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Biosynthesis of silver nanoparticles mediated by entomopathogenic bacteria: *Bacillus cereus* VCRC 641 against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*

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Abstract

Mosquito-borne diseases are illnesses caused by the transmission of viruses, parasites, and protozoa to humans by mosquito vectors. Mosquitoes spread various diseases, including, dengue fever, malaria, chikungunya, West Nile fever, Zika and yellow fever. Bacterial DNA and cellular proteins were shown to help produce AgNPs, with bacterial DNA acting as a stabilising agent and cellular proteins acting as both a reducing and stabilising agent. As a result, the bacterial lysate, which contains a lot of DNA and proteins, could help in the creation of AgNPs that are larvicidal against mosquito larvae. Therefore, this work was aimed to study the larvicidal effect of synthesized AgNPs by *Bacillus cereus* VCRC 641 cell lysate. The present study demonstrates the larvicidal efficacy of silver nanoparticles (AgNPs) produced by *Bacillus cereus* VCRC 641 against the diseases-causing mosquitoes *Aedes aegypti, Culex quinquefasciatus*, and *Anopheles stephensi.* Silver nano particles were synthesized using *Bacillus cereus* to enhance its mosquitocidal efficacy. The level of toxicity in the AgNPs synthesized *B. cereus* was increased 117 fold in comparison with the cell lysate.

Keywords: AgNPs; *Bacillus cereus; Aedes aegypti; Culex quinquefasciatus; Anopheles stephensi*

1. Introduction

Mosquito-borne diseases are illnesses caused by the transmission of viruses, parasites, and protozoa to humans by mosquito vectors. Mosquitoes spread various diseases, including, dengue fever, malaria, chikungunya, West Nile fever, Zika, and yellow fever [1]. According to WHO, around 4.3 billion people die each year from malaria and dengue, and the development of these mosquito-borne diseases is increasing due to global climate change and people moving away from endemic areas [2,3]. As a result, finding some efficient and cost-effective solutions for preventing and reducing mosquito-borne diseases are a challenge to health researchers. Mosquitoes can be controlled in a variety of ways, including environmental management and removal. Elimination of mosquito larvae is one of the efficient way to reduce vector borne diseases. Insecticides and insect growth regulators are compounds which are used to kill mosquitoes. Insecticides are toxic compounds that disrupt the normal functioning of the nervous system to kill insects. DDT was once a commonly used pesticide, but it is now banned in many countries due to its adverse effects on humans and other non-target living things, as well as its long persistence in the environment. Pyrethroid insecticides, which are extensively used for larval control, are another type of insecticides that are now not recommended due to its high resistance development in insect larvae. Due to their high efficacy in killing insect larvae and short half-life in the

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environment, organophosphates (such as temephos and fenthion) are the currently used insecticides. Anti-juvenile hormones and chitin synthesis inhibitors are well-known growth regulators used to kill insects. Anti-juvenile hormones (such as methoprene and pyriproxyfen) have been shown to impede larval development. Chitin synthesis inhibitors (such as diflubenzuron and triflumuron) have the potential to harm larvae during the moulting process [4]. Biological control agents, as an alternative to toxic chemicals, have generated a lot of interest in mosquito control because of their safety, cost-effectiveness, and eco-friendliness [5]. Proteinaceous toxins produced by certain bacteria such as *Bacillus thuringiensis* are used to kill mosquito larvae [6]. The other mosquitocidal bacteria include *Bacillus amyloliquefaciens* [7], *Bacillus subtilis* [8], *Bacillus sphaericus* [9], *Lysinibacillus sphaericus* [10] and *Bacillus cereus* [11]. Multiple crystal (Cry) proteins and cytolytic (Cyt) toxins in this *Bacillus* induce critical toxicity in mosquito larvae. Cry proteins primarily target the outer membrane of gut cells, causing cell membrane leakage, gut function disruption, and larval mortality. Cyt toxins cause larval mortality through cytolytic action at the cell membrane [8, 9, 11, 12]. BinA and BinB proteins are the main toxins of *L. B. sphaericus*, which have cytopathological effects on the midgut and cause larval mortality [13]. Toxin resistance was later reported in various insect species due to reduced toxin binding to mid-gut cells [14]. Therefore, search for a new, cost-effective mosquitocidal agent has been a research challenge. Because of their strong activity against mosquito larvae and low cost of manufacture, silver nanoparticles (AgNPs) have been recommended as an alternative larvicidal agent for mosquito control [15]. Several plant extracts and bacterial culture media were used as alternative biological reducing and stabilising agents in the synthesis reaction to produce green AgNPs [16, 17]. Chemical components included in plant extracts were found to increase the larvicidal efficacy of synthesized AgNPs [18]. In addition to plant extracts, bacterial culture filtrate, particularly bacterial strains that release larvicidal compounds into cultured media, were employed to enhance AgNPs production, such as *Bacillus thuringiensis* [19], *Bacillus amyloliquefaciens* [20], and *Bacillus subtilis* [21]. The LC_{50} of these synthesized AgNPs was 0.10 ppm against *Aedes aegypti* [19], 1.29 ppm against *Culex pipiens* [20], and 2.00 ppm against *Culex quinquefasciatus* [21]. However, AgNPs were produced from bacteria that did not give larvicidal toxin, such as *Escherichia coli*, and showed effective mosquito-larvicidal action with an LC_{50} of 5 ppm against *C. pipiens* [22]. Furthermore, there was a controversy about the toxicity of $AgNO_3$ on mosquito larvae. Some reserachers stated that, the LC_{50} of $AgNO_3$ was 39.7 ppm against *Aedes aegypti* [23] and 1,078 ppm against *C. pipiens* [24]. Others found no larvicidal action of $AgNO_3$ at 50 ppm against *C. pipiens* and 2,153 ppm against *C. quinquefasciatus* and *Ae. aegypti* [25, 26]. Furthermore, the majority of research focused on green synthesised AgNPs made with cultured bacterial medium; nevertheless, the application of bacterial lysate to enhance AgNPs generation for mosquito larval control has yet to be examined. Bacterial DNA and cellular proteins were shown to help produce AgNPs, with bacterial DNA acting as a stabilising agent [27,28] and cellular proteins acting as both a reducing and stabilising agent [29,30,31]

2. Material and methods

2.1. *B. cereus* VCRC 641 cell lysate preparation

Bacillus cereus VCRC-641 overnight culture (50µl) was inoculated into 100ml LB broth and allowed to grow for 72hours in the Orbitek incubator shaker. Further, the culture centrifuged for 30 minutes at 10,000 rpm. Subsequently, wet biomass was obtained and quantified. About 1gm of wet biomass was homogenized with 10 ml of sterile water. Further the homogenized mixture was subjected to sonication under ice cold condition (5 cycles, 10 min) in sonicator (Biomatrix, India). The sonicated sample was centrifuged and the aqueous phase (cell lysate) was collected. Protein content of the cell lysate was estimated [32]. According to the estimated protein in the sample, mosquitocidal bioassay was carried out with the cell lysate as such (0.01, 0.05, 0.1, 0.5, 1.25, 2.5 and 5 milligrams).

2.2. Synthesis of silver nano particles and toxicity bioassay

The cell lysate obtained from 72-hour culture was used for synthesis of silver nano particles. According to the prescribed methodology, cell lysate was treated with a 100mM solution of silver nitrate ($AgNO_3$) [28]. The samples were incubated under light source (Fluorescent light) until the colour changed into brown (Figure 1). Mosquitocidal bioassay was carried out using synthesized AgNPs by *Bacillus cereus* VCRC 641 against all three major species of mosquito larvae (*Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi*).

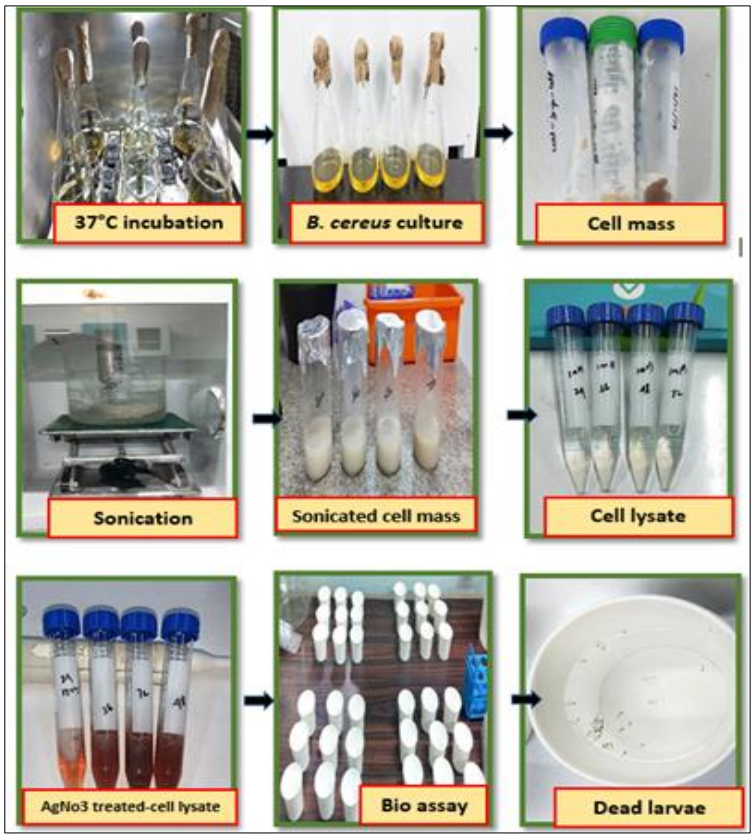
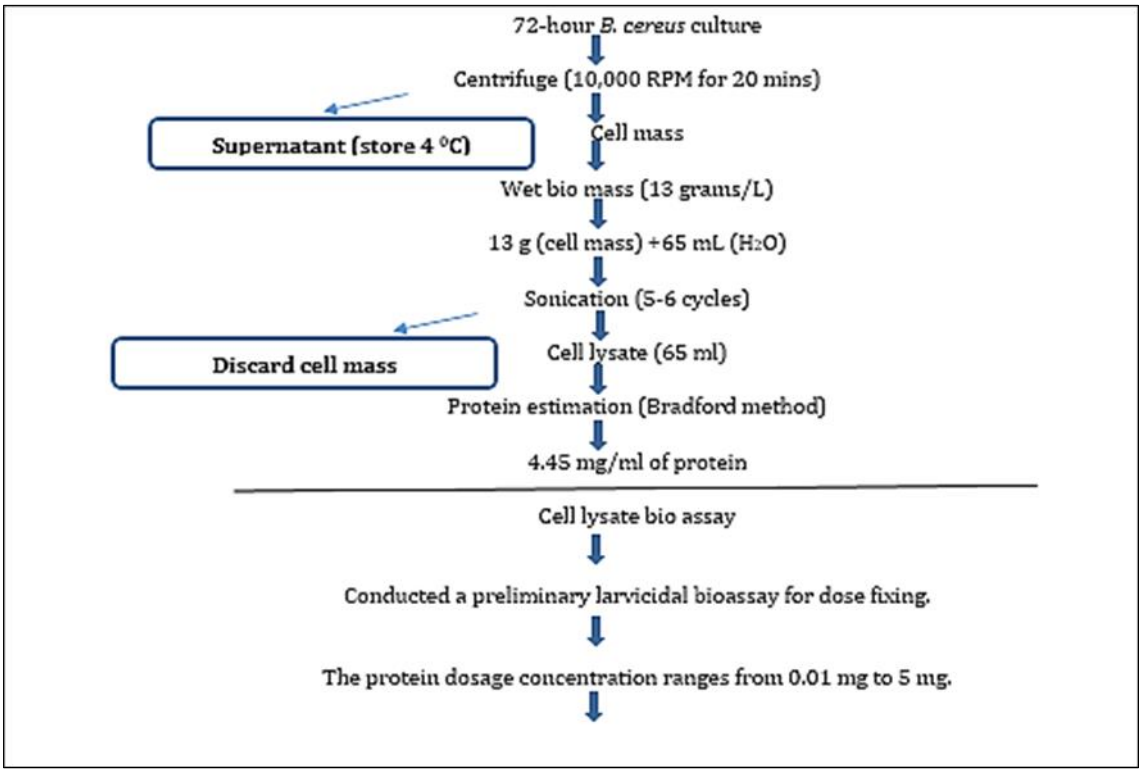


Figure 1 Synthesis of AgNPs form *Bacillus cereus* VCRC-641

2.3. Flow chat for AgNPs production and bio assay experiment



According to the protein concentration dosages were fixed for extensive bioassay

↓

Calculation

1ml=4.45 mg

↓

5 mg/4.45 mg=1.123 ml of lysate

↓

1.123 ml of lysate contains 5mg of protein

↓

Like that we have to calculate all the doses

↓

Totally necessary for one bioassay 5+2.5+1.25+0.5+0.1+0.05+0.01 mg of protein. Total protein consumption was 9.41 mg. In 2.11 ml of lysate, 9.41 mg of protein were suspended.

↓

Calculation (for one bio assay)

Protein (mg) - Lysate volume

5.0 - 1.123 ml

2.5 - 0.561 ml

1.25 - 0.280 ml

0.5 - 0.112 ml

0.1 - 0.022 ml

0.05 - 0.011 ml

0.01 - 0.002 ml

9.41 mg - 2.11 ml

Then, the same quantity of protein from the lysate must be treated with AgNO3 to synthesize silver nanoparticles

100mM AgNO3 treatment

↓

0.2 ml of AgNO3 and 2.75 ml of water should be added to 5 mg of lysate protein

↓

For one bioassay, we need to consume 9.41 mg of lysate protein, which is equal to 2.11 ml of lysate solution.

↓

Calculation

For 5 mg (1.123 ml) of cell lysate protein have to add 0.250 ml of 100mM AgNO3 Accordingly, for 9.41 mg (2.11 ml) of cell lysate protein requires 0.470 ml of 100mM AgNO3

9.41mg/5 mg= 1.882 mg×0.250 ml=0.470 ml (100mM $AgNO_3$)

Therefore, for 9.41mg of lysate protein have to add 5.175ml of water since 5mg of lysate protein needs 2.75ml of water

↓

9.41 mg/5 mg=1.882mg×2.75ml=5.175 ml (H_2O)

↓

9.41mg of lysate protein (2.11ml of lysate) + 0.470ml (100mM$AgNO_3$) + 5.175ml (H_2O) = 8.4ml

↓

Keep the above mixture in light source

↓

Appearance of Brown color

↓

Bio assay

Dosage calculation

Prior to the suspension of 9.41 mg of protein in 2.11 ml of lysate

↓

Because we added $AgNO_3$ and H2O to the cell lysate, there is 9.41 mg of protein suspended in 8.4 ml of solution here

Bio assay with synthesized AgNPs

↓

5, 2.5, 1.25, 0.5, 0.1, 0.05 and 0.01milligram.

Calculation

9.41 mg of protein are present in 2.11 ml of lysate prior to $AgNO_3$ treatment; 9.41 mg/ml = 4.45.

Protein concentration after $AgNO_3$ treatment is 9.41 mg per 8.4 ml, which is 1.120 mg per ml (9.41 mg/8.4 ml)

Dosage for one bioassay

Protein (mg) - AgNPs synthesized cell lysate

5 - 4.46 ml

2.5 - 2.23 ml

1.25 - 1.11 ml

0.5 - 0.446 ml

0.1 - 0.089 ml

0.05 - 0.044 ml

0.01- 0.008 ml

9.41 - 8.4 ml

3. Results and discussion

B. cereus cell lysate after synthesis of AgNPs showed a significant increase in toxicity when compared with cell lysate alone. Toxicity of the cell lysate of *Bacillus cereus* before $AgNO_3$ treatment was shown in Table 1. The level of toxicity in the AgNPs synthesized *B. cereus* was increased 117 fold when compared to cell lysate alone as shown in the Table 2.

Table 1 LC50 and LC90 values of Bacillus cereus cell lysate before AgNO3 treatment

Mosquito species	LC50 (mg/L) (95% UCL-LCL)	LC90 (mg/L) (90% UCL-LCL)	Std. Error	χ2 (df)
Aedes aegypti	0.9447 (1.239-0.719)	2.1644 (2.896-1.751)	0.160	98.7
Culex quinquefasciatus	1.0913 (1.379-0.8755)	2.1474 (2.776-1.773)	0.1752	119.4
Anopheles stephensi	1.044 (1.349-0.814)	2.258 (2.982-1.841)	0.154	104.4

Table 2 LC50 and LC90 values of Bacillus cereus cell lysate after AgNO3 treatment

Mosquito species	LC50 (mg/L) (90% UCL-LCL)	LC90 (mg/L) (90% UCL-LCL)	Std. Error	χ2 (df)
Aedes aegypti	0.0257 (0.0438-0.0075)	0.0996 (0.1669-0.0753)	4.673	65.8
Culex quinquefasciatus	0.0093 (0.0085-0.1356)	0.0335 (0.0950-0.0183)	12.26	26.9
Anopheles stephensi	0.0270 (0.0415-0.0052)	0.0828 (0.1240-0.0646)	5.424	71.03

Numerous gram-positive and gram-negative bacteria isolated from varied settings are capable of producing AgNPs [33]. Silver nanoparticles (AgNPs) have significantly increased in relevance with rising demand in recent years due to their comprehensive kind of uses in energy, biosensors, nanomedicine, catalysis, antibacterial, electronics and antifungal capabilities [34].

Bacterial synthesis of AgNPs is gaining popularity among biological techniques due to its sustainability, biocompatibility, affordability, non-toxicity, and simplicity of usage for large manufacturing [35, 36, 37, 38]. According to a recent publication, certain mosquitocidal Bacilli cell-free filtrates were used to produce silver nanoparticles (AgNPs), and these results raise the possibility that nitrate reductase may play a role in the transformation of silver ions into silver nanoparticles as well as in maintaining their stability. The studied AgNPs had multiple bioactivities. Nano metals with bactericidal, mosquitocidal, fungicidal, and virucidal properties are studied by researchers [39].

Due to its enhanced penetrating capability, non-toxicity, vast surface area, and remarkable strength, nanotechnology has emerged as one of the most promising methods for managing insects and pests. When AgNPs cluster and enter the larval membrane of mosquitoes, they connect to DNA or proteins that contain sulphur, which results in the denaturation of organelles and enzymes. Silver nanoparticles have been demonstrated to affect the larvae of *Aedes aegypti* and *Anopheles stephensi* [23, 40, 41, 42].

Finally, the current study provided a highly efficient, simple, environmental friendly and low cost approach for biosynthesis of AgNPs, which were synthesized from *Bacillus cereus* VCRC 641 toxins to enhance their efficacy against *Aedes aegypti* (LC_{50} = 0.0257mg/L), *Culex quinquefasciatus* (LC_{50} = 0.0093mg/L) and *Anopheles stephensi* (LC_{50} = 0.0270mg/L). It was observed that the toxicity assay results of AgNPs were 117 times higher than cell mass toxicity.

Similar kind of research was done by Shanmugasundaram *et al*., 2013, the biosynthesized AgNPs, which were derived from the action bacterium *Streptomyces* sp., M25, exhibited significant larvicidal activity against the malarial vector, *An. subpictus* (LC_{50} = 51.34 mg/L), filarial vector, *Cx. quinquefasciatus* (LC_{50} = 48.98 mg/L), and dengue vector, *Ae. aegypti* (LC_{50} = 60.23 mg/L), respectively.

4. Conclusion

Silver nano particles were synthesized using *Bacillus cereus* to enhance its mosquitocidal efficacy. The level of toxicity in the AgNPs synthesized *B. cereus* was increased 117 fold in comparison with the cell lysate.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors agree no conflict of interest.

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