



Biogenic Synthesis of Silver Nanoparticles and Valuation of their Antimicrobial Activity against Dengue Larvae

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Abstract

Dengue is a viral illness that is spread by a female mosquito *Aedes aegypti*. The occurrence of dengue has increased about 30 times. Half of the world's population is endangered by this disease as statistics provide a data of 50-million cases reported annually, while currently the disease is endemic in more than 100 countries. It can be controlled by number of ways but most preferred and environmental friendly method is biological control. For this purpose, Silver nanoparticles were successfully synthesizing by using *Bacillus thuringiensis*. The Surface Plasmon Resonance (SPR) property of synthesized nanoparticle was studied by UV-Vis spectroscopy and the peak of the spectra was found to be at 420 nm. The XRD study gives results that the nanoparticle form in the process is crystalline in nature. The larvicidal activity of silver nanoparticles concludes that the silver nanoparticles show significant larvicidal activity against Dengue vector *A. aegypti*. The biosafety analysis proved Bt-AgNPs safe for other biological species.

Keywords: Dengue; Nanoparticle; *Bacillus thuringiensis*; Silver

Introduction

Dengue is a viral illness that is spread by a female mosquito *A. aegypti*. This disease has swiftly stretched around the globe in the recent decade. In 1950s, severe dengue (formerly known by the name of "dengue hemorrhagic fever") was first investigated during dengue epidemics in Thailand and Philippines. Now a day, it is affecting Asian as well as Latin American countries and has become a leading cause behind hospitalization and mortality among all age groups in proposed regions [1]. In the last five decades, the occurrence of dengue has increased about 30 times. Half of the world's population is endangered by this disease as statistics provide a data of 50-million cases reported annually, while currently the disease is endemic in more than 100 countries.

Dengue fever is a rigorous, flu-like sickness that can affect a man at neonate, adolescent, and adult stage of the age, most often resulting in severe dengue fever. Severe dengue is a fatal illness which results in bleeding, respiratory distress, fluid accumulation, plasma leaking, or organ impairment. Cautionary signs appear in 2 to 7 days (following a temperature underneath 38°C) after the emergence of first symptom and comprises of persistent vomiting, severe abdominal tenderness, muscle fatigue, bleeding gums, restlessness, and rapid breathing. The next 24 h to 48 h are of critical importance. This time duration may prove lethal for the patient for whom appropriate medical attention is required to circumvent disease complexities and death risk. Dengue is extensively spread, specially throughout the tropics, with local variations due to temperature, rain fall, rapid and unplanned urbanization.

Ultrafine particles possessing at least one of its all dimensions in between 1 nm to 100 nm size range are called nanoparticles. Production of nanoparticles is possible through number of processes like biological, physical, and chemical method. Most preferred process over the others is biological method also known as "green synthesis". Because of its requirement of both weak and strong reducing agents, chemical method is harmful and toxic to environment along with its low production rate, while on the contrary biological method is eco-friendly and nontoxic. Biological agents like yeast, actinomycetes, fungi, and bacteria are used for the biological synthesis of nanoparticles. Enzyme that hydrolyses metal are secreted by biological agents thus giving an enzymatic reduction of metal ions.

Biological control comprises of targeting a specific species with specific organisms that ultimately reduce the targeted species' population by parasitizing, preying, and competing with it. WHO have reported that a diversified range of larvivorous fishes and predatory freshwater crustaceans (small copepods) can be used against the initial larval stages of *A. aegypti*. Numerous microbes also were found to work as biological control agent with the potential of AgNPs synthesis. *Bacillus* species are known for their ability to construct bio pesticides and antibiotics, for whose production *B. thuringiensis* is known to be the best. Since more than 40 years, producing effective, less hazardous, highly specific activity against the wide spectrum of insect pests. The main purpose to conduct the present study was to use the modern nanotechnology concept with a biological synthesis approach for the development of a larvicide against dengue larva.

Materials and Methods

This study was held at the Centre of Environment Protection Studies (CEPS), PCSIR Laboratories Complex, Lahore. CEPS is a distinguished department of Pakistan Council of Scientific and Industrial Research (PCSIR). The Labs are well equipped and provided with all the required standards. All the regular as well as random checks were made to ensure high level accuracy and to minimize personal and technical errors, which played a vital role in the accomplishment of the research tasks.

Preparation of working solution of AgNO₃

From a 1 M solution of AgNO₃ solution 1 mM solution was prepared.

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For this purpose, 16.987 g of AgNO_3 was completely dissolved in 100 ml of distilled water, 1 ml of which was poured into 1000 ml of distilled water to make 1 mM solution.

Storage of AgNO_3 solution: Silver nitrate solution is photosensitive. In the presence of light, it gets hydrolyzed to give nitric acid and silver oxide. To avoid this problem, prepared solution was stored in an amber bottle for further processing.

Isolation of *B. thuringiensis*

B. thuringiensis strain was obtained from CEPS, PCSIR Lahore. Where the isolation of particular bacterial strain was done locally from different soil samples and maintained on different Nutrient agar slants and plates. Pure culture was thus obtained by streak plate method, these pure colonies further processed with gram staining and spore staining. Once staining was done, characterization was performed with morphological evidences and chemical test analysis to identify the particular species of bacteria. After the confirmation of bacterial strain. These potent strains were thus subjected to further massive production through optimized fermentation media. The isolated strain was grown in optimized fermentation media at 36°C for 72 h.

Extraction and washing of biomass

This *B. thuringiensis* culture was centrifuged at 150 rpm for 15 min. Supernatant was discarded and biomass was used as a starting material for the production of nanoparticles. Biomass was washed twice in 0.5% solution of NaCl. Subsequent washing was performed with TE buffer and distilled water (once each). Finally, it was suspended in 3 ml of injection water.

Synthesis of AgNPs

The washed biomass (1 ml) was poured into 99 ml of 1 mM aqueous silver nitrate solution. The pH was adjusted to 8.5 by NaOH. The flask containing this solution was placed in a horizontal shaking water bath at 150 rpm for 3 days at 36°C in dark. The appearance of yellowish brown colour was indicative of the production of AgNPs [2]. The solution was transferred into china dish for drying in an oven set at 50°C. Dried particles were collected and then processed for further characterization.

Characterization of Bt- AgNO_3

Bio reduction of silver ion is monitored in the aliquot by change of the colour of solution. Moreover, three different techniques were used to characterize AgNPs.

UV-Vis spectroscopy: A small amount of AgNPs sample was diluted in distilled water. This sample was further subjected to spectral analysis. Ag reduction was monitored by observing UV-Vis spectrum between the range of 300 nm - 600 nm. UV-Vis spectroscopy is generally recognized to examine size and shape of nanoparticles.

XRD-measurement: Purified silver nanoparticles were obtained by repeated centrifugation of AgNPs solution at 5000 rpm for 20 min followed by re-suspension of silver nanoparticles in 10 ml of deionized water. The dried AgNPs were then processed for XRD measurement by X-ray diffractometer. XRD- analysis is popular for its use to confirm structure of the subjected material. The crystalline domain size was calculated from the width of XRD peaks using the Scherrer formula.

$$D = 0.94 \lambda / \beta \cos \theta$$

D=average crystalline domain size

λ =X- wavelength

β =FWHM (full width at half maximum)

θ =Diffraction angle

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Larvicidal activity of Bt-silver nanoparticles

In order to investigate about larvicidal activity of *B. thuringiensis*, bioassay was performed according to protocol prescribed by World Health Organization. For this purpose, 15 larvae of 4th instar *A. aegypti* were taken in cups each containing 100 ml dechlorinated tap water. Different concentrations (1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm) of Bt-AgNPs were suspended in all cups, which were then covered with a mosquito net. Larval feed (a drop of yeast) was also added in all cups. The experiment was run along with a control. Normally *A. aegypti* larvae were much motile, the larvae which exhibited no motility after being probed with the help of a needle were termed as dead. To record mortality data, first reading was taken at 12 h and the next at 24 h. Each concentration was tested in three replicates.

Statistical analysis

Microsoft Excel 2016 was used to analyze data for percentage mortality calculation. Probit analysis was further performed to calculate LC50 and LC90

Results and Discussion

The present research deals with the biological synthesis of Bt-AgNPs, characterizations of these nanoparticles and their larvicidal activity against dengue larva *A. aegypti*. The details of this investigations presented wide infra, and open ways for a different and effective dengue vector control approach.

Biosynthesis of Bt-AgNPs

1 mM solution of AgNO_3 along with biomass of *B. thuringiensis* culture was subjected to a reduction reaction. The change in colour from watery to brown exhibit the formation of silver nanoparticles (Figure 1a). Clear flasks are shown to indicate the color of solution. The experiment was run in an amber bottle to avoid light reaction (Figure 1b). A negative control comprising AgNO_3 solution without Bt was also run and no color change was observed in this flask (Figures 1c and 1d).

Characterization of synthesized silver nanoparticles

After the visual confirmation of silver nanoparticle synthesis, the Bt-AgNPs sample was further characterized with the help of different analysis techniques.

UV-Visible spectroscopy: The primary characterization of the colloidal dark brown Bt-AgNPs solution was done with the assistance of UV-Vis spectroscopic analysis. This analysis confirmed the AgNPs

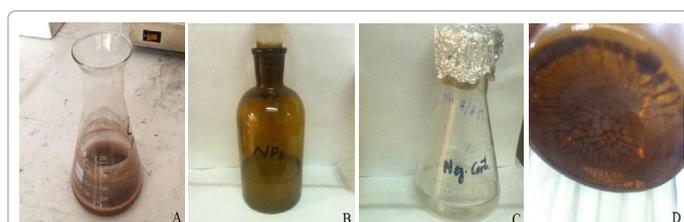


Figure 1: Visual observation of Bt-AgNPs synthesis. **A:** Dark brown color of solution due to nanoparticle synthesis, **B:** Bt-AgNPs solution in amber bottle, **C:** No change of color in negative control and **D:** Nanoparticle obvious at the bottom of the bottle.

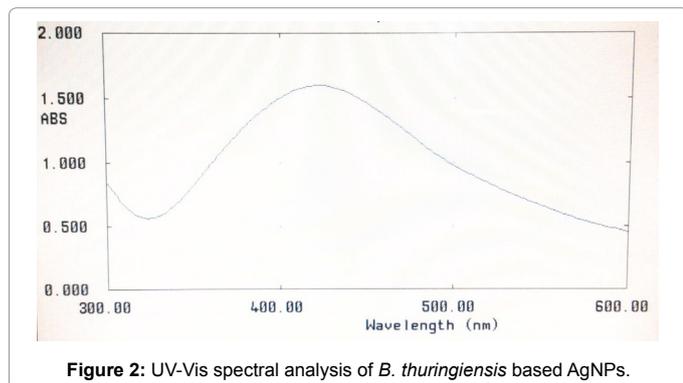


Figure 2: UV-Vis spectral analysis of *B. thuringiensis* based AgNPs.

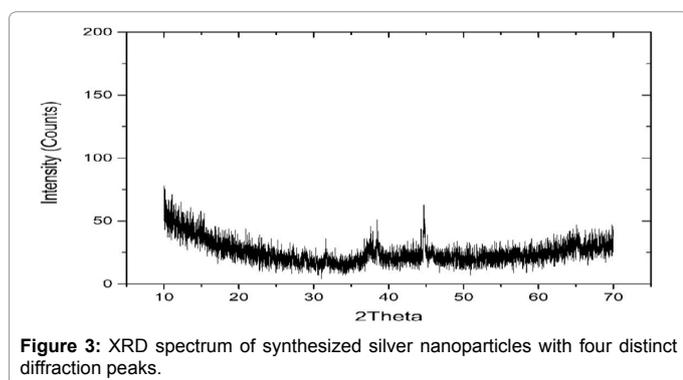


Figure 3: XRD spectrum of synthesized silver nanoparticles with four distinct diffraction peaks.

formation and stability of these particle. A strong, intense, and broad peak was observed at 420 nm by the absorption spectrum of AgNPs. This type of peak is referred to the SPR (Surface plasmon resonance) of the particles (Figure 2).

X-ray diffraction study: The figure is showing four intense peaks in the whole spectrum of 2θ ranging from 10 to 70. These sharp Bragg peaks might have resulted due to crystalline nature of silver nanoparticles. The estimated particle size using scherrers formula is estimated to be 19 nm (Figure 3).

Confirmation of larvicidal activity

For the confirmation of larvicidal activity of *B. thuringiensis* mediated silver nanoparticles, bioassays were conducted in replicates. The percentage larvicidal activity of every single experiment was obtained by taking mean of the replicate value of larvicidal activity. This helps to omit the effect of personal or systematic errors. The Table 1 here under explains percentage larvicidal activity with respect to different test concentration (conc.) at different time intervals.

Larvicidal activity of Bt-AgNPs at 1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm was tested against 15 larvae each which exhibited 100%, 97%, 85%, 75.6%, 47.3%, 32% mortality rate, respectively after 12 h. At the time of 2nd reading (24 h) the same test subject of 1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm, 0.001 ppm were found to exhibit 100%, 100%, 89%, 85%, 52% and 32% larvicidal activity respectively.

It was observed that there is a direct relationship between mortality

S. No.	Testing material parameter	Replica	Final conc. of test material larvae dose setting stage	Volume of test block	A. aegypti larvae growth stage	No. of larvae	1 st reading	Lethal %	mean	2 nd reading	Lethal %	Mean
Experiment No. 1												
1	Bt-AgNPs	R-1	1 ppm	100 ml	4 th instar	15	12 h	100	100%	24 h	100	100%
2	Bt-AgNPs	R-2	1 ppm	100 ml	4 th instar	15	12 h	100		24 h	100	
3	Bt-AgNPs	R-3	1 ppm	100 ml	4 th instar	15	12 h	100		24 h	100	
Experiment No. 2												
1	Bt-AgNPs	R-1	0.5 ppm	100 ml	4 th instar	15	12 h	100	97%	24 h	100	100%
2	Bt-AgNPs	R-2	0.5 ppm	100 ml	4 th instar	15	12 h	100		24 h	100	
3	Bt-AgNPs	R-3	0.5 ppm	100 ml	4 th instar	15	12 h	93		24 h	100	
Experiment No. 3												
4	Bt-AgNPs	R-1	0.1 ppm	100 ml	4 th instar	15	12h	87	85%	24 h	93	89%
5	Bt-AgNPs	R-2	0.1 ppm	100 ml	4 th instar	15	12h	81		24 h	87	
6	Bt-AgNPs	R-3	0.1 ppm	100 ml	4 th instar	15	12h	87		24 h	87	
Experiment No. 4												
8	Bt-AgNPs	R-1	0.05 ppm	100 ml	4 th instar	15	12 h	73	76%	24 h	81	85%
9	Bt-AgNPs	R-2	0.05 ppm	100 ml	4 th instar	15	12 h	73		24 h	87	
10	Bt-AgNPs	R-3	0.05 ppm	100 ml	4 th instar	15	12 h	81		24 h	87	
Experiment No. 5												
11	Bt-AgNPs	R-1	0.01 ppm	100 ml	4 th instar	15	12 h	54	49%	24 h	54	52%
12	Bt-AgNPs	R-2	0.01 ppm	100 ml	4 th instar	15	12 h	47		24 h	47	
13	Bt-AgNPs	R-3	0.01 ppm	100 ml	4 th instar	15	12 h	47		24 h	54	
Experiment No. 6												
14	Bt-AgNPs	R-1	0.05 ppm	100 ml	4 th instar	15	12 h	34	32%	24 h	34	32%
15	Bt-AgNPs	R-2	0.05 ppm	100 ml	4 th instar	15	12 h	34		24 h	34	
16	Bt-AgNPs	R-3	0.05 ppm	100 ml	4 th instar	15	12 h	27		24 h	27	
Experiment No. 7 (Control)												
17	Bt-AgNPs	R-1	No treatment	100 ml	4 th instar	15	12 h	0	0%	24 h	0	0%
18	Bt-AgNPs	R-2	No treatment	100 ml	4 th instar	15	12 h	0		24 h	0	
19	Bt-AgNPs	R-3	No treatment	100 ml	4 th instar	15	12 h	0		24 h	0	

Table 1: Analysis of larvicidal activity at different concentration of Bt-AgNPs.

and test subject concentration. As the concentration level decreases mortality rate was also observed to decrease. Maximum larvicidal activity was observed at 1 ppm and 0.5 ppm of Bt-AgNPs. Moreover, this experimentation also illustrate that better results have been observed at 24 h.

Larvicidal activity after 12 h of treatment: Figure 4 is the graphical illustration of Bt-AgNPs activity at 12 h of treatment. Control was found to exhibit no mortality. While 100% mortality was observed with 1 ppm of test material concentration. Mortality rate was found to decrease as the concentration of test material decreases.

Larvicidal activity after 24 h of treatment: Figure 5 showed 100% mortality both at 1 ppm and 0.5 ppm and with a drop-in concentration of test material, larvicidal activity also decreased. At 0.005 ppm, the mortality rate was less than 50% which is not considered as a significant result.

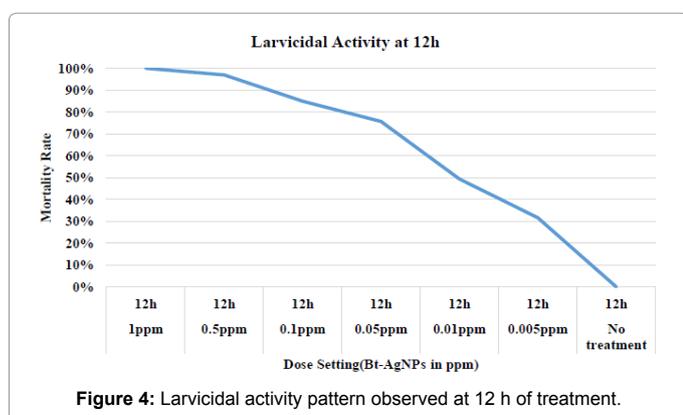


Figure 4: Larvicidal activity pattern observed at 12 h of treatment.

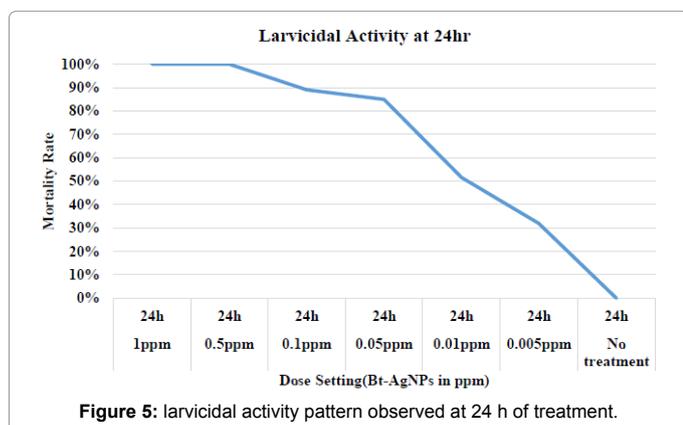


Figure 5: larvicidal activity pattern observed at 24 h of treatment.

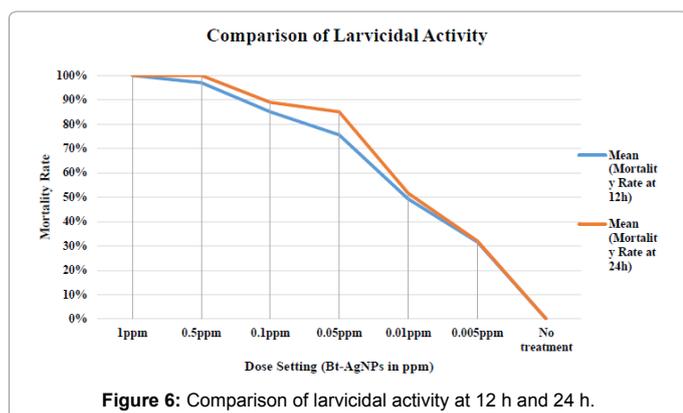


Figure 6: Comparison of larvicidal activity at 12 h and 24 h.

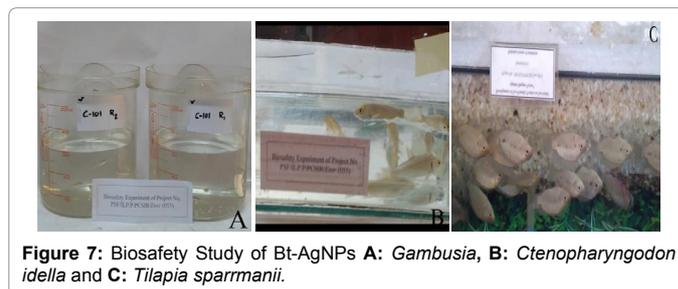


Figure 7: Biosafety Study of Bt-AgNPs A: *Gambusia*, B: *Ctenopharyngodon idella* and C: *Tilapia sparrmanii*.

Comparison of larvicidal activity at different time intervals: Figure 6 displac a comparison between mortality rate at 12 h and 24 h. It is obvious from the graph that an increase of mortality rate is experienced at 24 h. For 1 ppm concentration, the larvicidal activity rate remain same both at 12 h and 24 h. For 0.5 ppm, the mortality value was 97% at 12 h and 100% at 24 h. With 0.1 concentration, the mortality rate was 85% at 12 h and 89% at 24 h. When the concentration of the test material was 0.05 ppm, the larvicidal potential was 75.60% at 12 h and 85% at 24 h. At 0.01 ppm concentration, the larvicidal activity at 12 h was 49.30% and at 24 h it was 52%. Below 0.01 ppm the larvicidal activity was less than 50% both after 12 h and 24 h. Hence considered insignificant.

LC50 and LC90 Count for the tested concentrations of Bt-AgNPs: LC50, lethal concentration that kills 50% of the exposed larvae; LC90, lethal concentration that kills 90% of the exposed larvae.

The Table 2 explains the LC50 and LC90 values are 0.009 ppm and 0.104 ppm for the tested concentration 1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm of Bt-AgNPs.

Biosafety study of Bt-AgNPs

An experiment was conducted to investigate the biosafety potential of Bt-AgNPs. Three Different types of fishes (*Tilapia sparrmanii*, *Ctenopharyngodon idella*, *Gambusia affinis*) were taken. These fishes were exposed to different concentrations of Bt-AgNPs (1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm). No toxic effect was observed on fishes, all were alive even after 24 h of Bt-AgNPs exposure (Figures 7a-7c).

The world is facing endless challenges in the control of dengue disease. The present study enlightened the use of micro-organism to fabricate nanotechnology based biomaterials for the control of dengue disease. Silver nanoparticles are reported to exhibit a promising anti-fungal and anti-bacterial activities. Biologically synthesized AgNPs are being evaluated for their use in the progress of human health field. Microbe based synthesis of silver nanoparticles is generally preferred over other chemical approaches, as it is based on ecofriendly mode of synthesis. The intracellular and extracellular fabrication of AgNPs by bacteria *Brevibacterium casei*, *Staphylococcus aureus*, *B. licheniformis*, *E. coli* have been documented [3-5]. Similarly, *B. licheniformis* have been described to synthesize by reaction mixture sonification [3].

The current research exploited the biomass of *B. thuringiensis*, a soil-dwelling biological pesticide, for the silver nanoparticle synthesis. *B. thuringiensis* is well known for its larvicidal potential in the field of mosquito control. However, its use is being neglected because of

Concentration of Bt-AgNPs (ppm)	LC ₅₀ (ppm)	95% Fiducial CI		LC ₉₀ (ppm)	95% Fiducial CI	
		Lower	Upper		Lower	Upper
1, 0.5, 0.1, 0.05, 0.01, 0.005	0.009	0.004	0.020	0.104	0.048	0.225

Table 2: LC50 and LC90 count for Bt-AgNPs.

resistance toward cry toxin. Pugazhenthiran et al. [6] have documented that the Ag resistant *Bacillus* species form silver nanoparticles when cultured along with silver nitrate solution. The present research manifested that the yellowish brown colour of solution exhibit the reduction of silver ion, which is an authentic indication of silver nanoparticle formation. Banu et al. [7] have reported the change of colour from clear to brown as an indication of AgNPs synthesis. The yellowish brown colour of solution undoubtedly verify the AgNO₃ reduction by *B. thuringiensis* generating AgNPs [2,8,9]. Hence literature supports the present research finding of the color change of the solution.

In the present investigation, the Bt-AgNPs exhibited a characteristic peak around 420 nm. This broad peak is because of surface plasmon resonance of the electron present on the surface of the particular nanoparticles. Hence most of the literature have demonstrate the absorption peak of silver nanoparticles at 420 nm [2,4,7]. Hence literature is supporting UV-Vis analysis of this research. Correspondingly, Kalishwaralal et al. [5] reported the *B. licheniformis* mediated silver nanoparticle ranging in size of around 50 nm.

In the present research XRD analysis of the Bt-AgNPs sample exhibit four peaks, which Declared the particles to be crystalline in nature. The particle size of AgNPs is 19 nm. Dash, [2] have reported four intense peaks for XRD analysis of AgNPs. Jain et al. [10] have also reported four peaks and the particle size of BT-AgNPs as 15 nm, which is close enough to the present research finding.

Conclusion

The present investigation revealed that Bt-AgNPs exhibit 100% mortality at the concentration of 1 ppm and 0.5 ppm. Soni and Prakash [11] have reported the fabrication of AgNPs by using a popular fungus *C. tropicum*, calculate LC50 and LC90 values as 4 and 8.91 against Dengue vector, these values were comparatively much higher than the LC50 and LC90 values measured in the current research work. Priyadarshini et al. [12] have reported that *Euphorbia hirta* mediated AgNPs exhibit LC50 as (10.14 ppm, 27.89 ppm) and LC90 as (31.98 ppm, 69.84 ppm). The present research also reported a unique result that Bt-AgNPs exhibit better mortality effects after 24 h of treatment as compared to 12 h.

In the present study, biosafety analysis experiment showed that Bt-AgNPs have a nontoxic effect on *Tilapia sparrmanii*, *Ctenopharyngodon idella* and *Gambusia affinis*. So Bt-AgNPs can be considered as biologically safe. Recently, Salunkhe et al. [13] 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*. The AgNPs did not exhibit any noticeable toxicity on *Poecilia reticulata* after 24 h, 48 h, and 72 h of exposure. These results suggest that the synthesized AgNPs have the potential to be used as an ideal ecofriendly approach for the control of the *A. stephensi* and *C. quinquefasciatus* [14].

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.

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