

# Biosynthesis of silver nanoparticles using *Bacillus thuringiensis* against dengue vector, *Aedes aegypti* (Diptera: Culicidae)

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**Abstract** The present study reveals the larvicidal activity of silver nanoparticles (AgNPs) synthesized by *Bacillus thuringiensis* (Bt) against *Aedes aegypti* responsible for the diseases of public health importance. The Bt-AgNPs were characterized by using UV–visible spectrophotometer followed by scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) spectroscopy. A surface plasmon resonance spectrum of AgNPs was obtained at 420 nm. The particle sizes were measured through SEM imaging ranging from 43.52 to 142.97 nm. The Bt-AgNPs has also given a characteristic peak at 3 keV in EDX image. Interestingly, the mortality rendered by Bt-AgNPs was comparatively high than that of the control against third-instar larvae of *A. aegypti* (LC<sub>50</sub> 0.10 ppm and LC<sub>90</sub> 0.39 ppm) in all the tested concentrations, viz. 0.03, 0.06, 0.09, 0.12, and 0.15 ppm. Hence, Bt-AgNPs would be significantly used as a potent mosquito larvicide against *A. aegypti*.

## Introduction

One of the major public health problems in developing countries are vector-borne diseases. Especially, mosquito vectors are solely responsible for transmitting diseases such as malaria, dengue, chikungunya, Japanese encephalitis, and lymphatic filariasis. In this regard, *Aedes aegypti* (Diptera: Culicidae) is the main vector of chikungunya and dengue fever (Sourisseau et al. 2007). Till 10 October 2012, 151 districts of eight states/provinces of India have been affected by chikungunya fever (Pialoux et al. 2007). As chemical

insecticides create serious ecological problems such as mosquito resistance, elimination of beneficial insects, viz. predators, parasites, bees, and pollinators in the environment, implementation of biocontrol programs would be an ideal way to stabilize the life-threatening arthropod insects in an eco-friendly approach. An ideal way to prevent the prevalence of mosquito-borne diseases is to prevent them from emergence. Hence, larvicides play a vital role in controlling mosquitoes in their breeding sites.

Among the biological organism so far used for the mosquito control programs, bacteria such as *Bacillus* species are known for its mosquito larvicidal effect. Of which, *Bacillus thuringiensis* israelensis and *Bacillus sphaericus* are effective, but serious resistance as high as 50,000-fold has evolved where *B. sphaericus* is used against *Culex* mosquitoes (Soni and Prakash 2011). Recently, the laboratory resistance in the mosquitoes has been demonstrated to some isolates of *B. thuringiensis* (Surendran and Vennison 2011). These results suggest that the *B. thuringiensis* (Bt)-synthesized silver nanoparticles (AgNPs) have the potential to be used as an ideal eco-friendly approach for the control of the *A. aegypti*. This method is considered as a new approach to control dengue vector. Therefore, this study provides the first report on the mosquito larvicidal activity of *B. thuringiensis*-synthesized AgNPs against dengue vector.

Recently, Jiang et al. (2004) have emphasized that metals, especially Ag (silver), has long been recognized as having inhibitory effect towards many bacterial strains and microorganisms commonly present in medical and industrial process. Later, Sinha et al. (2009) have reported that the biosynthesis of silver nanoparticles is advantageous over chemical and physical methods. Recently, Santhoshkumar et al. (2011) reported 100 % mortality of *Culex quinquefasciatus* and *Anopheles subpictus* by *Nelumbo nucifera* leaf extract-synthesized AgNPs. Similarly, Soni and

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Prakash (2011) have also reported on higher (100 %) susceptibility of *A. aegypti* towards *Chrysosporium tropicum*-synthesized AgNPs. Salunkhe et al. (2011) have also reported on the larvicidal potential of AgNPs synthesized using fungus, *Cochliobolus lunatus*, against *A. aegypti* and *Anopheles stephensi*. In their study, the potentiality of AgNPs synthesized using aqueous leaf extract of *Eclipta prostrata* against fourth-instar larvae of *C. quinquefasciatus* and *Aedes albopictus* was established (Rajkumar and Rahuman 2011).

In connection to the above issues, the present investigation aims to study on the efficacy of Bt-AgNPs for the control of dengue vector, *A. aegypti*.

## Methods

### Isolation of *B. thuringiensis*

*B. thuringiensis* used in this study was isolated from rhizosphere soil (black soil) of cotton (Kalloorani, Aruppukottai, Virudhunagar district) Tamil Nadu, India, according to the method of Travers et al. (1987). The isolated strain was subjected to Amido black and carbol fuchsin staining. Smears were examined under a light microscope (Labomed) to observe the parasporal bodies of the bacterium (Fig. 1). The pure culture of Bt strain was then grown in Luria Bertani (LB Broth (Hi-Media)) broth in a 100-mL conical flask (Riviera™) at 37 °C for 36 h on an Orbit Incubator Shaker (Neolab, Neolab Instruments, Mumbai, India) at 150 rpm. The Bt culture was then centrifuged (Remi, Laboratory Centrifuge, Mumbai, India) at 5,000 rpm for 10 min, and the supernatant was used as a starting material for the synthesis of silver nanoparticles.

### Synthesis and characterization of silver nanoparticles

The supernatant was added to the reaction vessel (Riviera™) containing 1 mM (millimolar) silver nitrate (Laboratory Reagent, Reachem Laboratory Chemicals Private Ltd, Madras, Tamil Nadu, India) and placed on an Orbit Incubator Shaker (Neolab, Neolab instruments, Mumbai, India) at 150 rpm. The reaction between this supernatant and Ag<sup>+</sup> was carried out in dark conditions for 3 days. The bioreduction of the Ag<sup>+</sup> in the aliquot was monitored, and the spectrum was measured in the UV–vis spectrophotometer. Further, scanning electron microscope (SEM) (model JEOL JSM-6390) and energy-dispersive X-ray spectroscopy (EDX Oxford Instrument, INCA PentaFET X3, Karunya University, Coimbatore, Tamil Nadu, India) were performed for the detection and confirmation of elemental Bt-synthesized AgNPs. Milli-Q water was used to dissolve the Bt-AgNPs.

### Larvicidal activity of Bt-AgNPs against *A. aegypti*

The eggs were obtained from the Indian Council of Medical Research (ICMR), Madurai, Tamil Nadu, India, and reared in the laboratory condition supplemented with dog biscuits and yeast extract in the ratio 3:1. Bioassay was conducted with Bt-synthesized silver nanoparticles against third-instar larvae of *A. aegypti* based on a method of the World Health Organization (WHO 2005) with minor modifications. For the bioassay, a 25 third-instar larvae/concentration/replication was transferred into a 250-mL glass beaker containing different concentrations (0.03 to 0.15 ppm) of Bt-AgNPs, and the beaker was covered with a mosquito net. Five replications were maintained for each concentration. The setup was maintained at 27 ± 2 °C and 77 ± 4 % RH. The mortality of mosquito larvae was noted at 24-h intervals with and without Bt-AgNPs.

### Statistical analysis

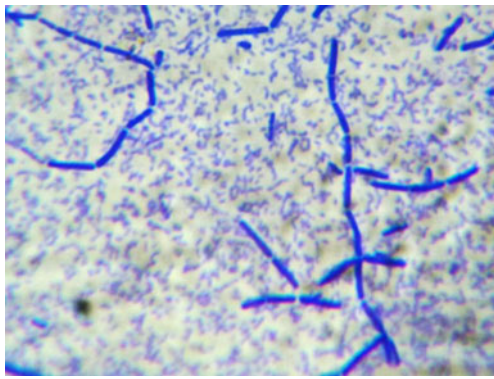
Mortality data was subjected to probit analysis to predict the LC<sub>50</sub> and LC<sub>90</sub> values by using SPSS 10.0. Percentage mortality was also calculated for the mortality data using Microsoft Excel 2007.

## Results

The present investigation describes the synthesis of AgNPs by using *B. thuringiensis* (Fig. 1) and its larvicidal potential against *A. aegypti*, the details of which are presented vide infra.

### Characterization of Bt-AgNPs

The culture supernatant along with AgNO<sub>3</sub> was subjected to reduction reaction and the change in the color to yellowish brown, indicating the formation of AgNPs (Fig. 2). UV–vis spectroscopy is an initial step for analyzing the formation of silver nanoparticles in aqueous solution. AgNPs have free electrons, which give rise to a surface plasmon resonance absorption band, due to the combined vibration of electrons of the metal nanoparticles in resonance with the light wave. A surface plasmon resonance spectrum of AgNPs was obtained at 420 nm (Fig. 3). Also, their presence has been well defined in SEM imaging (Fig. 4) and different sizes ranging from 43.52 to 142.97 nm. The Bt-AgNPs has also given a characteristic peak at 3 keV in EDX image, which indicates the reduction of Ag<sup>+</sup> to Ag<sup>0</sup> (Fig. 5).



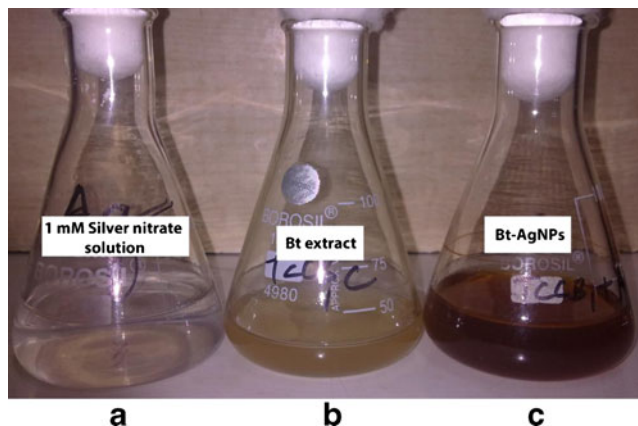
**Fig. 1** Microscopic view of *B. thuringiensis*

#### Larvicidal activity of Bt-AgNPs against *A. aegypti*

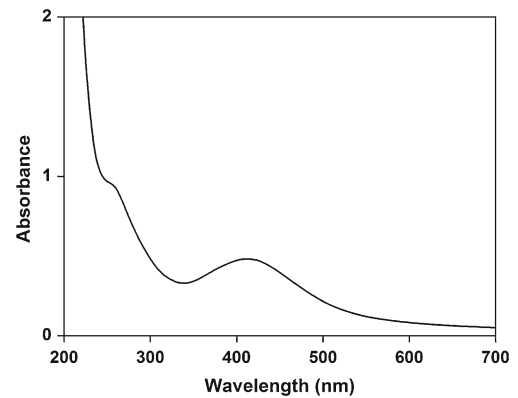
The efficacy ( $LC_{50}$  and  $LC_{90}$ ) of the Bt-AgNPs is shown in Table 1. The treated *A. aegypti* reacts with the higher susceptibility towards Bt-AgNPs. The  $LC_{50}$  and  $LC_{90}$  values of the tested concentrations are 0.10, 0.14, 0.23, 0.25, and 0.72 ppm, and 0.39, 0.56, 0.92, 0.85, and 4.11 ppm with respect to 0.15, 0.12, 0.09, 0.06, and 0.03 ppm of Bt-AgNPs. The percent mortality of the tested concentration is 13.60, 20.00, 39.20, 56.00, and 65.60 % with respect to the above ppm concentration at 24-h intervals. The mortality rate is directly proportional to the concentration, and while the concentration was increased, the death rates also increase.

#### Discussion

As silver nanoparticles have potent antibacterial and antifungal activity against microbial pathogens with broad-spectrum antibiotic resistance, evaluating them for the betterment of human health by controlling human vector (i.e., mosquitoes) by using nanotechnology-based biomaterials gains importance in recent years. Uses of microorganism for the synthesis of nanoparticles often have



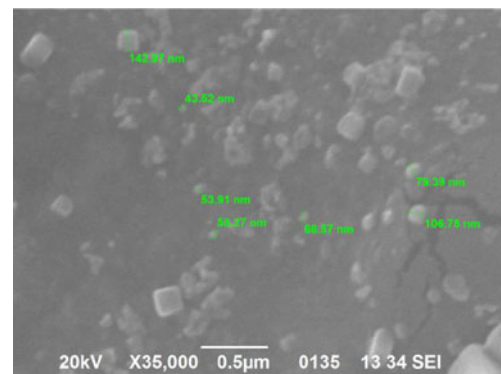
**Fig. 2** Photographs of a 1 mM  $AgNO_3$ , b Bt extract, and c Bt-AgNPs



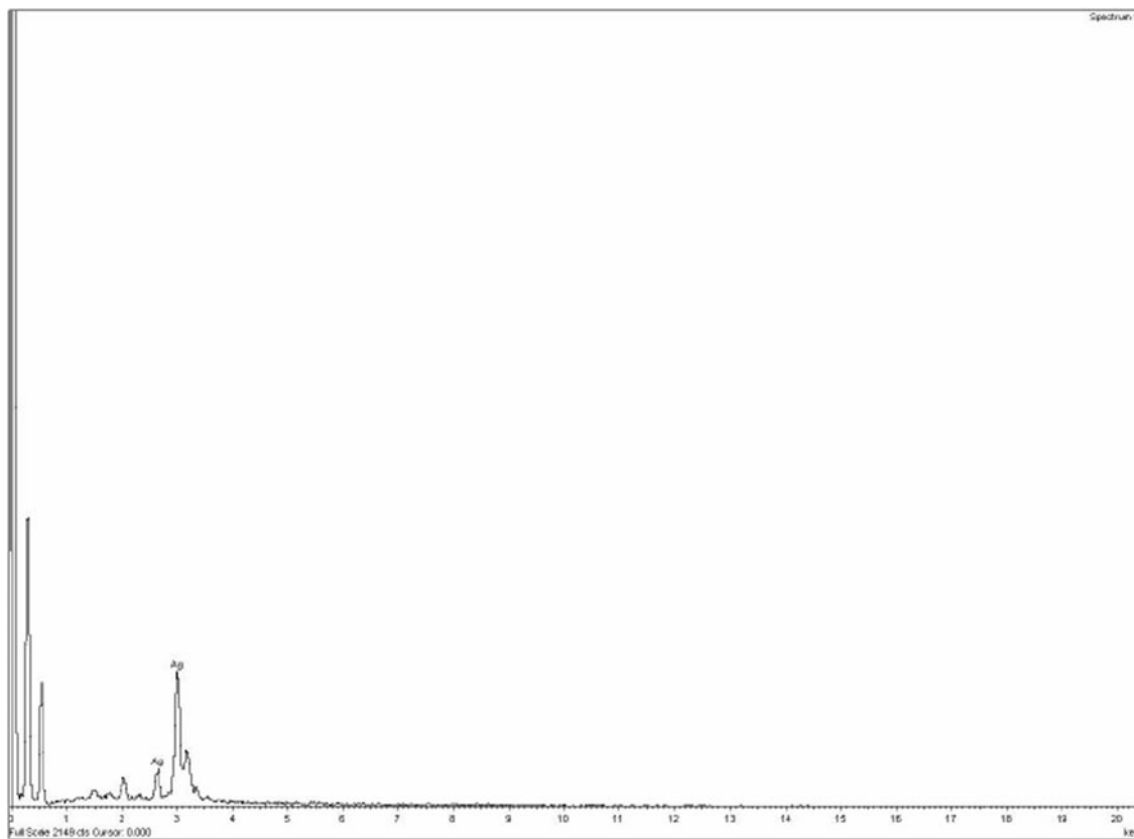
**Fig. 3** UV-visible spectra of silver nanoparticles

advantage over physical and chemical methods as it is an environmentally friendly process. The formation of extracellular and intracellular silver nanoparticles by bacteria *Pseudomonas stutzeri* AG259 (Tanja et al. 1999), *Klebsiella pneumoniae* (Ahmad et al. 2007), *B. licheniformis* (Kalimuthu et al. 2008), *Escherichia coli* (Gurunathan et al. 2009a, b), *Staphylococcus aureus* (Nanda and Saravanan 2009), and *Brevibacterium casei* (Kalishwaralal et al. 2010) has been investigated. The formation of extracellular silver nanoparticles by photoautotrophic cyanobacterium *Plectonema boryanum* has been described (Lengke et al. 2007).

Recently, a rapid method for synthesizing silver nanoparticles by treating the aqueous silver nitrate solution with culture supernatants of different strains of *Enterobacteria* such as *K. pneumonia* has been described (Shahverdi et al. 2007; Mokhtari et al. 2009). The process of synthesis was quite fast. Similarly, Kalimuthu et al. (2008) have investigated the process of synthesis with silver nanoparticles by using bacteria *B. licheniformis* and sonification of reacting mixture. The extracellular synthesis of silver and gold-silver nanoparticles by fungus *Fusarium oxysporum* biomass had a contribution on the formation of nanoparticles (Ahmad et al. 2003).



**Fig. 4** SEM image of Bt-AgNPs



**Fig. 5** EDX spectrum of synthesized silver nanoparticles

The present investigation exploited the culture supernatant of *B. thuringiensis*, an entomopathogenic endotoxic bacterium for synthesis of silver nanoparticles. *B. thuringiensis* is often known for its mosquito larvicide effects whereas the resistance against cry toxins neglected their usage in the recent decades. The present study exemplified that the formation of yellowish brown color was due to reduction of silver ions that indicated the formation of silver nanoparticles. Besides, they have given a characteristic band at 420 nm. While no absorption band was observed in both controls

**Table 1** Larvicidal activity (in parts per million) of *B. thuringiensis*-synthesized silver nanoparticles (Bt-AgNPs) against *A. aegypti*

Concentration of Bt-AgNPs (ppm)	LC <sub>50</sub>	LC <sub>90</sub>	% Mortality
Control	0.00	0.00	0.00
0.15	0.10	0.39	65.60 ± 0.047
0.12	0.14	0.56	56.00 ± 0.081
0.09	0.23	0.92	39.20 ± 0.094
0.06	0.25	0.85	20.00 ± 0.047
0.03	0.72	4.11	13.60 ± 0.094

LC<sub>50</sub>, lethal concentration that kills 50 % of the exposed larvae; LC<sub>90</sub>, lethal concentration that kills 90 % of the exposed larvae; % percent cumulative mortality values are means ± SD

(positive and negative). Thus, it indicates the complete reduction of silver ions. Silver nanoparticles showed yellowish brown color in aqueous solution due to excitation of surface plasmon vibration in silver nanoparticles (Vigneshwaran et al. 2007). However, the bioreduction of the Ag<sup>+</sup> could be associated with metabolic processes utilizing nitrate by reducing nitrate to nitrite and ammonium (Lengke et al. 2007). The reduction of silver ions by *F. oxysporum* strains has been attributed to a nitrate-dependent reductase and a shuttle quinone extracellular process. The extracellular biosynthesis of silver nanoparticles by using the filamentous fungus *Aspergillus fumigatus* has been investigated (Bhainsa and D'Souza 2006). Similarly, the presence of Cry toxin in the culture supernatant has been clearly evidenced using SEM imaging, which may be responsible for the bioreduction of silver ion into silver. Kalishwaralal et al. (2008) revealed the size of the silver nanoparticles in the range of 50 nm by supernatant of *B. licheniformis*. Similarly, herein scanning electron microscopic image has confirmed the presence of nanoparticles, and the particle size ranged between 43.52 and 142.97 nm. The EDX has confirmed the presence of elemental silver by the sharp peaks at a range of 3 keV which is typical for the absorption of metallic silver nanoparticles.

The present investigation showed high potent larvicidal property of Bt-AgNPs with low lethal (0.10 ppm)



concentration against *A. aegypti*. Soni and Prakash (2011) reported on the potentiality of AgNPs synthesized by a fungus *C. tropicum* and found LC<sub>50</sub> and LC<sub>90</sub> values of 4 and 8.91 ppm against third-instar larvae of *A. aegypti*, which were much higher to the concentration recorded in the present investigation. Similarly, Santhoshkumar et al. (2011) also obtained LC<sub>50</sub> and LC<sub>90</sub> values of 0.69 and 1.10 ppm as well as 2.15 and 3.59 ppm of AgNPs synthesized by leaf extract of *N. nucifera* against *C. quinquefasciatus* and *A. subpictus*, which were comparable to the results obtained in the present study. The concentration of *Euphorbia hirta*-synthesized AgNPs (3.125, 6.25, 12.5, 25, and 50 ppm) were tested against larvae of *A. stephensi*. The highest larval mortality was found in the synthesized AgNPs against the first- to fourth-instar larvae and pupae of values LC<sub>50</sub> (10.14, 16.82, 21.51, and 27.89 ppm, respectively), LC<sub>90</sub> (31.98, 50.38, 60.09, and 69.94 ppm, respectively) (Agalya Priyadarshini et al. 2012). Subarani et al. (2013) results showed that the maximum efficacy of synthesized AgNPs against the fourth-instar larvae of *A. stephensi* (LC<sub>50</sub>, 12.47 and 16.84 mg/mL; and LC<sub>90</sub>, 36.33 and 68.62 mg/mL) on 48 and 72 h of exposure and against *C. quinquefasciatus* (LC<sub>50</sub>, 43.80 mg/mL; and LC<sub>90</sub>, 120.54 mg/mL) on 72-h exposure. The AgNPs did not exhibit any noticeable toxicity on *Poecilia reticulata* after 24, 48, and 72 h of exposure. These results suggest that the synthesized AgNPs have the potential to be used as an ideal eco-friendly approach for the control of the *A. stephensi* and *C. quinquefasciatus*. Recently, Salunkhe et al. (2011) have reported on the larvicidal potential of the AgNPs synthesized using the fungus *C. lunatus* against *A. aegypti* and *A. stephensi*, and they have also reported on the nontoxic effect of AgNPs against *P. reticulata*, which inhabitates the habitat of *A. aegypti*.

Thus, the present investigation enlightened the potential of the Bt toxin, which has energized the synthesized silver nanoparticles as potential larvicidal novel material against *A. aegypti*. The larvicidal efficiency of the silver nanoparticles often encouraged unearths the vulnerable parts and possible entry paths. Besides, it has been clearly understood that they are often involved in the cessation of neuro as well as enzyme secretion, which was often well understood from the prolonged larval duration during the course of investigation. From this study, it is suggested that the synthesized silver nanoparticles had potential to kill the larvae of *A. aegypti*. Hence, further investigation studying on the impact received by the physiological portion of the larvae and its growth-stimulating hormone is required.

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