay was carried out using the 5, 6, 7, and 8ml dilution of bacterial biomass of isolates. The containers were covered with mosquito net to avoid external contamination. The mortality rate readings were made after 24 and 48h exposure. All concentrations were tested in triplicates and control was maintained. The average number of dead larvae was recorded. If mortality in the control treatment exceeded 10%, the test was discarded and repeated. The LC_{50} calculations were performed by the log probit analysis (Finney, 1971). The same procedure was followed for *Bti*.

2.5 Calculation of relative mortality rate index

The relative mortality rate index was calculated by relative susceptibility described by Rodrigues et al. (1999) with some modifications.

$$\text{Relative susceptibility (RS)} = \frac{(LC_{50}) \text{ standard}}{LC_{50} \text{ bacterial isolate}}$$

Where the standard value is the highest value of LC_{50} in the bacterial isolates

3 Results and Discussion

3.1 Identification of the bacterial isolates

The bacterial isolates were confirmed as *Peanibacillus macerans and Bacillus subtilis* by MTCC, Chandigarh.

3.2 Larvicidal activity of bacterial isolates and *Bti* against third instar larvae of *A. aegypti*

Third instar larvae of *A. aegypti* was selected for this study. Mulla (1990) reported that first larval instars were difficult to handle, which might have cause high mortality rates due to the handling procedures. The fourth instar larvae that feed very little or have ceased to feed are less susceptible, since their ingestion of the toxin is minimal during this short period. Karch and Coz (1984) and Rodrigues et al. (1999) concluded that third and fourth larval instars were less affected than earlier instars. In the present study, larvicidal activity of bacterial isolates against third instar larvae of *A. aegypti* was recorded (Fig. 1). The highest mortality rate (87 %) was obtained at 48h using the highest concentration of *P. macerans*. The lowest mortality (16 %) rate was recorded at the 1mg concentration of *Bti* for 24h treatment. The cell number of *B. subtilis*, *P. macerans* and *Bti* were 19.9 ±6.1×10^6 /ml, 13.2 ± 4×10^6 /ml and 9.9 ± 3.5×10^6 /ml, respectively.

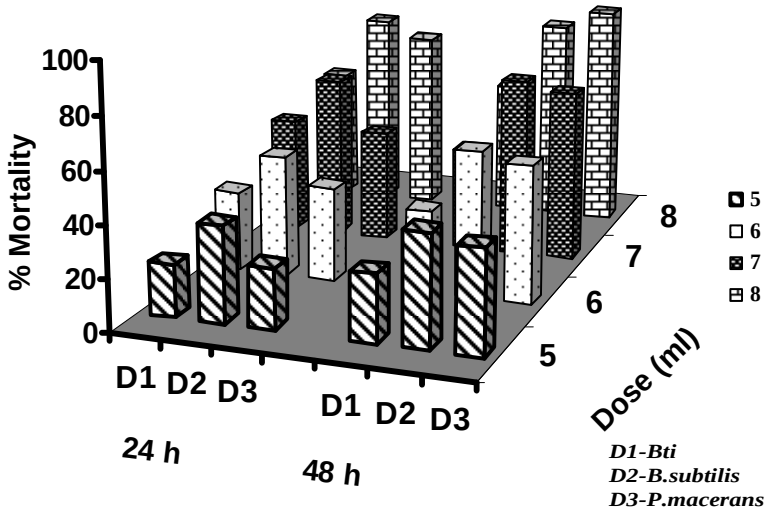

Fig. 1 Percent mortality of the bacterial isolates against *A. aegypti*

The LC_{50} values were calculated using probit analysis (Table 1). The LC_{50} values of *P. macerans* and *B. subtilis* are 70.99, 50 ×10^6 cells /ml and 58.97, 49 ×10^6 cells /ml for 24 h and 48h treatment, respectivlely. But the LC_{50} values of *Bti* were recorded as 152.02 and 50 ×10^6 cells /ml for 24 and 48h, respectively. It is clearly stated that LC_{50} values were 53% and 1% lower than *P. macerans* and 61 % and 2 % lower than *B. subtilis* for 24hrs and 48hrs, respectively when compared the standard culture. Genetically engineered microorganism, *Asticcacaulis excentricus* showed the LC_{50} 6.83 × 10^5 cells / ml (Armengol et al., 2005) against for *A. aegypti*. Otieno-Ayayo et al. (2008) studied the purified toxins of *Bacillus thuringiensis* subsp. *israelensis* against larvae of seven mosquito species. Recombinant *E. coli* with *Bacillus thuringiensis* subsp *israelensis* and *Bacillus thuringiensis spahricus* were used to control *Aedes*, *Culex* and *Anopheles* larvae (Otieno-Ayayo et al., 2008). The *Bti* strain was isolated and its crude protein's larvicidal activity was recorded by Alam et al. (2008). The LC_{50} and LC_{90} value of the culture filterate of keratinophilic fungus *Trichophyton mentagrophytes* against *A. aegypti* was 110 ± 11.5 and 200 ± 20.7 μL/mL, respectively (Murugesan et al., 2009).

3.3 Relative mortality rate index

Relative mortality index was calculated by the method of Rotrigues. In the present study, *Bti* was chosen as the standard for calculation of relative mortality index because the highest LC_{50} values for 24 and 48hrs were obtained in the bioassay. The relative susceptibility values of *A. aegypti* to the bacterial isolates considering *Bti* as standard are shown in Table 2. According to the results, the values are lower for *P. macerans* in relation to

B. subtilis which indicates that *B. subtilis* is 17% and 0.98 % more sensitive than the *P. macerans* for 24 and 48 hrs treatment, respectively.

Table 1 Toxicities of bacterial isolates against *A. aegypti* larvae

S. No	*Bti* $\times 10^6$ cells /ml		*P. macerans* $\times 10^6$ cells /ml		*B. subtilis* $\times 10^6$ cells /ml	
Reading Hours	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
LC_{50}	152.02	50	70.99	50	58.97	49
LC_{90}	296.82	176	119.09	99	103.2	109

Table 2 Relative mortality index of *A. aegypti* against isolated microoragnisms

Bacterial isolates	*Peanibacillus macerans*		*Bacillus subtilis*	
Reading Hours	24hrs	48hrs	24hrs	48hrs
LC_{50}	2.14	1.01	2.58	1.02
LC_{90}	2.49	1.78	2.88	1.62

The percent mortality rate was found to be dose dependent. So for there is no report of on larvicidal activity of *B. subtilis* and *P. macerans* except the pupicidal activity if *B. subtilis*. This is the first report for larvicidal activity if *B. subtilis* and *P. macerans.* There is no report of *B. subtilis* and *P. maecerans* for mosquito control. Pupicidal activity of *B. subtilis* was done (Geetha and Manonmani, 2008). But it is the first report for larvicidal activity of *B. Subtilis* and *P. macerans*.

4 Conclusion

Biological control of mosquito larvae with biocontrol agents would be a more-effective and eco-friendly approach, avoiding the use of synthetic chemicals and related damage to the environment. These results get substantial confirmation from the findings of other works. This study revealed that the *B. subtilis* and *P. macerans* has a potent mosquito larvicidal activity and could be selected for further studies particularly these pertaining to its effect on growth and development of mosquitoes. Further studies like constructions of genetically modified *P. macerans and B.subtilis* for the better result are at present being in this direction.

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References

Alam KA, Khan SA, Seheli K, et al. 2008. Mosquitocidal activity of Bti producing Cry protein against Aedes aegypti mosquito. Research Journal of Environmental Sciences, 2: 46-51

Armengol GO, Enrique GS, Orduz N, et al. 2005. Expression of the *Bacillus thuringiensis* mosquitocidal toxin Cry11Aa in the aquatic bacterium *Asticcacaulis excentricus*. Current microbiology, 51: 430-433

Bellows TS. 2001. Restoring population balance through natural enemy introductions. Biological Control, 21: 199-205

Cavados Fonseca RN, Chaves JQ, Rabinovitch L. 2001. Identification of entomopathigenic *Bacillus* isolated from Simulium (Diptera: Simuliidae) larvae and adults. Memórias do Instituto Oswaldo, Rio de Janeiro, 96(7): 1017-1021

CDC-Centres for disease Control and Prevention. 2008. http:/www.cdc.gov/ncidod/dvbid/dengue/dengue-qa.html. www.cdc.gov/ncidod/dvbid/Chikungunya/CH_Symptoms Treatment.html

Das BP. 2003. *Chilodonella uncinata*-a protozoa pathogenic to mosquito larvae. Current Science, 85: 483-489

Finney DJ. 1971. Probit Analysis (3rd ed). Cambridge University Press, London, UK

Geetha I, Manonmani AM. 2008 Mosquito pupicidal toxin production by *Bacillus subtilis* subsp. subtilis Biological Control, 44(2): 242-247

Headrick DH, Goeden RD. 2001 Biological control as a tool for ecosystem management. Biological Control. 21: 249-257

Karch S, Coz J. 1984. Acceleration de lactivite larvicide de *Bacillus sphaericus sur Culex pipiens* par ingestion de cadavres de larves de moustiques intoxiqués par ce bacille. Cah. O.R.S.T.O.M., sér. Ent. méd. Parasit., 22(3): 175-177

Kaushik R, Saini P. 2008. larvicidal activity of leaf extract of *Millingtonia hortensis* (Family: Bignoniacear) against Anopheles stephensi, Culex quinquefasciatus and *Aedes aegypti*. Journal of Vector Borne Diseases, 45: 66-69

Kaushik R, Saini P. 2009. Screening of some semi-arid region plants for larvicidal activity against *Aedes aegypti* mosquitoes. Journal of Vector Borne Diseases, 46: 244-246

Khyami-Horani H, Katbeh-Bader A, Mohsen ZH. 1999. Isolation of endospore-forming bacilli toxic to Culiseta longiareolata (Diptera: Culicidae) in Jordan. Letters in Applied Microbiology, 28: 57-60

Milam CD, Farris JL, Wilhide JD. 2000. Evaluating mosquito control pesticides for effect on target and nontarget organisms. Archives of Environmental Contamination and Toxicology, 39: 324-328

Mulla MS. 1990. Activity field efficacy and use of *Bacillus thuringiensis israelensis* against mosquitoes. In: Bacterial Control of Mosquitoes and Black Flies: Biochemistry, Genetics and Applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (Barjac H, Sutherland DJ, eds). 134-160, New Brunswick, Rutgers University Press, USA

Murugesan AG, Prabu SC, Selvakumar C. 2009 Biolarvicidal activity of extracellular metabolites of the keratinophilic fungus *Trichophyton mentagrophytes* against larvae of *Aedes aegypti* – a major vector for chikungunya and dengue. Folia Microbiologica, 54(3): 213-216

Ohkuma M, Shimizu H, Thongaram T, et al. 2003. An alkaliphilic and xylanolytic *Peanibacillus* species isolated from the gut of a soil feeding termite. Microbes and Environments, 18(3): 145-151

Otieno-Ayayo Z, Zaritsky A, Wirth MC, et al. 2008. Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis ssp. israelensis*. Environmental Microbiology, 10: 2191-2199

Parthiban M, David BV. 2007. Mosquito. In: Manual of Household and Public Health Pests and Their Control. Namrutha Publications, Chennai, India

Radhika D, Ramathilaga A, Prabu CS, et al. 2011. Evaluation of larvicidal activity of soil microbial isolates (Bacillus and Acinetobactor Sp.) against Aedes aegypti (Diptera: Culicidae) - the vector of Chikungunya and Dengue. Proceedings of the International Academy of Ecology and Environmental Sciences, 1(3-4): 169-178

Reinert SF, Harbaach RE, Kitching IJ. 2004. Phylogeny and classification of Aedini (DiotirAa: Culicidae), based on morphological character of all life stages. Zoological journal of the Linnean Society, 142: 289-368

Rodrigues IB, Tadei WP, Dias JS. 1999. Larvicidal activity of bacillus sphaericus 2362 against *Anopheles nuneztovari*, *Anopheles darlingi* and *Anopheles braziliensis* (Diptera: culicidae). Rev. Inst. Med. Trop. S. Paulo, 41: 2

Rozendaal JA. 1997. Mosquitoes and other biting Diptera. In: Vector Control - Methods for use by individuals and communities. World Health Organization, Geneva, Switherland