

Extracellular synthesis of silver nanoparticles using *Bacillus megaterium* against malarial and dengue vector (Diptera: Culicidae)

A. Najitha Banu¹ · C. Balasubramanian²

Received: 16 June 2015 / Accepted: 16 July 2015 / Published online: 2 August 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract Biosynthesis of silver nanoparticles has provoked nowadays and alternative to physical and chemical approaches. In the present study, silver nanoparticles (AgNPs) were synthesized extracellular method using *Bacillus megaterium*. The AgNPs formations were confirmed initially through color change, and the aliquots were characterized through UV–visible spectrophotometer, followed by scanning electron microscopy (SEM), energy-dispersive X-ray (EDX) spectroscopy, and Fourier transform infrared (FTIR) spectra. The surface plasmon resonance band was shown at 430 nm in UV–vis spectrophotometer. The bioreduction was categorized through identifying the compounds responsible for the AgNP synthesis, and the functional group present in *B. megaterium* cell-free culture was scrutinized using FTIR. The topography and morphology of the particles were determined using SEM. In addition, this biosynthesized AgNPs were found to show higher insecticidal efficacy against vector mosquitoes. The LC₅₀ and LC₉₀ were found to be 0.567, 2.260; 0.90, 4.44; 1.349, 8.269; and 1.640, 9.152 and 0.240, 0.955; 0.331, 1.593; 0.494, 2.811; and 0.700, 4.435 with respect to the first, second, third, and fourth instar larvae of *Culex quinquefasciatus* and *Aedes aegypti*. All the calculated χ^2 values are highly significant compared with the tabulated value. Therefore, *B. megaterium*-synthesized silver nanoparticles would be used as a potent larvicidal agent against *Cx. quinquefasciatus* and *Ae. aegypti*.

Keywords Silver nanoparticle · *Bacillus megaterium* · Bioreduction · Pathogenicity · LC₅₀ and LC₉₀

Introduction

Mosquito vectors are only responsible for transmitting diseases such as malaria, dengue, chikungunya, Japanese encephalitis, dengue, and lymphatic filariasis, which are mostly transmitted by three genera namely *Culex*, *Aedes*, and *Anopheles*. An estimated 100 million annual dengue cases occur each year in tropical cities, in which more than 2.5 billion people (almost half of the global population) are at risk (Gubler and Trent 1993). They are transmitted mainly by the *Aedes aegypti* mosquito and also by *Aedes albopictus*. Biologically, dengue viruses are highly adapted to the mosquito and are maintained by vertical transmission. Dengue virus produces from a subclinical infection to a mild self-limiting disease, the dengue fever (DF), and a severe disease that may be fatal, the dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The mosquito vectors are present in tropical and subtropical regions of the earth that determines the prevalence of dengue virus in a region. Dengue has been an urban disease but now has spread to rural areas of India as well. Prior to 1970, only nine countries had experienced cases of DHF; since then, the number has increased more than 4-fold and continues to rise. The WHO published a global map of the distribution of the dengue epidemic activity during the year 2006 that shows whole India affected by dengue (Kumar et al. 2001; Arunachalam et al. 2004; Chaturvedi and Nagar 2008). Till 10 October 2012, 151 districts of eight states/provinces of India have been affected by chikungunya fever (Pialoux et al. 2007; Yang et al. 2009).

The factors considered responsible for global resurgence of DF/DHF are unprecedented population growth, unplanned

✉ A. Najitha Banu
najirila2010@gmail.com

¹ Department of Zoology, M.K.M. Group of Colleges, Hodal, Haryana, India

² Postgraduate and Research Department of Zoology, Thiagarajar College, Madurai, Tamil Nadu, India

and uncontrolled urbanization, increased air travel, absence of an effective mosquito control program, and deterioration of Public Health infrastructure. The risk factors for infection with dengue viruses are the increased density of the mosquito vector, re-infestation with *Ae. aegypti* of a new geographical area, warm and humid climate, increased population density, water storage pattern in houses, storage of junk in open spaces, including tires, coconut shells, etc. that trap rainwater and introduction of new serotype of the virus, etc. Vaccines or antiviral drugs are not available for dengue viruses; the only effective way to prevent epidemic DF/DHF is to control the mosquito vector, *Ae. aegypti* and prevent its bite (Chaturvedi and Nagar 2008). According to the WHO report in 2012 More than 1.3 billion people in 72 countries worldwide are threatened by lymphatic filariasis, commonly known as elephantiasis. Over 120 million people are infected, with about 40 million disfigured and incapacitated by the disease (WHO 2012).

Extensive utilization of insecticides for the control of pest all over the world resulted in the development of resistance to insecticides among an increasing number of species (Guneidy et al. 1988). Repeated application of chemical insecticides creating serious ecological problems such as mosquito resistance, ecological imbalance in the ecosystem, 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 (Kumar 1984; Najitha Banu et al. 2014).

In the current scenario, biologically synthesized silver nanoparticles were used as mosquito larvicides. Many researchers were reported the microbes like *Aspergillus niger*, *Cladosporium tropicum*, *Agaricus bisporus*, *Escherichia coli*, *Penicillium* sp. and *Vibrio* sp., *A. niger* 2587, *Bacillus thuringiensis*, *Beauveria bassiana* etc., synthesized silver nanoparticles dynamically control the mosquito larvae (Soni and Prakash 2012, 2013; Dhanasekaran and Thangaraj 2013; Najitha Banu et al. 2014; Najitha Banu and Balasubramanian 2014). Therefore, in this study, *B. megaterium* was used as reducing and capping agent for synthesis of metal (Ag) nanoparticles for controlling public health promising mosquito vector.

Materials and methods

Extracellular synthesis of AgNPs using *Bacillus megaterium*

The pure culture of *B. megaterium* was freshly inoculated into on a liquid media (Luria-Bertani broth (LB broth (Hi-Media))

in an Erlenmeyer flask. The flask containing medium was incubated in orbital shaker at 150 rpm in 25 ± 2 °C (Neolab Instruments, Mumbai, India) for 24 h. After 24 h of growth, the culture-containing medium was centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into sterile Erlenmeyer flask, and the pellet was discarded. For synthesis of silver nanoparticles, 50-mL aqueous solution of 1 mM silver nitrate (AgNO_3) (Laboratory Reagent, Reachem Laboratory Chemicals Private Ltd, Madras, Tamil Nadu, India) (0.017 g/100 mL) was treated with 50 mL of culture supernatant solution in a 250-mL Erlenmeyer flask (pH adjusted to 8.5). The whole mixture was placed in a shaker at 40 °C with 150 rpm for 3 days and maintained in the dark. Control experiment was conducted only with broth without inoculating bacteria, to check for the role of bacteria in the synthesis of nanoparticles. The reduction of Ag^+ ions was monitored by sampling an aliquot (2 mL) of the solution at intervals of 24 h and measured under the UV–vis spectra.

Characterization of silver nanoparticles

The *B. megaterium*-synthesized silver nanoparticles were subjected to structural and functional characteristics analysis. The structural characteristics analysis included the UV–vis spectroscopy and energy-dispersive X-ray analysis (EDX), and the functional characteristics analysis included Fourier transform infrared spectroscopy at Karunya University, Coimbatore and Madras University Chennai.

Purification of silver nanoparticles

The primary detection of synthesized silver nanoparticles was carried out in the mixture by observing the color change of the medium. The silver nanoparticles solution thus obtained was purified by repeated centrifugation at 10,000 rpm for 10 min or the yellowish brown color of *B. megaterium*-synthesized silver nanoparticle solutions were poured into the watch glasses and dried under hot air oven (Technico Laboratory Products Pvt. Ltd, Chennai) at 25–30 °C. Supernatant was discarded, and the pellet was dissolved in double-distilled water or milli-Q water. The dried AgNP samples were scraped and then stored in a screw-capped vials for further characterization analysis.

UV–vis absorbance spectroscopy analysis

The samples used for analysis were diluted with 2 mL double-distilled water and subsequently measured by the UV–vis spectrum at regular intervals by using a quartz cuvette with water/methanol as reference (Rajesh et al. 2009). UV–vis spectroscopy analyses of silver nanoparticles were carried out on Perkin Elmer (Lambda 35) UV–vis spectrophotometers at a scanning speed of 200–800 nm.

EDX analysis

The presence of elemental silver was determined through EDX spectra analysis. The samples were dried at room temperature and then analyzed for samples composition of the synthesized nanoparticles. EDX analysis was carried using EDX-oxford instrument, INCA PENTAFET X3 were confirmed for the presence of elemental silver in the particles as well as to detect other elementary composition of the particle (Jegadeeswaran et al. 2012; Najitha Banu and Balasubramanian 2014).

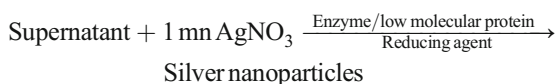
SEM analysis of silver nanoparticles

Scanning electron microscopic (SEM) analyses was done using (Model-JEOL.JSM-6390) SEM machine. Thin films of the samples were prepared on carbon copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper, and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

FTIR analysis of silver nanoparticles

The interaction between protein and silver nanoparticles was analyzed by Fourier transform infrared (FTIR) analysis. To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution 10 mL after reaction was centrifuged at 5000 rpm for 60 min and the pellet was obtained. This is followed by redispersion of the pellet of AgNPs into 1 mL of deionized water. Thereafter, the purified suspension was freeze-dried to obtain dried powder. The FTIR spectra were recorded using Bruker Tensor-27 FT-IR spectrometer with OPUS software in the range 4000–400 cm^{-1} , at a resolution of 4 cm^{-1} . The pellet for analysis was made by taking equal amounts of bacterial-AgNPs and KBr (1:1 ratio), and the background calibrations have been carried out using pure KBr pellet.

The protein (low molecular), enzyme (nitrate reductase), and other compound present in the microbial extracts work as reducing agents and are responsible for conversion of silver nitrate to silver nanoparticles. The reaction may be written as



Characterization and identification of mosquito larvae

Culex quinquefasciatus and *Ae. aegypti* egg rafts were collected from Thiagarajar College campus, and egg masses were procured from the Centre for Research in Medical Entomology (CRME), Madurai, Tamil Nadu, India. The

identified mosquito larvae were kept in plastic and enamel trays containing tap water, maintained and reared in the laboratory condition supplemented with dog biscuits and yeast extract in the ratio of 3:1 as per the method of Kamaraj et al. (2009).

Laboratory evaluation of microbial synthesized AgNPs against human mosquito vectors

Bioassay was conducted with *B. megaterium*-synthesized silver nanoparticles against the first, second, third, and fourth instar larvae of *Cx. quinquefasciatus* and *Ae. aegypti* based on a method of the World Health Organization (WHO 2005) with minor modifications. For bioassay, 25 larvae/concentration/replication were transferred into 250-mL glass beaker (Borosil®) containing 0.03- to 1.0 - μm concentration. Five replications of microbial AgNPs were maintained separately, each was covered with a mosquito net. The setup was maintained at 27 ± 2 °C and 77 ± 4 % RH. Mortality and survival rate were registered after a 24-h exposure period with and without bacterial AgNPs. The moribund and dead larvae were collected, and larval mortality was calculated for each concentration. Five replicates were maintained in each concentration. Percent mortality was calculated using the formula (1) and corrections for mortality when necessary were done using Abbot's (1925) formula (2).

Percentage of mortality:

$$\frac{\text{No. of dead larvae}}{\text{No. of larvae introduced}} \times 100 \quad (1)$$

Corrected percentage of mortality:

$$\frac{1 - n \text{ in } T \text{ after treatment} \times 10}{n \text{ in } C \text{ after treatment}} \times 100 \quad (2)$$

where n is the number of larvae, T is the treated, and C is the control.

Statistical analysis

Mortality data was subjected to probit analysis to predict the LC_{50} , LC_{90} , chi-squared, slope and intercept value by using EPA 1.5. Percentage mortality was also calculated for the mortality data using Excel 2007.

Results

The present investigation describes the biological synthesis of silver nanoparticles (AgNPs) using *B. megaterium* (Fig. 1) and its larvicidal potentials were evaluated against the different instars of *Cx. quinquefasciatus* and *Ae. Aegypti*.



Fig. 1 Microscopic view of *Bacillus megaterium*

Bacteria-mediated synthesis of AgNPs

The synthesis efficiency of *B. megaterium* was initially found through color change of the aqueous solution. Supernatant was subjected to the reduction reaction, when 1 mM AgNO_3 was mixed with culture supernatant color will change pale yellow to brown and it was confirmed the reduction occurs (Ag^+ to Ag^0) in the aqueous solution. There was no similar color change in both positive and negative control (culture supernatant and 1 mM AgNO_3) (Fig. 2).

Characterization of AgNPs

UV–vis spectroscopy

The *B. megaterium*-synthesized AgNPs were characterized structurally through UV–vis spectrophotometer. This is an initial step for analyzing the formation of silver nanoparticles in aqueous solution. A surface plasmon resonance spectrum of AgNPs was obtained at 430 nm (Fig. 3). Further, *B. megaterium*-synthesized AgNPs were centrifuged at 10,000 rpm for 20 min or dehydrated using hot air oven 35 to 40 °C. After which the pellet was redispersed in deionized water to get rid of any uncoordinated biological molecules. The purified pellets were then freeze-dried, powdered and characterized by XRD, SEM, EDX, and FTIR analyses.

EDX spectroscopy analysis

The presence of elemental silver can be seen in the graph presented by the EDX analysis. This is indicating the reduction of silver ions in the aqueous solution. EDX analysis proved the chemical purity of the synthesized silver nanoparticles. The EDX analysis obtained in the present study confirmed that the presence of silver



Fig. 2 AgNO_3 , 1 mM; culture supernatant and AgNPs

nanoparticles in the bacterial supernatant, given a characteristic peak at 3–4 keV in EDX image, which indicates the reduction of Ag^+ to Ag^0 (Fig. 4).

SEM analysis

SEM provided further insight to study the morphology and size details of the silver nanoparticles. The freeze-dried silver nanoparticles were mounted on specimen stubs with double-sided tape, coated with gold in a sputter coater and examined under a HITACHI S-4500 SEM at 12–16 kV with a tilt angle of 45°. Representative SEM micrographs of the *B. megaterium*-synthesized silver nanoparticles magnified at $\times 5000$ and $\times 10,000$ times. The SEM results showed that the size ranged from 46.10 to 80.62 nm (Fig. 5).

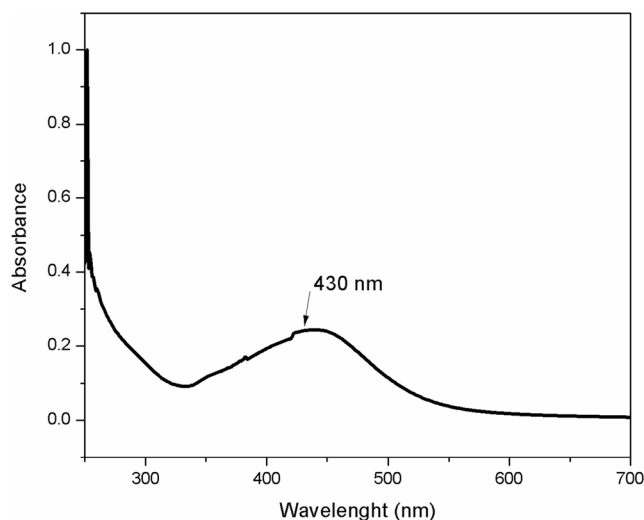


Fig. 3 UV–visible spectra of *B. megaterium*-synthesized silver nanoparticles

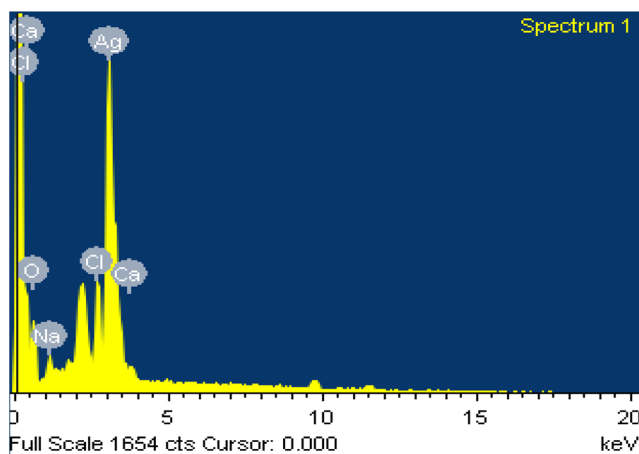


Fig. 4 Energy-dispersive X-ray spectra (EDX) *B. megaterium*-synthesized AgNPs

FTIR spectroscopy analysis

FTIR spectroscopic study was carried out to investigate the possible mechanism behind the formation of silver nanoparticles and offer information regarding the functional groups. The representative spectra of silver nanoparticles are shown in Fig. 6. Vibrational assignments/functional groups corresponding to the absorption peaks are enumerated in Table 1. The very strong broad absorption peaks at 3403.41, (O–H stretch and bending); very strong and sharp peaks at 2923.52 cm^{-1} (NO_2) and some variable stretching and bending peaks were observed, which may be from AgNO_3 solution, the metal precursor involved in the AgNP synthesis process. Strong interaction of water with the surface of silver could be the reason for the O–H stretching and C=C, =C–H, O–H, C=O, N–H functional groups, which may be present between the amino acid residues and protein synthesized during microbial AgNPs.

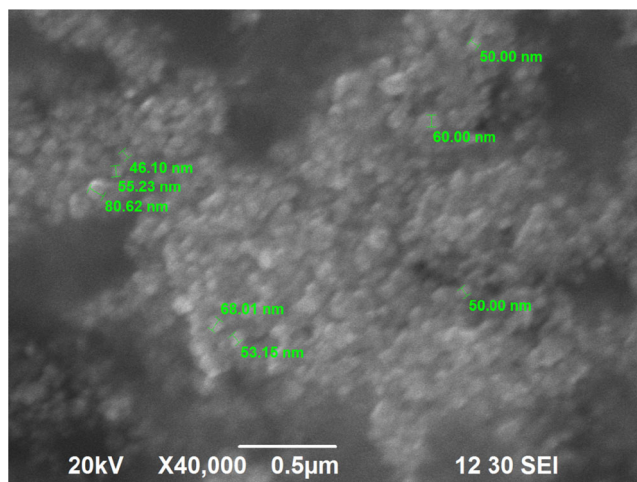


Fig. 5 SEM image of *B. megaterium*-synthesized AgNPs

Toxicity of bacteria-synthesized AgNPs against mosquito larvae

In vitro study on the *B. megaterium* AgNPs was done for its insecticidal activity against *Cx. quinquefasciatus* and *Ae. aegypti* at different concentrations (0.03, 0.06, 0.09, 0.12, 0.15, 0.25, 0.50, and 1.00 ppm, respectively) level. All larval stage of *Cx. quinquefasciatus* and *Ae. aegypti* were found to be more susceptible to the synthesized silver nanoparticles.

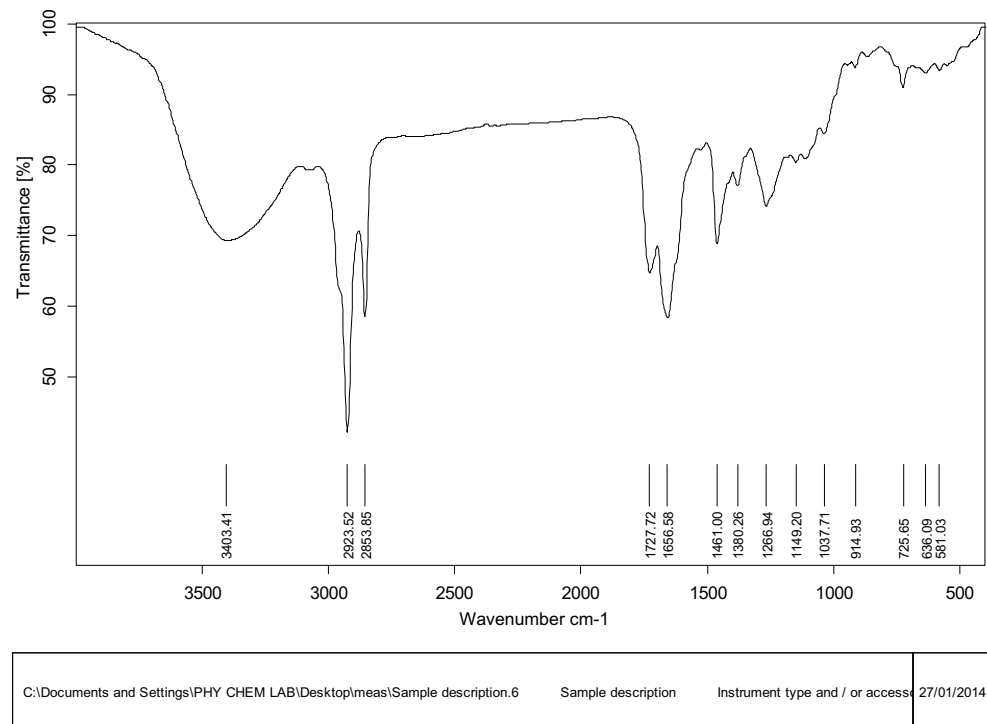
The *Bm*-AgNPs, conferred the lowest LC_{50} and LC_{90} values (0.567 and 2.260 ppm) against first instar larvae of *Cx. quinquefasciatus* at 1 ppm concentration. For second instars (0.900 and 4.444 ppm), third instars (1.349 and 8.269 ppm), and fourth instars (1.640 and 9.152 ppm) were observed after 24 h posttreatment. The percentage mortality and chi-squared, intercept and slope of the all the concentration and larval stages are shown in Tables 2 and 3.

The larvae of *Ae. aegypti* were found highly susceptible to the synthesized silver nanoparticles. The first instar larvae of *Ae. aegypti* were found highly susceptible to the synthesized silver nanoparticles and have shown the 87.6 ± 0.450 % mortality. In case of second instars (80.2 ± 0.115), third instars (66.0 ± 0.360), and fourth instars (53.8 ± 1.058), respectively. There was no mortality observed in the control group. Compared to *Cx. quinquefasciatus*, the lowest LC_{50} and LC_{90} values (0.240 and 0.955 ppm) were obtained when *Ae. aegypti* is treated with *B. megaterium* AgNPs. Which was confirmed that all the instars of *Ae. aegypti* are more susceptible to synthesized AgNPs. The tested AgNP concentration was directly proportional to the death rate instars while concentration was increase larval mortality also increase. The percentage mortality is shown in Table 4. All these chi-squared values, intercept, and slope of the all the concentrations and larval stages were shown in Table 5.

Discussion

Mosquitoes are generally held responsible for spreading serious diseases like malaria, filariasis, dengue, chikungunya, and Japanese encephalitis. It is known that larvicide plays a vital role in controlling mosquitoes in their breeding sites. There is a global interest in the use and manipulation of entomopathogens for biological control of insects. They are considered for natural mortality and environmentally safe agents. Entomopathogenic bacteria and fungi can be used as biolarvicide, as they actively control the mosquito vectors. However, studies on the effects of extracellular metabolites on mosquito larvae appear to be very limited in comparison with the use of spores of bacteria and mycelia of fungi to control the mosquito larvae. Unlike other mosquito larval control agents, the entomopathogens are unique.

Fig. 6 FTIR spectrum of *B. megaterium*-synthesized AgNPs



The drawback with use of chemical insecticides is the failure of many vector control campaigns resulting in the vector resurgence in epidemic zones. The commercial use of bacterial larvicides to control nuisance and vector mosquitoes has grown rapidly over the past two decades. These are now being globally used instead of synthetic chemical insecticides in many countries (Becker and Ludwig 1993; Becker 2000; Fillinger and Lindsay 2006). However, the resistance that has been started emerging in the mosquitoes (Wirth et al. 2010) provokes researchers to search for other organisms. It is now believed that any widely used insecticide will sooner or later encounter the resistance problem. Also, further, it has raised a question that what measures should be taken to counter this problem? The probable solution of the problem depends on more acumen in researches, especially in the

discovery and development of new species and candidate metabolites from effective organisms for suitable control of mosquitoes (Cruz et al. 2010).

The secondary metabolites of entomopathogenic fungi *Chrysosporium* sp. (Verma and Prakash 2010; Soni and Prakash 2010) and *Fusarium* (Peter et al. 1989; Prakash et al. 2010) have been screened as a potential larvicides successfully. However, the use of metabolites for controlling mosquito takes longer time. The fungus-derived metabolites work slowly but effectively. There is always need of fast and immediate working products for controlling mosquito population.

As silver nanoparticles have potent antibacterial and antifungal activity against broad spectrum antibiotic resistance microbial pathogens, evaluating them for the betterment of

Table 1 FTIR functional groups analysis for *Bacillus megaterium*-AgNPs

Vibration assignment/functional groups	Observed wavenumber (cm ⁻¹)	Visible intensity
O-H γ δ	3403.41	Strong broad beak
CH ₃ γ as + CH ₂ γ as + CH γ + CH (ketones) γ + OH γ	2923.52	Very sharp beak
C-H γ	2853.85	Sharp beak
C=O γ	1727.72	Small sharp beak
NH α + NO ₂ γ as + NO γ	1656.58	Sharp beak
-C-H γ	1461.00	Sharp beak
C-N γ	1266.64	Small sharp

α in-plane bending, β out-of-plane bending, ω wagging, τ twisting, γ stretching, δ bending, γ s symmetric stretching, γ as asymmetric stretching

Table 2 Larvicidal activity (in parts per million) of *Bacillus megaterium*-synthesized silver nanoparticles (Bm-AgNPs) against *Culex quinquefasciatus*

Concentration (ppm)	% Mortality of larval instars			
	First instar	Second instar	Third instar	Fourth instar
0.03	**	**	**	**
0.06	**	**	**	**
0.09	2.6±1.205	**	**	**
0.12	6.8±0.529	5.8±0.611	3.6±0.832	1.6±0.611
0.15	15.2±0.6	11.6±0.264	8.8±1.222	4.4±0.503
0.25	28.6±0.416	19.6±0.986	18.6±0.416	12.8±0.757
0.50	41.4±1.026	30.2±1.105	24.8±0.642	20.6±0.305
1.00	68.8±1.442	51.4±0.346	36.2±0.642	30.8±0.461
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

% Percent mortality values are means±SD; ** no mortality

human health by controlling human vector (i.e., Mosquitoes) using nanotechnology based biomaterials gain importance in recent years. Uses of microorganism for the synthesis of nanoparticles often have advantage over physical and chemical methods as it is an environment-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), *E. coli* (Gurunathan et al. 2009a, b), *Staphylococcus aureus* (Nanda and Saravanan 2009), *Brevibacterium casei* (Kalishwaralal et al. 2010), Phychrophilic bacteria (Shivaji et al. 2011), and *B. thuringiensis* (Najitha Banu et al. 2014) has been investigated.

The present investigation exploited the culture supernatant of *B. megaterium* for synthesis of silver nanoparticles. *B. thuringiensis* was often known for its mosquito larvicide effects whereas the resistance against cry toxins neglected their usage in the recent decades (Surendran and Vennison 2011; Cadavid-Restrepo et al. 2012; Chenniappan and Ayyadurai 2012). 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 and exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver

nanoparticles which correlated with the results obtained by Mukherjee et al. (2001), Vigneshwaran et al. (2007), Soni and Prakash (2012), and Najitha Banu et al. (2014).

In the UV–vis absorption spectrum, a strong, broad peak, located at about 420 nm, was observed for nanoparticles synthesized using the culture supernatant. Observation of this peak, assigned to a surface plasmon, is well documented for various metal nanoparticles with sizes ranging from 2 to 100 nm (Sastry et al. 1997, 1998). Kalimuthu et al. (2008) reported that the silver nanoparticle synthesis by *B. licheniformis*, where cultures in the stationary phase showed the maximum synthesis of AgNps. The significant results were obtained in the present study, *B. megaterium* have given a characteristic band at 430 nm. While no absorption peaks were observed in both controls (positive and negative). Many research groups have been observed the absorption maxima of colloidal silver solution between 410 and 440 nm which is assigned to surface plasmon of various metal nanoparticles (Sarkar et al. 2010).

Chandran et al. (2006) reported that the SEM image showed relatively spherical shape nanoparticles formed with diameter range 48–67 nm. The SEM analysis by Khandelwal et al. (2010) showed the particles between 25 and 50 nm as well as the cubic structure of the nanoparticles. Vivek et al. (2011) observed that the SEM analysis of silver nanoparticles

Table 3 LC_{50} , LC_{90} , intercept, slope, and chi-squared (χ^2) value of *Bacillus megaterium*-synthesized silver nanoparticles (Bm-AgNPs) against *Cx. quinquefasciatus*

Larval instar	LC_{50} (lower and upper confidence limit)	LC_{90} (lower and upper confidence limit)	Intercept	Slope	χ^2
First instar	0.567 (0.488–0.676)	2.260 (1.701–3.272)	2.133	5.526	7.690
Second instar	0.900 (0.730–1.181)	4.444 (2.951–7.829)	1.848	5.084	10.637
Third instar	1.349 (1.010–2.023)	8.269 (4.723–18.839)	1.627	4.788	12.347
Fourth instar	1.640 (1.197–2.596)	9.152 (5.060–22.592)	1.716	4.630	7.457

LC_{50} lethal concentration that kills 50 % of the exposed larvae, LC_{90} lethal concentration that kills 90 % of the exposed larvae

*Significant at 0.05 % level

Table 4 Larvicidal activity (in parts per million) of *Bacillus megaterium*-synthesized silver nanoparticles (Bm-AgNPs) against *Ae. aegypti*

Concentration (ppm)	% Mortality of larval instars			
	First instar	Second instar	Third instar	Fourth instar
0.03	4.2±0.529	1.6±0.450	**	**
0.06	9.6±0.818	7.8±0.366	5.8±0.360	2.2±0.305
0.09	15.8±0.929	14.2±0.416	11.6±0.450	8.0±0.4
0.12	20.0±1.040	17.6±0.808	14.2±0.251	11.6±0.450
0.15	33.6±0.8	27.8±0.929	18.4±0.305	14.2±0.416
0.25	58.2±0.115	46.2±0.416	37.6±0.450	34.4±1.249
0.50	78.4±0.4	61.8±0.360	50.4±0.832	40.2±1.311
1.00	87.6±0.450	80.2±0.115	66.0±0.360	53.8±1.058
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

% Percent mortality values are means±SD; ** no mortality

synthesized by the help of *Gelidiella acerosa* extract having average mean size of the silver nanoparticles and seems to be spherical in morphology. Similarly, herein, scanning electron microscopic image has confirmed the presence of nanoparticles and the particles size ranged between 46.10 and 80.62 nm.

The EDX profile showed a strong elemental signal along with oxygen, which may have originated from the biomolecules bound to the surface of the nanoparticles (Jea and Beom 2009). The EDX analysis obtained in the present study confirmed the presence of AgNPs in the aqueous solution and showed strong signal energy peaks for silver atoms in the range of 3–4 keV which is typical for the absorption of metallic silver nanoparticles. EDX analysis of silver nanoparticles showed the signal characteristics of elemental silver, due to the surface plasmon resonance property. Silver nanoparticles show absorption band peak at approximately 3 keV (Magudapathy et al. 2001; Arunachalam et al. 2012; Behera et al. 2013). In earlier study, the formation of individual spherical-shaped AgNPs in the range 2.5–4 keV by using *Alfalfa* has been reported by Gardea-Torresdey et al. (2003).

FTIR spectra clearly indicates that the biomolecules especially proteins present in filtrate are responsible for synthesis and stabilization of AgNPs (Dhanasekaran and Thangaraj 2013). Sen (2004) reported that the very strong absorption

peaks at 1624 and 1600 cm^{-1} and the strong absorption peaks at 1383 and 1352 cm^{-1} represents the presence of NO_2 which may be from AgNO_3 solution, the metal precursor involved in the Ag nanoparticle synthesis process. Strong interaction of water with the surface of silver could be the reason for the O–H stretching mode peaks at 2926, 2812, and 2717 cm^{-1} and O–H in plane bending mode peaks at 1383 and 1352 cm^{-1} . The similar results were obtained from the present study, very strong broad absorption peaks at 3403.41 (O–H stretch and bending); very strong and sharp peaks at 2923.52 cm^{-1} (ketones). The FTIR results thus indicate that the secondary structure of the proteins is not affected as a consequence of reaction with the Ag^+ ions or binding with the silver nanoparticles. This result suggests that the biological molecules could possibly perform a function for the formation and stabilization of silver nanoparticles in an aqueous solution. It is well known that proteins can bind to silver nanoparticles through free amine groups in the proteins (Gole et al. 2001) and therefore stabilization of the silver by surface-bound protein is a possibility (Logeswari et al. 2013).

Very few researches were recorded by synthesis of silver nanoparticles using bacteria against pest. Najitha Banu et al. (2014) reported that the *B. thuringiensis* synthesized silver nanoparticles against *Ae. aegypti*. Similarly, the present study *B. megaterium* was used for synthesis of silver nanoparticles

Table 5 LC_{50} , LC_{90} , intercept, slope, and chi-square (χ^2) value of *Bacillus megaterium*-synthesized silver nanoparticles (Bm-AgNPs) against *Ae. aegypti*

Larval instar	LC_{50} (lower and upper confidence limit)	LC_{90} (lower and upper confidence limit)	Intercept	Slope	χ^2
First instar	0.240 (0.214–0.271)	0.955 (0.771–1.249)	2.134	6.323	5.861
Second instar	0.331 (0.289–0.385)	1.593 (1.213–2.259)	1.877	5.901	1.995
Third instar	0.494 (0.417–0.606)	2.811 (1.976–4.487)	1.697	5.519	5.508
Fourth instar	0.700 (0.569–0.912)	4.435 (2.882–8.005)	1.598	5.2447	10.175

LC_{50} lethal concentration that kills 50 % of the exposed larvae, LC_{90} lethal concentration that kills 90 % of the exposed larvae

*Significant at 0.05 % level

against *Cx. quinquefasciatus* and *Ae. aegypti*. AgNPs have been synthesized by using the aqueous extract of leaf and bark of *Ficus religiosa* against *Cx. quinquefasciatus* (Soni and Prakash 2015).

Sundaravadivelan and Nalini (2012) exemplified the effect of *Pedilanthus tithymaloides* leaf synthesized silver nanoparticles against the dengue vector *Ae. aegypti* and has been observed their LC₅₀ values 0.046, 0.051, 0.046, 0.167, and 0.054 % (I–IV instars and pupa) have been observed at 0.25 % concentration level, which has the lowest concentration compare to aqueous stem extract alone, and its LC₅₀ values 1.529, 1.282, 1.450, 2.210, and 1.455 % have also been noticed after 24-h exposure. The larvicidal efficacies of synthesized AgNPs using aqueous leaf extract of *Vinca rosea* (L.) (Apocynaceae) against the larvae of malaria vector *An. stephensi* Liston and filariasis vector *Cx. quinquefasciatus* Say (Diptera: Culicidae) have been determined (Subarani et al. 2013). In their larvicidal activity test, the results showed that the maximum efficacy has been observed in synthesized AgNPs against the fourth instar larvae of *An. stephensi* (LC₅₀ 12.47 and 16.84 mg/mL and LC₉₀ 36.33 and 68.62 mg/mL) on 48 and 72 h of exposure and against *Cx. quinquefasciatus* (LC₅₀ 43.80 mg/mL and LC₉₀ 120.54 mg/mL) on 72-h exposure, and aqueous extract showed 100 % mortality against *An. stephensi* and *Cx. quinquefasciatus* (LC₅₀ 78.62 and 55.21 mg/mL and LC₉₀ 184.85 and 112.72 mg/mL) on 72-h exposure at concentrations of 50 mg/mL, respectively. The AgNPs did not exhibit any noticeable toxicity on *Poecilia reticulata* after 24, 48, and 72 h of exposure. Above results were comparatively not significant for the present investigation; the LC₅₀ and LC₉₀ values are 0.240, 1.219; 0.337, 2.210; 0.430, 2.453; and 0.652, 2.916 ppm for *Cx. quinquefasciatus* and 0.065, 0.558; 0.075, 0.707; 0.0998, 0.959; and 0.137, 1.278 ppm for *Ae. aegypti* treated with *B. megaterium*-synthesized silver nanoparticles.

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, *B. thuringiensis* var. *israelensis* and *B. 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; Cadavid-Restrepo et al. 2012; Chenniappan and Ayyadurai 2012). So, novel mosquito control agent is necessary for control the mosquito population at bottom level. Nowadays, botanical extract-synthesized silver nanoparticles are mostly used for pest control. Compared to the present investigation, the bacteria-synthesized silver nanoparticles were more actively participated to controlling mosquito larval population. This is probably the first report with synthesized silver nanoparticles using *B. megaterium* for control of *Cx. quinquefasciatus* and *Ae.*

aegypti. The microbe-mediated silver nanoparticles have rapid impact on mosquito population and thus conclude that the bacteria-mediated synthesis of silver nanoparticles distinctively plays a role for vector control tactic.

Acknowledgments The authors gratefully acknowledge the management of M.K.M. Group of Colleges, Hodal and Thiagarajar College (Autonomous), Madurai, for providing the facilities to perform the research works in the PG and Research Department of Zoology and Microbiology. Author ANB thanked UGC-MANF, India, for the financial support, and CRME (ICMR), Madurai, kindly supplied eggs and larvae required during our work. We thank the Department of Chemistry, Madras University and Karunya University, Coimbatore, for the instrumental analysis.

References

- Abbott WS (1925) A method of computing the effectiveness of an insecticide. *J Econ Entomol* 18:265–266
- Ahmad RS, Sara M, Himid RS, Hossein J, Ashraf-Asadat N (2007) Rapid synthesis of silver nanoparticles using culture supernatants of Enterobacteria: a novel biological approach. *Process Biochem* 42: 919–923
- Arunachalam N, Murty US, Kabilan L, Balasubramanian A, Thenmozhi V, Narahari D, Ravi A, Satyanarayana K (2004) Studies on dengue in rural areas of Kurnool District, Andhra Pradesh, India. *JAMCA* 20:87–90
- Arunachalam R, Dhanasingh S, Kalimuthu B, Uthirappan M, Rose C, Mandal AB (2012) Phytosynthesis of silver nanoparticles using *Coccinia grandis* leaf extract and its application in the photocatalytic degradation. *Colloids Surf B* 94:226–230
- Becker N (2000) Bacterial control of vector-mosquitoes and blackflies. In: Charles JF, Delecluse A, Nielson-Le Roux C (eds) *Entomopathogenic bacteria: from laboratory to field application*. Kluwer, Dordrecht, the Netherlands, pp 383–398
- Becker N, Ludwig M (1993) Investigations on possible resistance to *Aedes vexans* field populations after 10-year application of *Bacillus thuringiensis israelensis*. *JAMCA* 9:221–224
- Behera SS, Jha S, Arakha M, Panigrahi TK (2013) Synthesis of silver nanoparticles from microbial source—a green synthesis approach, and evaluation of its antimicrobial activity against *Escherichia coli*. *Int J Engine Res Appl* 3:058–062
- Cadavid-Restrepo G, Sahaza J, Orduz S (2012) Treatment of an *Aedes aegypti* colony with the Cry11Aa toxin for 54 generations results in the development of resistance. *Mem Inst Oswaldo Cruz* 107:74–79
- Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M (2006) Synthesis of gold nanotriangles and silver nanoparticles using *Aleo vera* plant extract. *Biotechnol Prog* 2:577–583
- Chaturvedi UC, Nagar R (2008) Dengue and dengue haemorrhagic fever: Indian perspective. *J Biosci* 33:429–441
- Chenniappan K, Ayyadurai N (2012) Synergistic activity of Cyt1A from *Bacillus thuringiensis* subsp. *israelensis* with *Bacillus sphaericus* B101 H5a5b against *Bacillus sphaericus* B101 H5a5b-resistant strains of *Anopheles stephensi* Liston (Diptera: Culicidae). *Parasitol Res* 110:381–388
- Cruz D, Fale PL, Mourato A, Va PD, Serralheiro ML, Lino AR (2010) Preparation and physicochemical characterization of Ag nanoparticles biosynthesized by *Lippia citriodora* (Lemon Verbena). *Colloids Surf B* 81:67–73
- Dhanasekaran D, Thangaraj R (2013) Evaluation of larvicidal activity of biogenic nanoparticles against filariasis causing *Culex* mosquito vector. *Asian Pacific J Trop Dis* 3:174–179

- Fillinger U, Lindsay SW (2006) Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicide in rural Kenya. *Trop Med Int Health* 11:1629–1642
- Gardea-Torresdey JL, Gomez E, Peralta-videa J, Parson JG, Troiani P, Santiago HE, Jose-Yacamán M (2003) *Alfalfa* sprouts: a natural source for the synthesis of silver nanoparticles. *Langmuir* 13:1357
- Gole A, Dash C, Ramachandran V, Mandale AB, Sainkar SR, Rao M, Sastry M (2001) Pepsin-gold colloid conjugates: preparation, characterization and enzymatic activity. *Langmuir* 17:1674–1679
- Gubler DJ, Trent DW (1993) Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. *Infect Agents Dis* 2:383–393
- Guneidy A, Ebeid A, Salem H (1988) Development and reversion of malathion resistance in adult *Culex pipiens*. *Indian J Entomol* 50: 45–54
- Gurunathan S, Kalishwaralal K, Vaidyanathan R, Venkataraman D, Pandian SRK, Muniyandi J, Hariharan N, Eom SH (2009a) Biosynthesis, purification characterization of silver nanoparticles using *Escherichia coli*. *Colloids Surf B* 74:328–335
- Gurunathan S, Lee KJ, Kalishwaralal K, Sheikpranbabu S, Vaidyanathan R, Eom SH (2009b) Antiangiogenic properties of silver nanoparticles. *Biomed Mater Res* 30:6341–6350
- Jea YS, Beom SK (2009) Rapid biological synthesis of silver nanoparticles using plant leaf extracts. *Bioprocess Biosyst Eng* 32:79–84
- Jegadeeswaran P, Shivaraj R, Venkatesh R (2012) Green synthesis of silver nanoparticles from extract of *Padina tetrastrum* leaf. *Digest J Nanomat Biostruc* 7:991–998
- Kalimuthu K, Babu SR, Venkataraman DM, Bilal Gurunathan S (2008) Biosynthesis of silver nanoparticles by *Bacillus licheniformis*. *Colloids Surf B* 65:150–153
- Kalishwaralal K, Deepak V, Pandian SRK, Kottaisamy M, BarathManiKanth S, Karthikeyan S, Gurunathan S (2010) Biosynthesis of silver and gold nanoparticles using *Brevibacterium casei*. *Colloids Surf B* 77:257–262
- Kamaraj C, Bagavan A, Rahuman AA, Zahir AA, Elango G, Pandiyan G (2009) Larvicidal potential of medicinal plant extracts against *Anopheles subpictus* Grassi and *Culex tritaeniorhynchus* Giles (Diptera: Culicidae). *Parasitol Res* 104:1163–1171
- Khandelwal N, Singh A, Jain D, Upadhyay MK, Verma HN (2010) Green synthesis of silver nanoparticles using *Argemone Mexicana* leaf extract and evaluation of their antimicrobial activities. *Digest J Nanomed Biostruc* 5:482–489
- Kumar (1984) Larvicidal activity of different product against *Culex quinquefasciatus* mosquito larvae. *J Environ Biol* 11:101–104
- Kumar A, Sharma SK, Padbidri VS, Thakare JP, Jain DC, Datta KK (2001) An outbreak of dengue fever in rural areas of northern India. *J Commun Dis* 33:274–281
- Logeswari P, Silambarasan S, Abraham J (2013) Eco-friendly synthesis of silver nanoparticles from commercially available plant products and their antibacterial properties. *Scientia Iranica F* 20:1049–1054
- Magudapathy P, Gangopadhyay P, Panigrahi BK, Nair KGM, Dhara S (2001) Electrical transport studies of Ag nanoclusters embedded in glass matrix. *Phys B Condens Matter* 299:142–146
- Mukherjee P, Ahmad A, Mandal D (2001) Bioreduction of AuCl₄⁻ ions by the fungus, *Ventricillium* sp. and surface trapping of the gold nanoparticles formed. *Angew Chem Int Ed Engl* 40(19):3585–3588
- Najitha Banu A, Balasubramanian C (2014) Myco-synthesis of silver nanoparticles using *Beauveria bassiana* against dengue vector *Aedes aegypti* (diptera: culicidae). *Parasitol Res* 113:2869–2877
- Najitha Banu A, Balasubramanian C, Vinayaga Moorthi P (2014) Biosynthesis of silver nanoparticles using *Bacillus thuringiensis* against dengue vector, *Aedes aegypti* (Diptera: Culicidae). *Parasitol Res* 113:311–316
- Nanda A, Saravanan M (2009) Biosynthesis of silver nanoparticles from *Staphylococcus aureus* and its antimicrobial activity against MRSA and MRSE. *Nanomed* 5:452–456
- Peter H, Math V, Roberts DW (1989) Enzymes involved in the synthesis of fungal toxins. *Proceeding of International Conference Biopesticide. Theory and Practice* 169–181
- Pialoux G, Gauzere B, Jaureguierry S (2007) Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis* 7:319–327
- Prakash S, Singh G, Soni N, Sharma S (2010) Pathogenicity of *Fusarium oxysporum* against the larvae of *Culex quinquefasciatus* (Say) and *Anopheles stephensi* (Liston) In laboratory. *Parasitology* 107:651–655
- Rajesh WR, Jaya RL, Niranjan SK, Vijay DM, Sahebrao BK (2009) Phytosynthesis of silver nanoparticle using *Gliricidia sepium* (Jacq.). *Curr Nanosci* 5:117–122
- Sarkar R, Kumbhakar P, Mitra AK (2010) Green synthesis of silver nanoparticles and its optical properties. *Digest J Nanomat Biostruc* 5: 491–496
- Sastry M, Mayya KS, Bandyopadhyay K (1997) pH dependent changes in the optical properties of carboxylic acid derivatized silver colloidal particle. *Colloids Surf A Physicochem Eng* 127:221–228
- Sastry M, Patil V, Sainkar SR (1998) Electrostatically controlled diffusion of carboxylic acid derivatized silver colloidal particles in thermally evaporated fatty amine films. *J Physical Chem B* 102:1404–1410
- Sen GNP (2004) Water induced stabilization of ZnS nanoparticles. *Solid State Commun* 132:791–794
- Shivaji S, Madhu S, Singh S (2011) Extracellular synthesis of antibacterial silver nanoparticles using psychrophilic bacteria. *Process Biochem* 49:830–837
- Soni N, Prakash S (2010) Effect of *Chrysosporium keratinophilum* metabolites against *Culex quinquefasciatus* after chromatographic purification. *Parasitol Res* 107:1329–1336
- Soni N, Prakash S (2011) Efficacy of fungus mediated silver and gold nanoparticles against *Aedes aegypti* larvae. *Parasitol Res* 110:175–184
- Soni N, Prakash S (2012) Efficacy of fungus mediated silver and gold nanoparticles against *Aedes aegypti* larvae. *Parasitol Res* 110(1): 175–184
- Soni N, Prakash S (2013) Possible mosquito control by silver nanoparticles synthesized by soil fungus (*Aspergillus niger* 2587). *Adv Nanoparticles* 2:125–132
- Soni N, Prakash S (2015) Different geometrical AgNPs for vector control and their added value of antibacterial activity. *J Parasitol Photon* 105:232–243
- Subarani S, Sabhanayakam S, Kamaraj C (2013) Studies on the impact of biosynthesized silver nanoparticles (AgNPs) in relation to malaria and filariasis vector control against *Anopheles sptephensi* Liston and *Culex quinquefasciatus* Say (Diptera: Culicidae). *Parasitol Res* 112: 487–499
- Sundaravadivelan C, Nalini M (2012) Biolarvicidal effect of phyto-synthesized silver nanoparticles using *Pedilanthus tithymaloides* (L.) Poit stem extract against the dengue vector *Aedes aegypti* L. (Diptera; Culicidae). *Asian Pacific J Trop Biomed* 1–8
- Surendran A, Vennison SJ (2011) Occurrence and distribution of mosquitocidal *Bacillus sphaericus* in soil. *Acad J Entomol* 4:17–22
- Tanja K, Ralph J, Eva O, Claes-Goran G (1999) Silver-based crystalline nanoparticles, microbially fabricated. *Proc Natl Acad Sci* 96:13611–13614
- Verma P, Prakash S (2010) Efficacy of *Chrysosporium tropicum* metabolite against mixed population of adult mosquito (*Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti*) after purification with flash chromatography. *Parasitol Res* 107:163–166
- Vigneshwaran N, Ashtaputrea NM, Varadarajana PV, Nachanea RP, Paralikara KM, Balasubramanyaa RH (2007) Biological Synthesis of Silver Nanoparticles using the Fungus *Aspergillus Flavus*. *Mater Lett* 61:1413–1418

- Vivek M, Senthil Kumar P, Steffi S, Sudha S (2011) Biogenic silver nanoparticles by *Gelidiella acerosa* extract and their antifungal effects. *Avicenna J Med Biotechnol* 3:143–148
- Wirth MC, Walton WE, Federici BA (2010) Evaluation of resistance to the *Bacillus sphaericus* bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subspecies *israelensis*. *Environ Microbiol* 12:1154–1160
- World Health Organization (2005) Guidelines for laboratory and field testing of mosquito larvicides WHO/CDS/WHOPES/GCDPP/13
- World Health Organization (2012) Lymphatic Filariasis. http://www.who.int/mediacentre/factsheets/fs_102/en/
- Yang T, Lu L, Fu G, Zhong S, Ding G (2009) Epidemiology and vector efficiency during a dengue fever outbreak in Cixi, Zhejiang Province, China. *J Vector Ecol* 34:148–154