REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1. Name and designation of ICMR- IF : **Dr.Subbiah Poopathi,**
Scientist-G
ICMR/DHR-IF Ref ID: 2019-00335
ICMR Ref: INDO/FRC/452(S-47)/2019-20-IHD dated 07/08/2019.

2. Address :

Dr.Subbiah Poopathi
Scientist-G
Head, Division of Microbiology and Molecular Biology
ICMR-Vector Control Research Centre,
Department of Health Research,
Ministry of Health & Family Welfare
Govt. of India
Puducherry - 605006

3. Frontline area of research in which training/research was carried out :Mosquito control using synthesis of silver nanoparticles from mosquitocidal bacteria.

4. Name & address of Professor and host institute :

Prof. Sineenat Siri
School of Biology, Institute of Science Suranaree University of Technology, 111, University Avenue, Muang District Nakhon Ratchasima, Thailand 30000
+66-44-223305

5. Duration of fellowship with exact date : **Three months** (from 1st September, 2019 to 30th November, 2019)

6. Highlights of work conducted :

i) Technique/expertise acquired : Please refer **ANNEXURE-1**
ii) Research results, including any papers, prepared/submitted for publication
iii) Proposed utilization of the experience in India :

30/12/19
Signature of ICMR-IF

ICMR Sanction No. INDO/FRC/452(S-47)/2019-20-IHD dated 07/08/2019.

Dr. S. POOPATHI, M.Sc., M.Phil., Ph.D.,
SCIENTIST - G
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Indian Council of Medical Research
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ANNEXURE -1

Highlights of work conducted during training program under ICMR-DHR-International Fellowship-2019.

i). Technique/expertise acquired:

Technique/expertise acquired on the field of synthesis of silver nanoparticles (AgNPs) from bacteria (*Bacillus mobilis*, *B.subtilis*, *B.cereus*) useful for mosquito control.

Justification for the completion of the training in abroad:

Nanotechnology is a cutting-edge field of science with the potential to revolutionize today's technological advances including industrial applications. It is being utilized for the welfare of mankind. Silver nanoparticles have been received much attention in mosquito vector control program, especially on the synthesis of plants and bacteria based bio-pesticides. From literature survey, it was observed that only very few studies have been carried out to implement the nanotechnology based biosynthesis of bacterial toxins for the control of mosquito vectors. Therefore attention have been paid in this area of research and a proposal was submitted to ICMR for the award of ICMR-DHR International fellowship. The proposal was approved and specialized training was acquired.

Training in host laboratory:

In order to study the synthesis of silver nanoparticles from these strains during the fellowship period of three months (September to November), I have under gone training under Prof. Sineenat Siri, School of Biology, Institute of Science, Suranaree University of Technology, Thailand. Following Table showing the name of the bacterial strains used for biosynthesis of silver nanoparticle for mosquito control. These experimental strains are eco-friendly and does not show any adverse effect against aquatic organisms except mosquito larvae.

Sl.No	Name of the strain	Strain code	Mosquitocidal activity
1.	*Bacillus cereus*	VCRC-B641	Cells & cell free culture medium (supernatant)
2.	*Bacillus mobilis*	VCRC-B633	Cells & cell free culture medium (supernatant)

3.	*Bacillus subtilis*	VCRC-B622	Cell free culture medium (supernatant)

Technique/expertise acquired

Project-1:

1. **"Production of silver nanoparticles (AgNPs) using cell lysate of *Bacillus cereus* and their activity against mosquito larvae".**

In this work, training and demonstration acquired on the experiments to study the effects of protein concentration of cell lysate of *B. cereus*, reaction time, and pH on the production of AgNPs. Also, the studies of UV-VIS spectrum, Transmission Electron Microscopy (TEM), High Resolution Transmission Electron Microscopy (HR-TEM), Selected Area Electron Diffraction-TEM (SAED-TEM), and Energy Dispersive X ray Analysis-TEM (TEM-EDX). The activity of AgNPs against *Culex quinquefasciatus* was tested in Thailand and their activity against C*x. quinquefasciatus, Anopheles stephensi*, and *Aedes aegypti* was also studied in India. During the training period about 80% of work was completed and continuation of further work is in progress for publication.

2. **Project 2: "Eco-friendly synthesis of silver nanoparticles using cell lysate of *Bacillus mobilis*"**

In this project, the training on the synthesis of AgNPs via the mediation of *B. mobilis* cell lysate was completed. The experiments to optimize the synthesis condition were also under gone. Approximately 30% of wok has been completed in the host laboratory and further works are in progress in the parent institute as continuation. A paper will be published after its completion.

3. **Project 3: "Bacterial extract derived from *Bacillus subtilis* to mediate the formation of silver nanoparticles:**

Similar experiments to optimize the synthesis condition of *B.subtilis* was carried out. Approximately 30% of wok has been completed. Further work will be carried out for future publication.

ii). Research results, including any papers, prepared/submitted for publication

A. Methodology on how the AgNPs synthesized:

Bacillus cereus VCRC-641 bacterial mother culture was inoculated into Luria Bertani broth and incubated for 24 hours, 48 hours and 72hours. All the cultures were centrifuged separately (10000 rpm for 20minutes) and the wet biomass was obtained (weight of each sample taken). All the three samples were sonicated after adding desired quantity of sterile water using a sonicator for 10 minutes under cold condition to avoid over-heating. Now the sonicated samples are centrifuged, the pellet is discarded and the supernatant is called the cell lysate and protein estimation was done for the cell lysates using Bradford's method. Then the preliminary bioassay was done for all the 3 samples (24, 48 and 72-hour cell lysates).

B. Method of $AgNO_3$ treatment

The calculated amount of cell lysate, $AgNO_3$ and water mixed thoroughly and kept under light source for 3 hours until dark brownish color appears.

C. Method of bioassay conducted with bacterial cell lysate:

Depending on the protein concentration and preliminary bio assay results of all the samples (i.e. 24, 48 &72 hours) the dosage of the lysate was fixed. The 24 hr lysate as such did not show larvicidal activity. So, for 48hrs and 72hrs the dosage was fixed as 0.01mg, 0.05mg, 0.1mg, 0.5mg, 1.25mg, 2.5mg and 5mg (of protein). That means the lysate quantity treated for bioassay contained the above mentioned protein concentration.

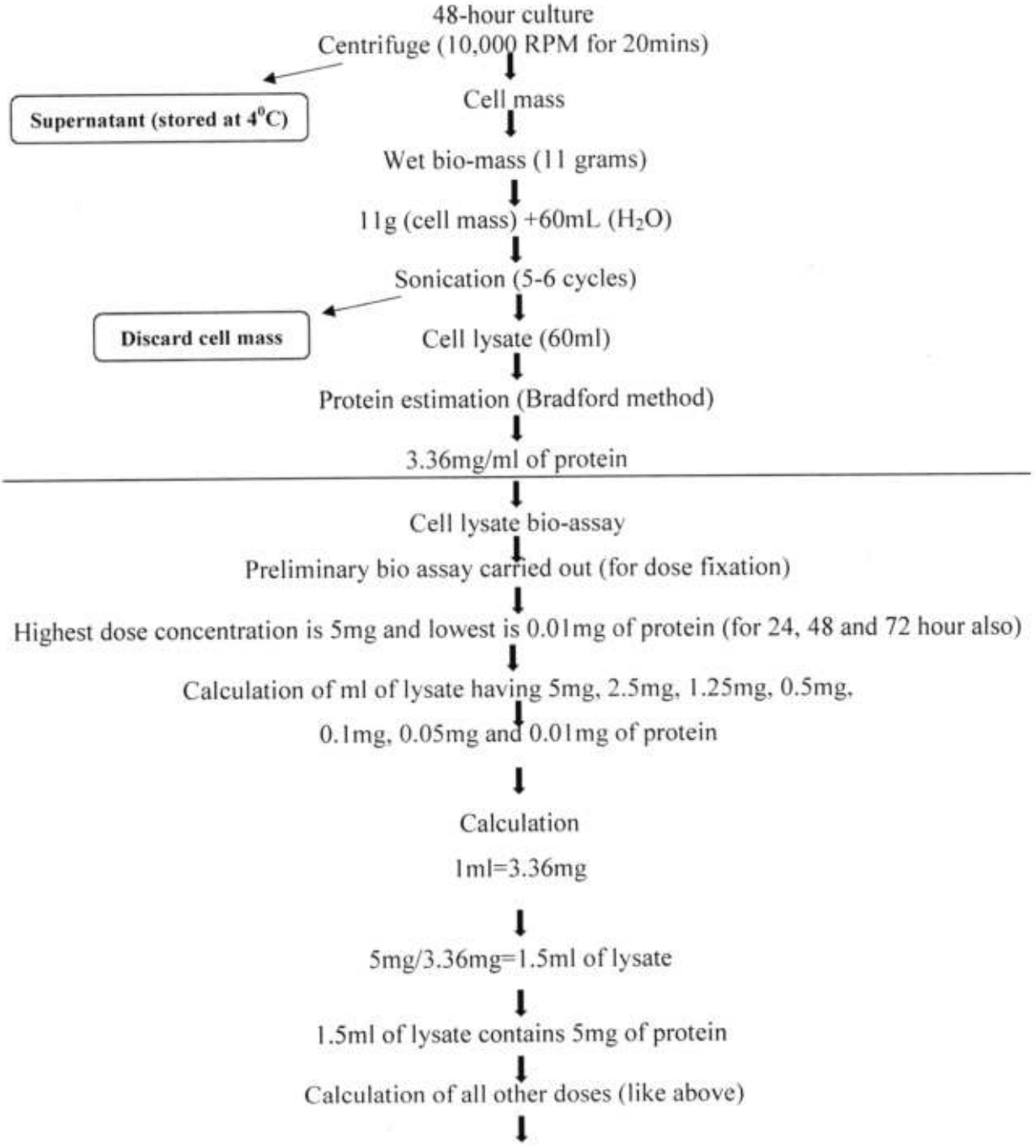
For the laboratory bioassay, 150 ml disposable paper cups with 100ml of tap water and 25 larvae (early 3rd instar stage) of 3 species *i.e., Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* were used. Seven doses of the lysate were treated to the larval cups (each dose 3 replicates) with 2 controls for each species of larva. After 24 hours the larval mortality was checked and the readings were recorded in a register. The bioassay was repeated for three consecutive days and the results were statistically calculated for LC50 and LC90 values.

D. Silver nitrate treated Bioassay

The cell lysate was treated with 100mM silver nitrate solution and used for the bioassay. The quantity of silver nitrate addition to the lysate depends on the protein concentration of each cell lysate. The method of conducting the bioassay experiment is the same as mentioned above and it is noted that the amount of sample used for treating the larva also remains same. Here also

the same 7 doses of AgNO3 treated lysate containing same protein concentration was used for the 3 species of larvae.

Flow chart showing synthesis of AgNPs, bio-assays and derivations



For one bio-assay required totally 5+2.5+1.25+0.5+0.1+0.05+0.01 mg of protein. Totally 9.41mg of protein was required. 9.41mg of protein was suspended in 2.8ml of lysate

⬇

Calculation (for one bio-assay)

Protein	-	**Lysate volume**
5mg	-	1.5ml
2.5mg	-	0.781ml
1.25mg	-	0.390ml
0.5mg	-	0.156ml
0.1mg	-	0.031ml
0.05mg	-	0.015ml
0.01mg	-	0.003ml
9.41mg	-	**2.8ml**

Same amount of lysate protein taken to $AgNO_3$ treatment for Silver Nano particles synthesis

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100mM $AgNO_3$ treatment

⬇

For, standard proportion (for 5mg of lysate protein add 0.250ml AgNO3 and 2.75ml H_2O

⬇

We need to take 9.41mg of lysate protein for one bioassay

That is equivalent to 2.8ml of lysate solution

⬇

Calculation (follow standard proportion volumes)

5mg (1.33ml) of protein requires 0.250ml of 100mM AgNO3 so 9.41mg (2.8ml) of protein requires 0.470ml of 100mM AgNO3

9.41mg/5mg= 1.882mg×0.250ml=0.470ml (100mM AgNO3)

5mg of lysate protein requires 2.75ml of H_2O so here 9.41mg of lysate protein requires 5.175ml of H_2O

⬇

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

⬇

Applying the standard proportion values for AgNO3 treatment

⬇

9.41mg of protein (2.8ml of lysate) + 0.470ml (AgNO3) + 5.175ml (H_2O) = 8.4ml

⬇

Light source (3hours)

↓

Brown color appears

↓

Bio-assay

↓

Dosage calculation

Take 2.8ml lysate for AgNO3 treatment, so 9.41mg of protein is suspended in 2.8ml of lysate

↓

Here 9.41mg of protein suspended in 8.4ml of solution because further more AgNO3 and H_2O added to the cell lysate.

↓

Do bio-assay with same protein concentration like earlier cell lysate bio-assay.

5mg, 2.5mg, 1.25mg, 0.5mg, 0.1mg, 0.05mg, 0.01mg.

↓

Calculation

Before AgNO3 treatment 2.8ml of lysate contains 9.41mg of protein, 9.41mg/2.8ml=3.36 therefore 3.36mg/ml

After AgNO3 treatment 8.4ml contains 9.41mg of protein, 9.41mg/8.4ml=1.120mg therefore 1.120mg/ml

Calculation for one bioassay

5mg	-	4.46ml
2.5mg	-	2.23ml
1.25mg	-	1.11ml
0.5mg	-	0.446ml
0.1mg	-	0.089ml
0.05mg	-	0.044ml
0.01mg	-	0.008ml
9.41mg	-	**8.4ml**

Figure.1. Synthesis of $AgNO_3$ from *Bacillus cereus* VCRC-641 during training program

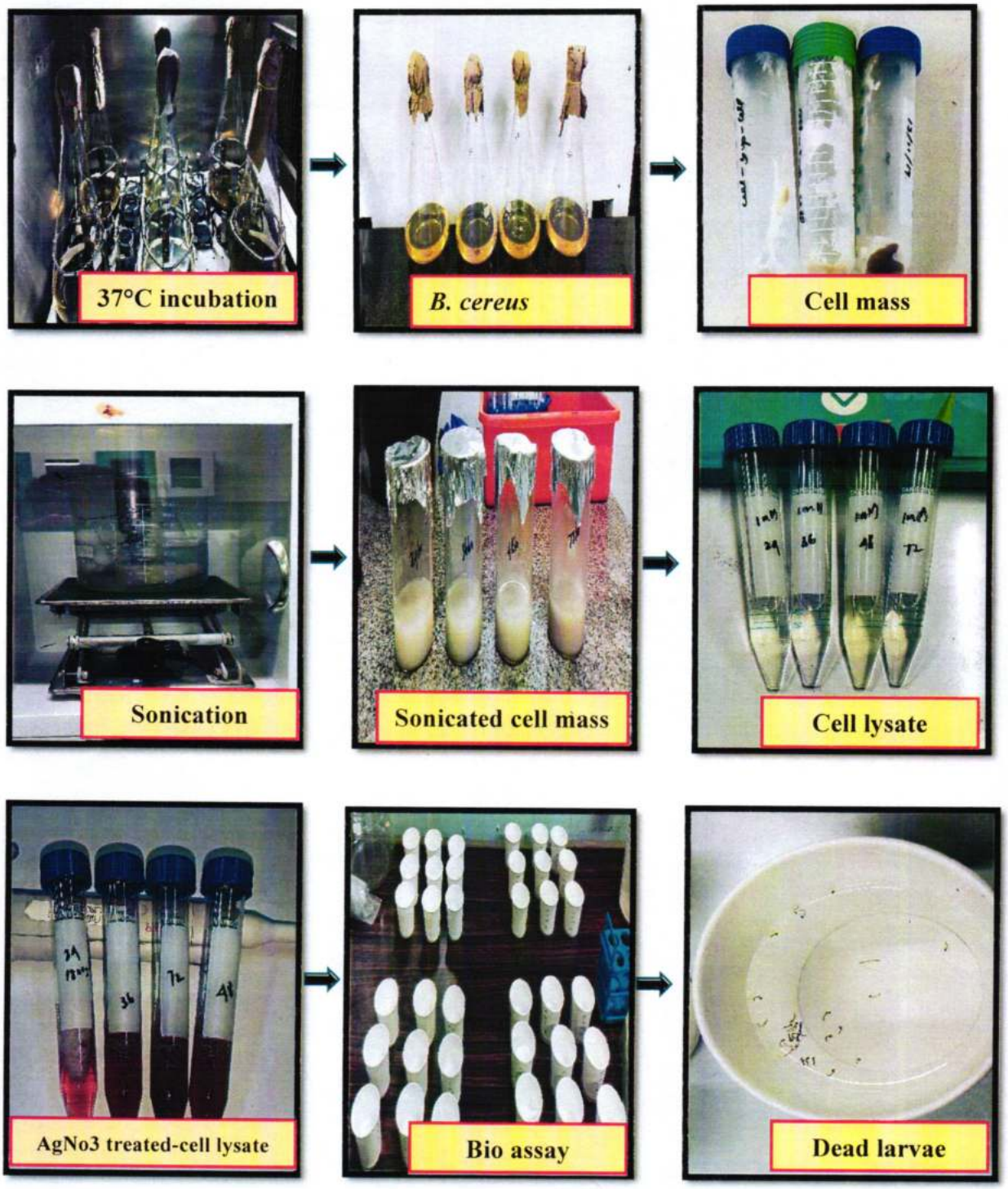


Figure. 2. SDS-PAGE showing the protein fractions from *Bacillus cereus*

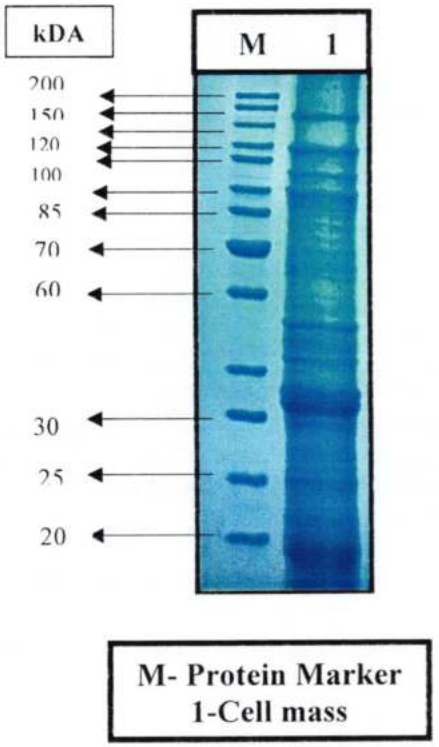
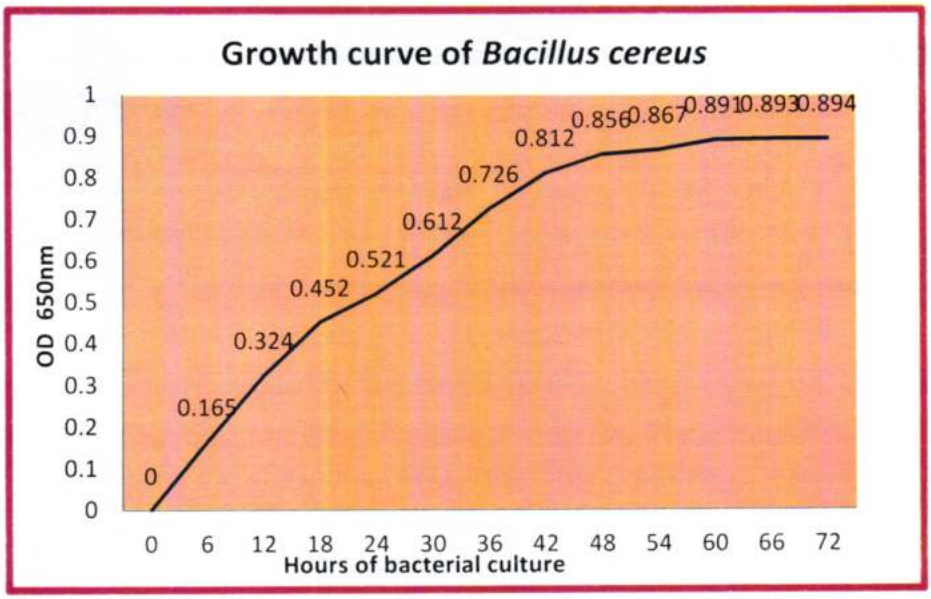


Figure.3. Growth curve of mosquitocidal bacteria (*Bacillus cereus* VCRC-B641)

Sample	Different hours (650nm)												
	0	6	12	18	24	30	36	42	48	54	60	66	72
1	0	0.165	0.324	0.452	0.521	0.612	0.726	0.812	0.856	0.867	0.891	0.893	0.864



Pl.Note: Maximum growth of bacteria completed up to 48 hours under 37^0C. Therefore 48 hour culture is taken into consideration of further studies.

Figure.4. Bacterial cell lysate of *B. cereus*. (a) Protein concentration of *B. cereus* cultured from LB medium at 37 C in an incubator shaker. (b) The protein profile of *B. cereus* cell lysate in a 12.5% SDS-PAGE.

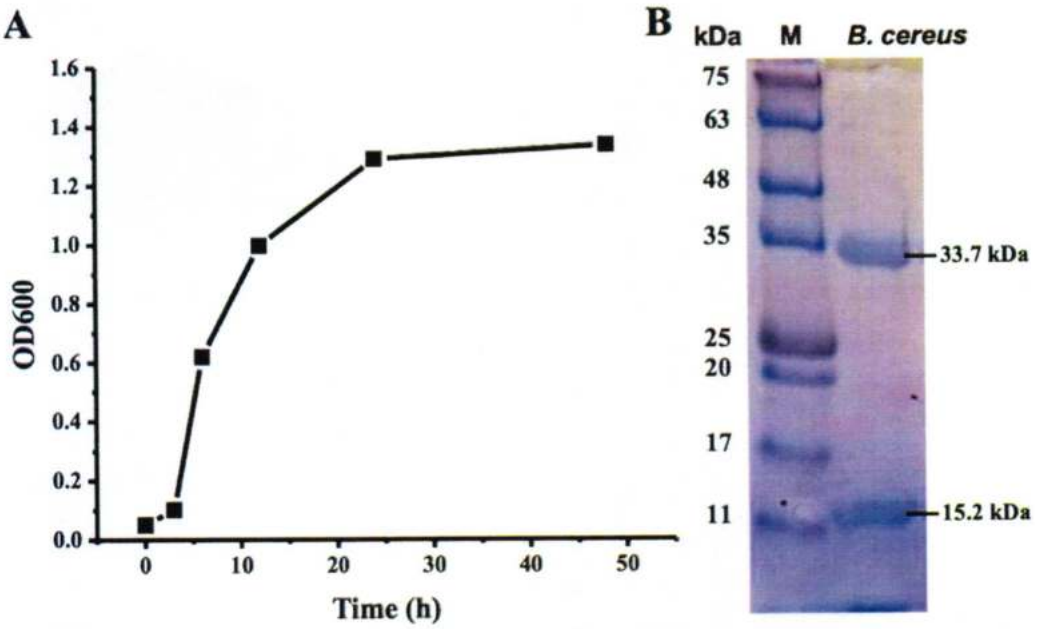


Figure. 5. Photoactivation on the formationof AgNPs. (a) The UV-Vis spectra of the synthesis reaction using different protein concentrations of the bacterial cell lysate under fluorescent light exposure for 2 h. (b) The UV-Vis spectra of the synthesis reaction in the condition without light exposure for 2 hour.

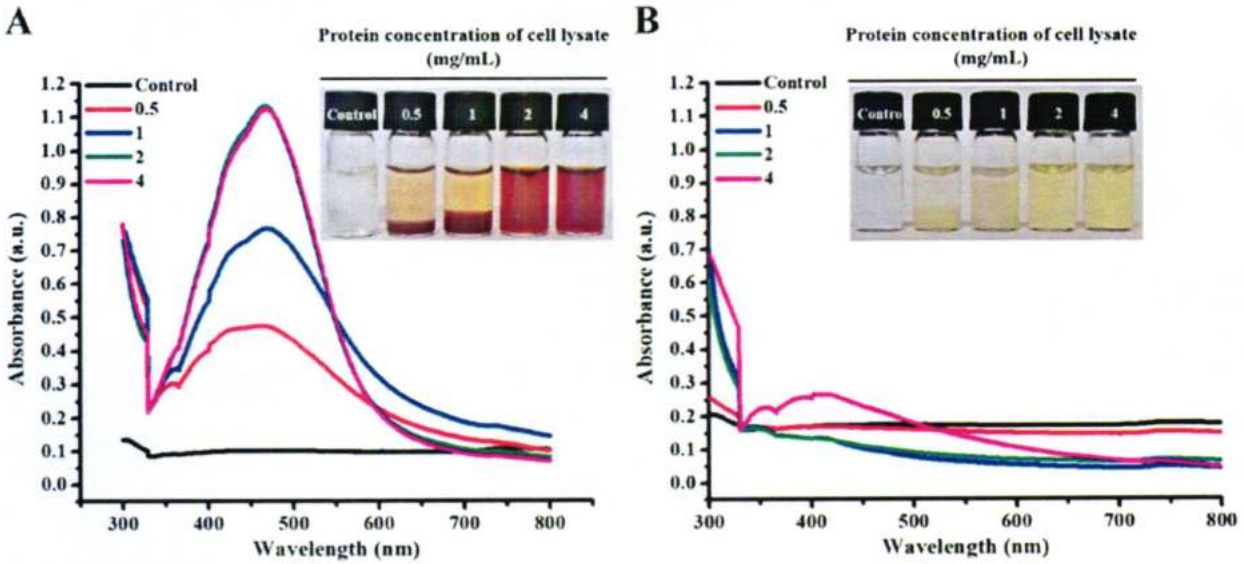


Figure. 6. Effects of reaction time and pH on the formation of AgNPs. (a) The UV-Vis spectra of the synthesis reaction in the time course of 240 min. (b) The UV-Vis spectra of the synthesis reaction using the pH 6, 7, 8, and 9.

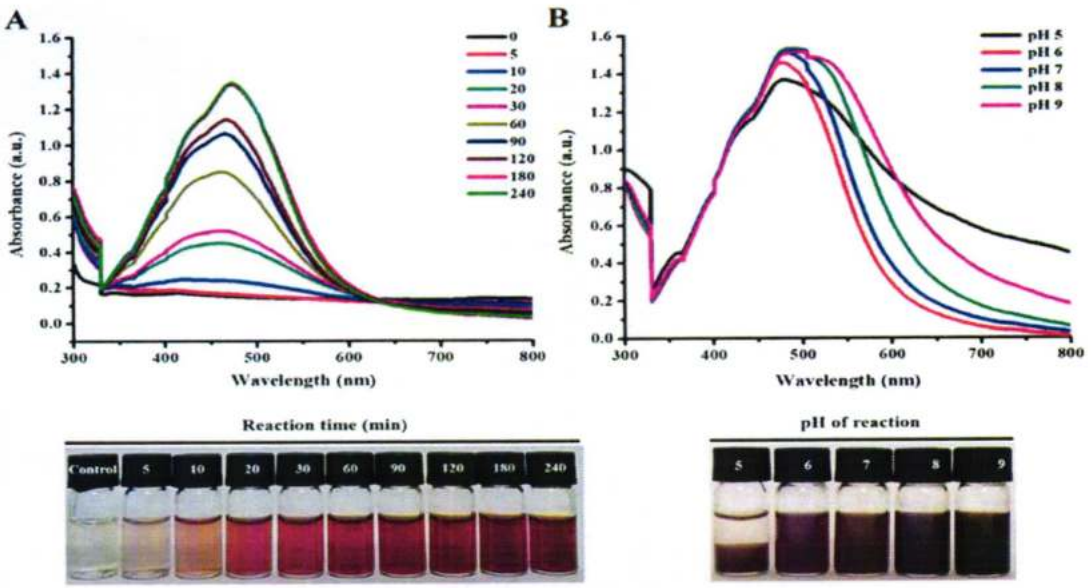


Figure 7. Transmission Electron Microscopy image and histogram of the size distribution of the produced AgNPs. (a) TEM images showing the amorphous morphology of AgNPs. (b) Size distribution of the synthesized AgNPs as measured from 100 particles.

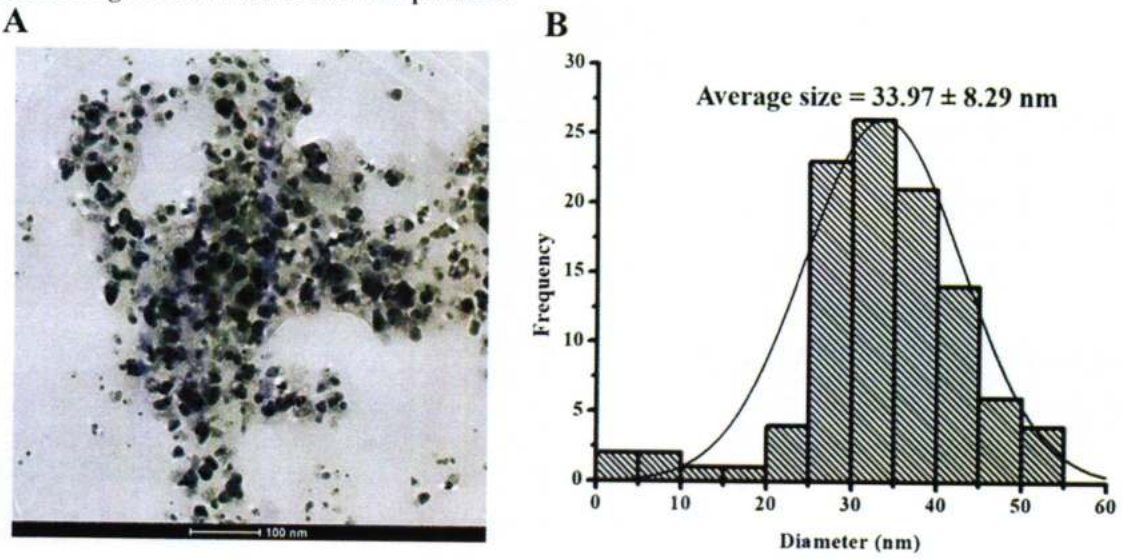


Figure 8. Characterization of the produced AgNPs by (a) HR-TEM, (b) SAED-TEM, and (c) EXD- TEM

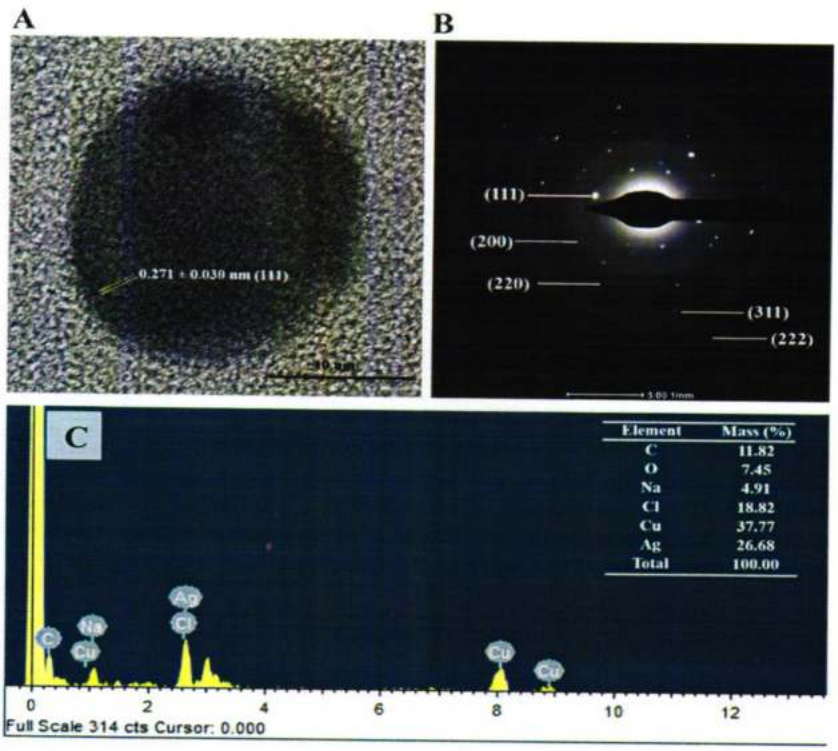


Table 1. *Bacillus cereus* VCRC – 641 cell lysate and AgNPs LC50 and LC90 values

Before AgNO3 treatment (As such Cell lysate):

Culture hours	Mosquito species	LC_{50} (mg/L) (90% UCL-LCL)	LC_{90} (mg/L) (90% UCL-LCL)	Std. Error	χ^2 (df)
24	*Aedes aegypti*	No mortality			
	Culex quinquefasciatus				
	Anopheles stephensi				
48	*Aedes aegypti*	1.4232 (2.910-0.768)	3.1819 (7.642-2.128)	0.1070	85.9
	Culex quinquefasciatus	1.1465 (1.4655-0.9081)	2.3767 (3.1113-1.948)	0.151	106.6
	Anopheles stephensi	1.8968 (4.672-1.033)	3.662 (11.035-2.379)	0.096	96.21
72	*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

100mM AgNO3 treated cell lysate:

Culture hours	Mosquito species	LC_{50} (mg/L) (90% UCL-LCL)	LC_{90} (mg/L) (90% UCL-LCL)	Std. Error	χ^2 (df)
24	*Aedes aegypti*	4.7688 (5.791-4.118)	6.9461 (9.023-5.893)	0.110	46.55
	Culex quinquefasciatus	4.4667 (5.430-3.824)	6.7944 (8.657-5.750)	0.089	54.1
	Anopheles stephensi	5.0230 (6.2834-4.2734)	7.4689 (9.9389-6.2260)	0.0981	42.43
48	*Aedes aegypti*	0.5245 (0.7135-0.3825)	1.2488 (1.7387-0.9891)	0.3009	112.5
	Culex quinquefasciatus	0.1217 (0.1977-0.0537)	0.4234 (0.6878-0.3120)	0.9293	94.8
	Anopheles stephensi	0.5603 (0.7344-0.4326)	1.1535 (1.5472-0.9322)	134.5	0.3393
72	*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

Conclusions:

The bio assay results clearly shown that there is increased mortality rate from $AgNO_3$ treated cell lysate than as such cell lysate.

For 24 hour 100mM $AgNO_3$ treated cell lysate giving 5 folds of increased mortality rate than as such cell lysate.

For 48 hour 100mM $AgNO_3$ treated cell lysate giving 10 folds of increased mortality rate than as such cell lysate.

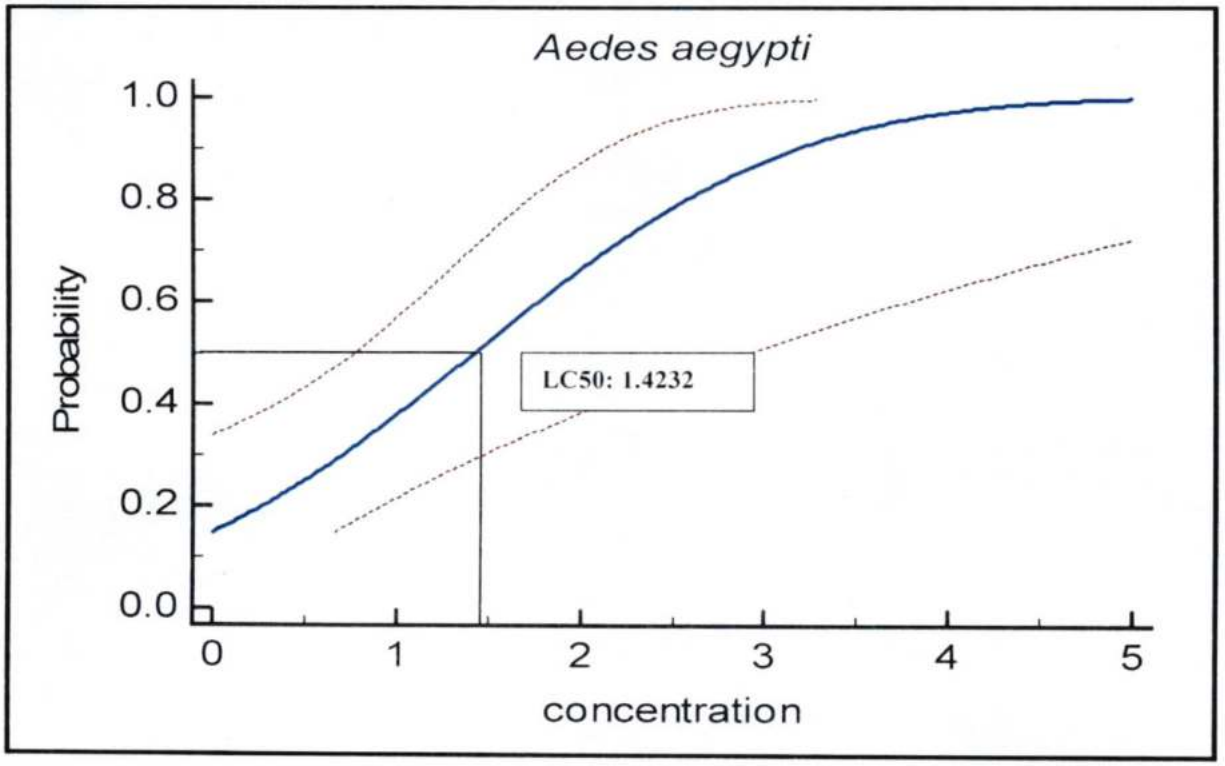
For 72-hour 100mM $AgNO_3$ treated cell lysate giving 117 folds of increased mortality rate than as such cell lysate.

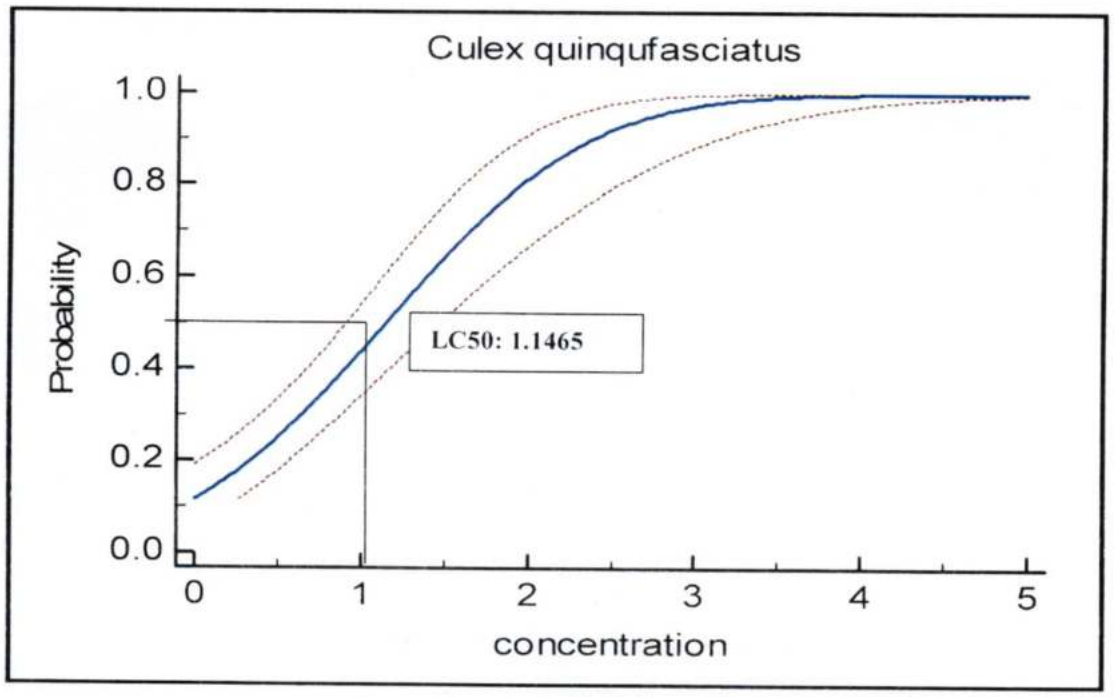
Graphical representation of bio assay results:

Before $AgNO_3$ treatment (As such cell lysate):

For 24-hour cell lysate- there is no mortality

Figure 9. Bioassays with *B.cereus* cell lysate after 48-hour culture.





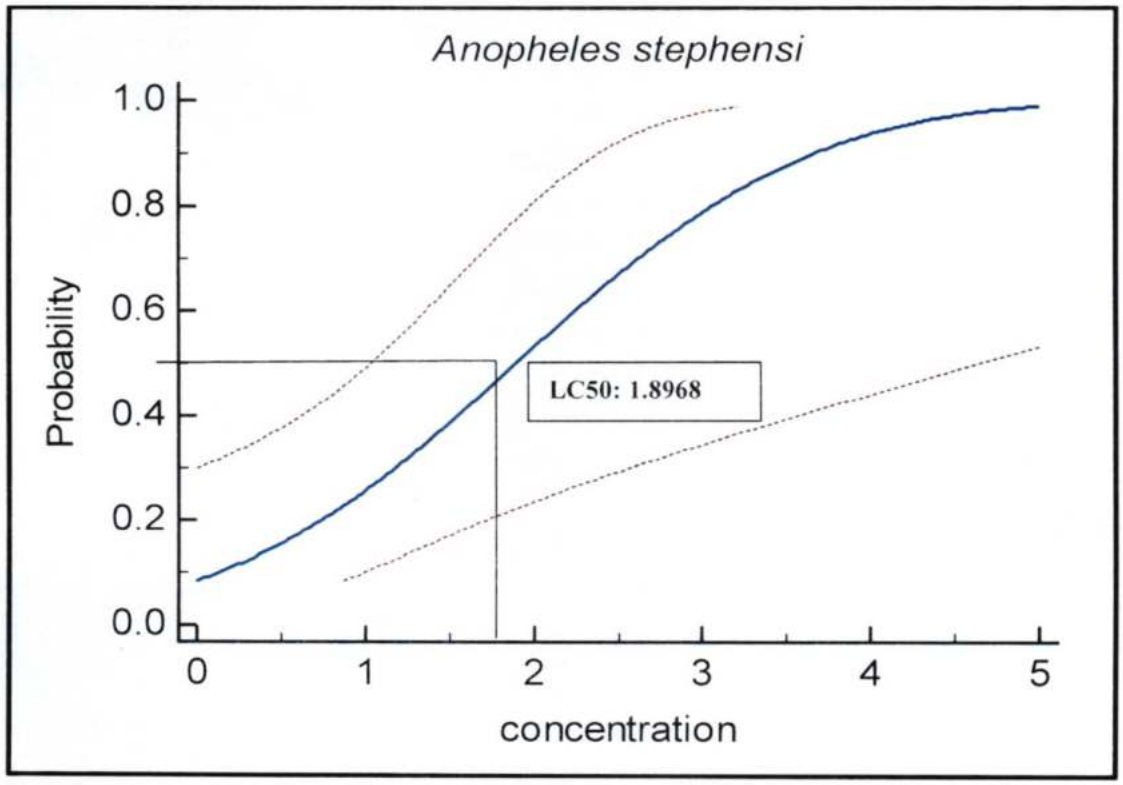
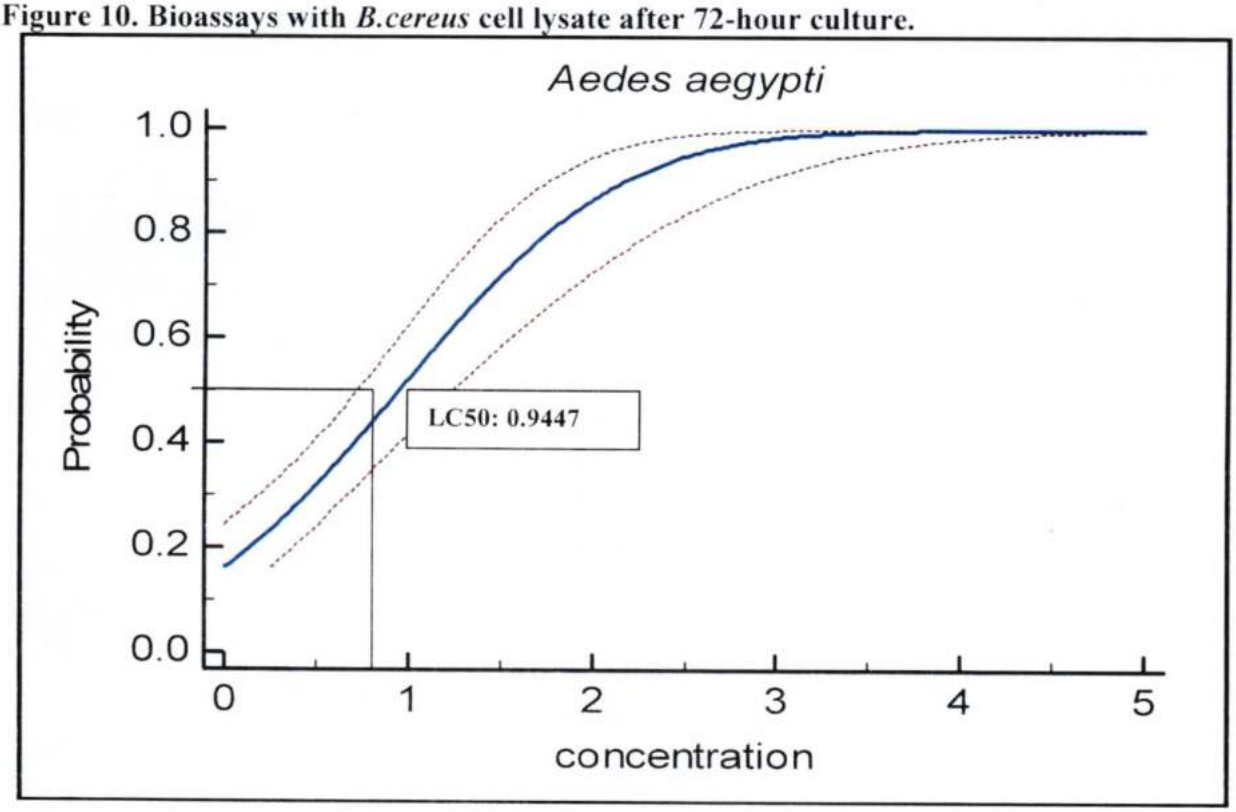
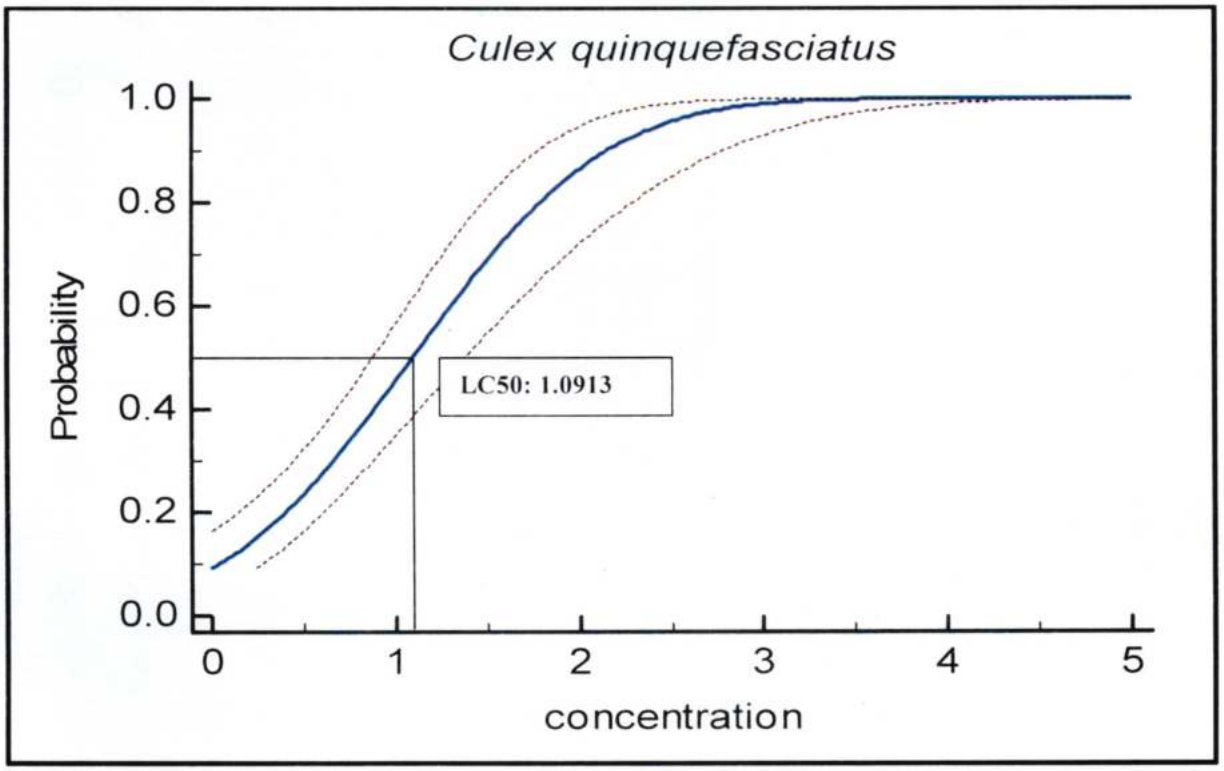


Figure 10. Bioassays with *B.cereus* cell lysate after 72-hour culture.




Culex quinquefasciatus
Probability
1.0
0.8
0.6
0.4
0.2
0.0
LC50: 1.0913
0
1
2
3
4
5
concentration

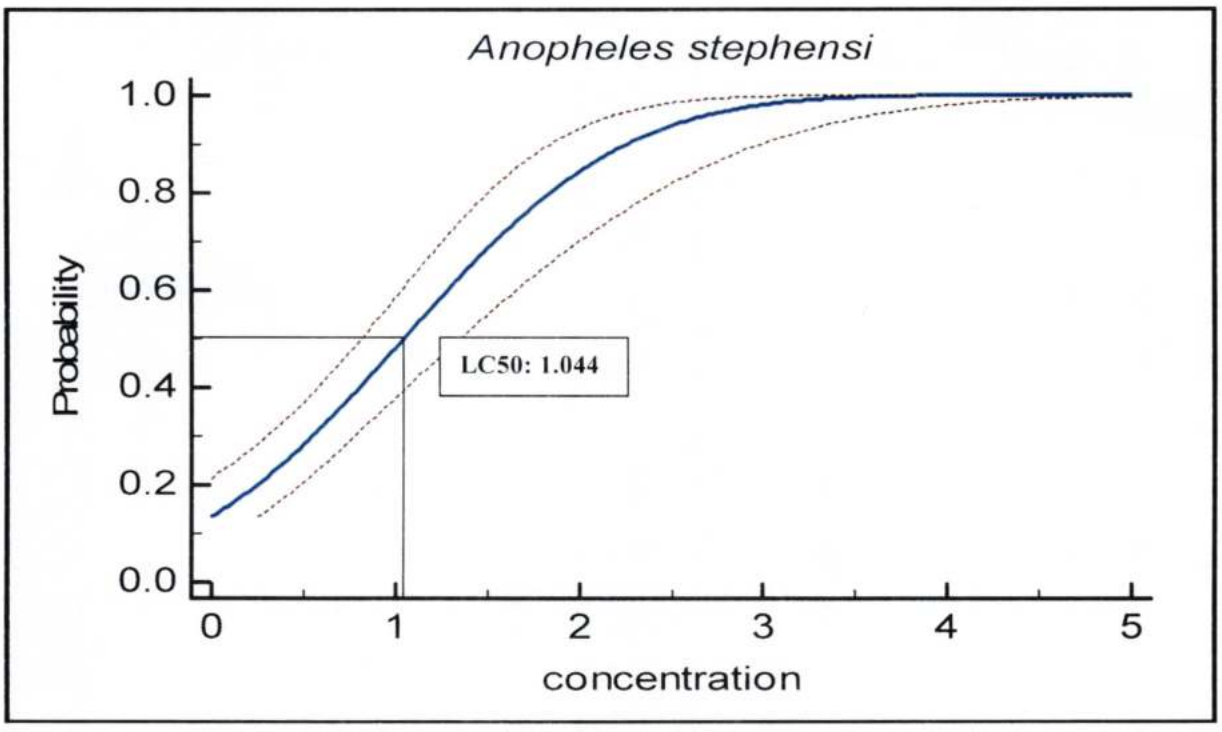
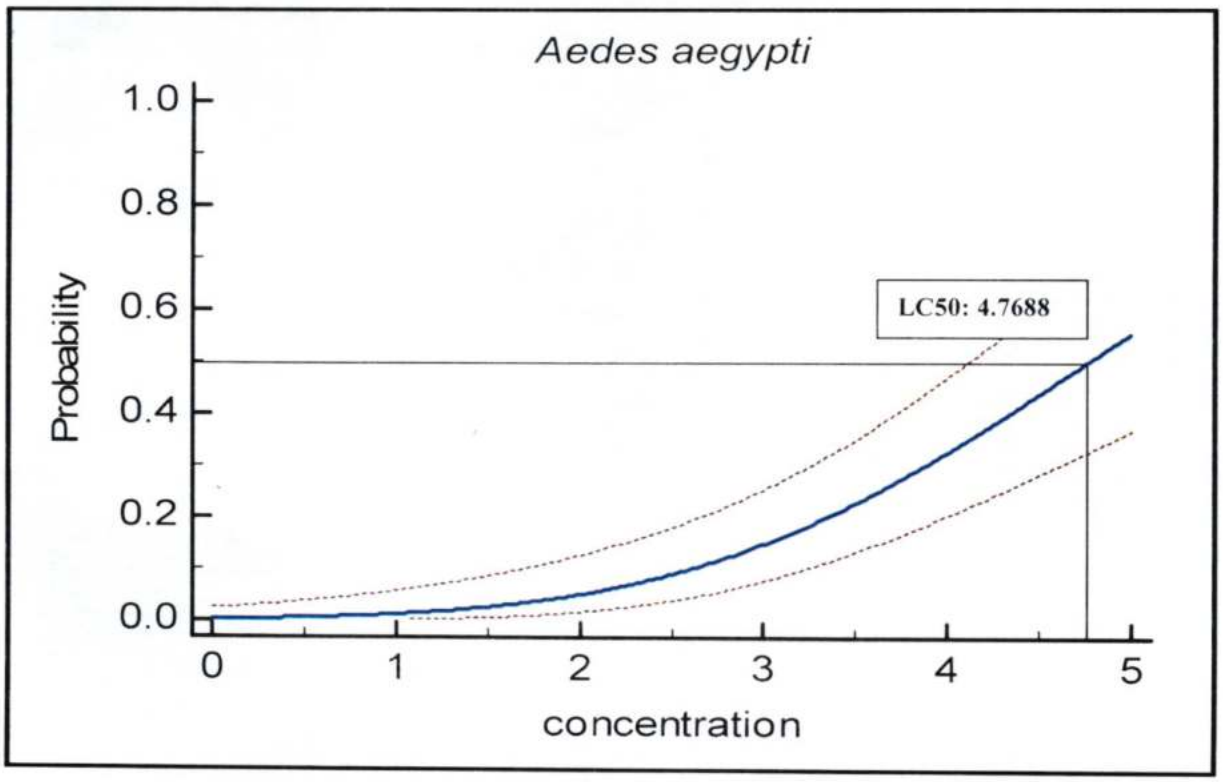
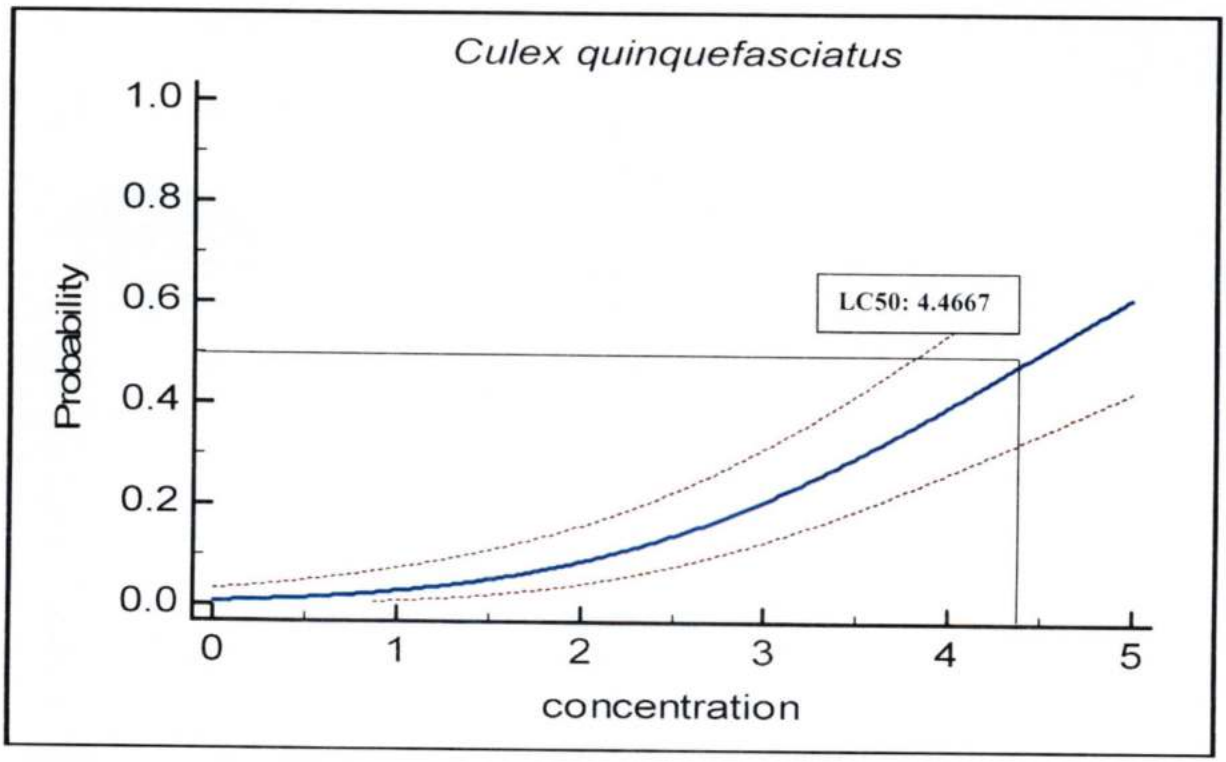

Anopheles stephensi
Probability
1.0
0.8
0.6
0.4
0.2
0.0
LC50: 1.044
0
1
2
3
4
5
concentration

Figure 11. Bioassays after synthesis of 100mM $AgNO_3$ from *B.cereus* 24 hour cell lysate.





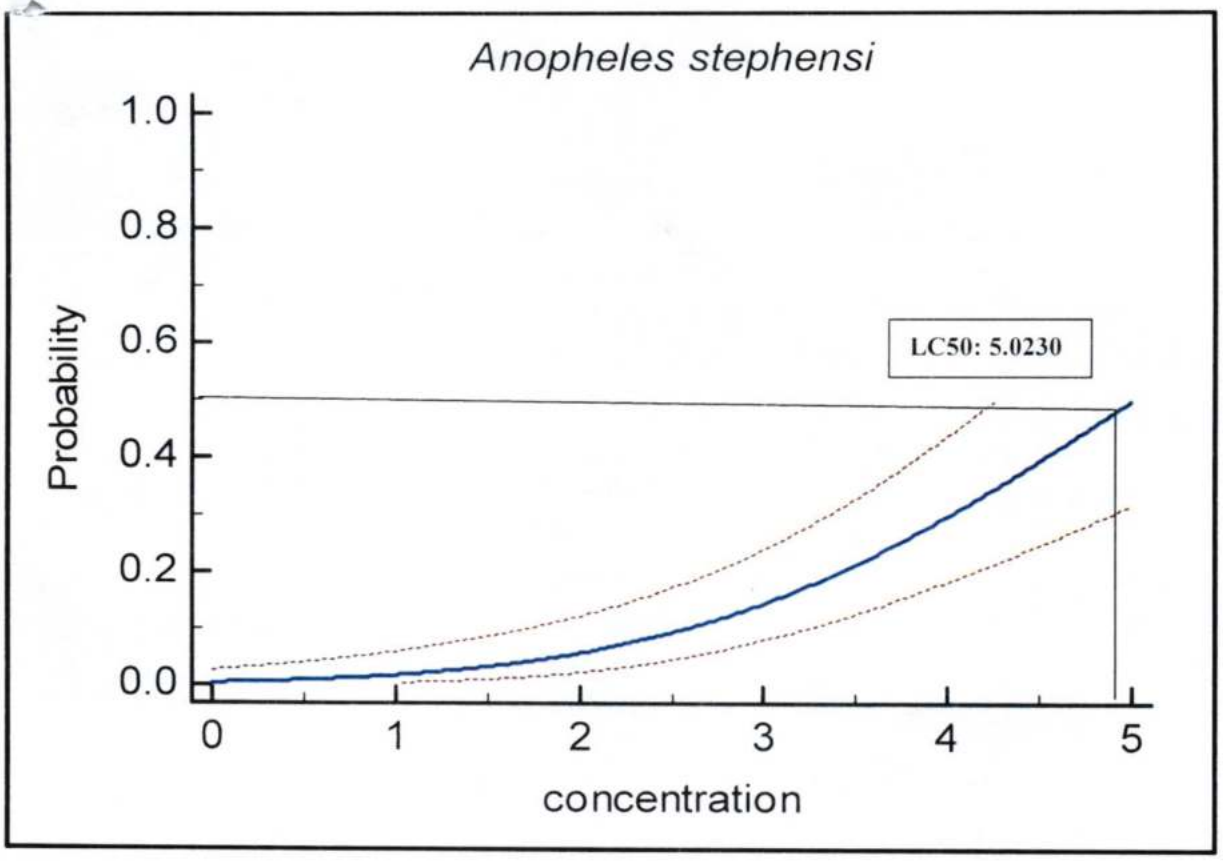
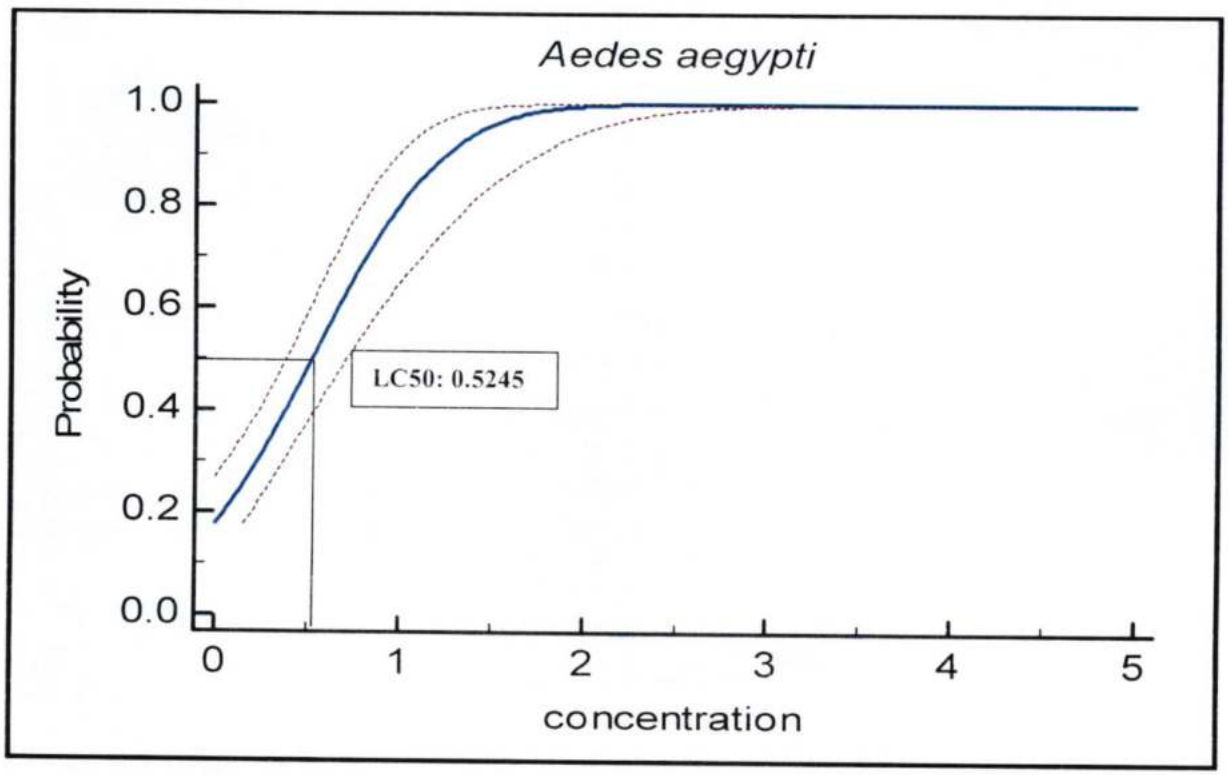
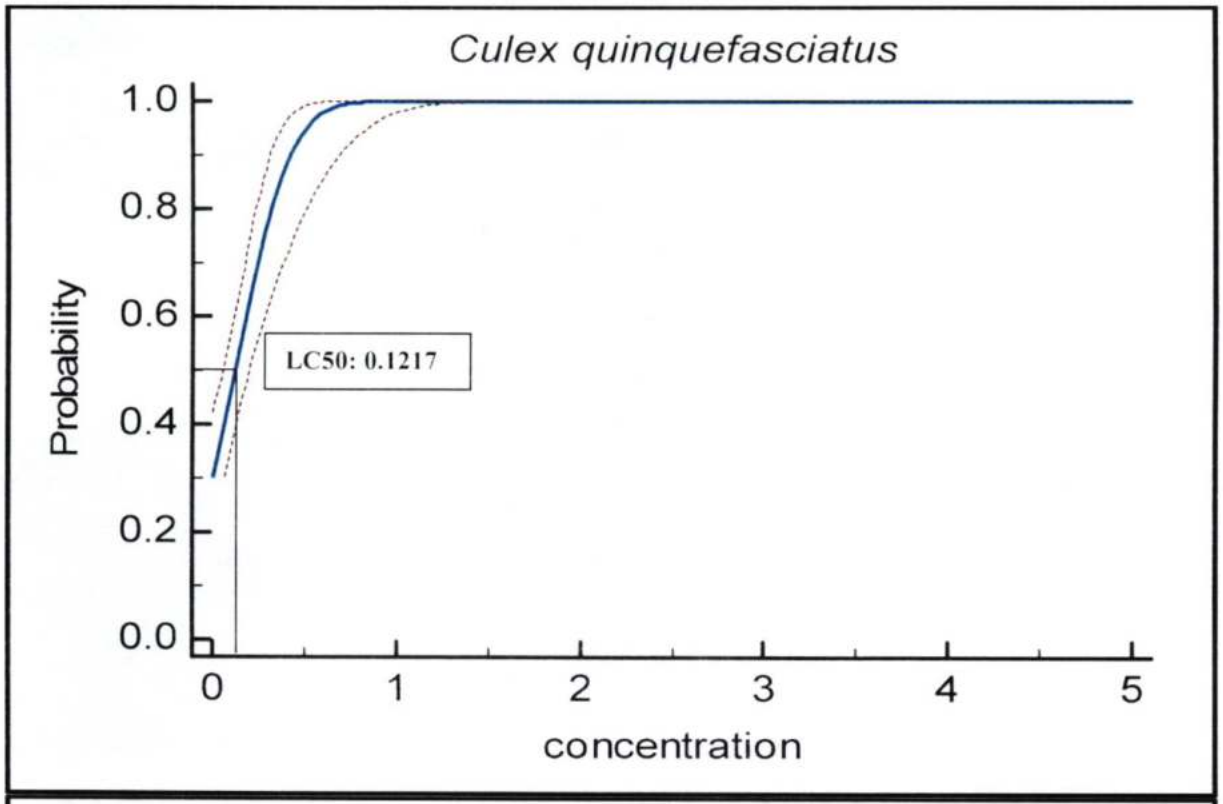


Figure 12. Bioassays after synthesis of 100mM $AgNO_3$ from *B.cereus* 48 hour cell lysate.




Culex quinquefasciatus
Probability
1.0
0.8
0.6
0.4
0.2
0.0
LC50: 0.1217
0
1
2
3
4
5
concentration

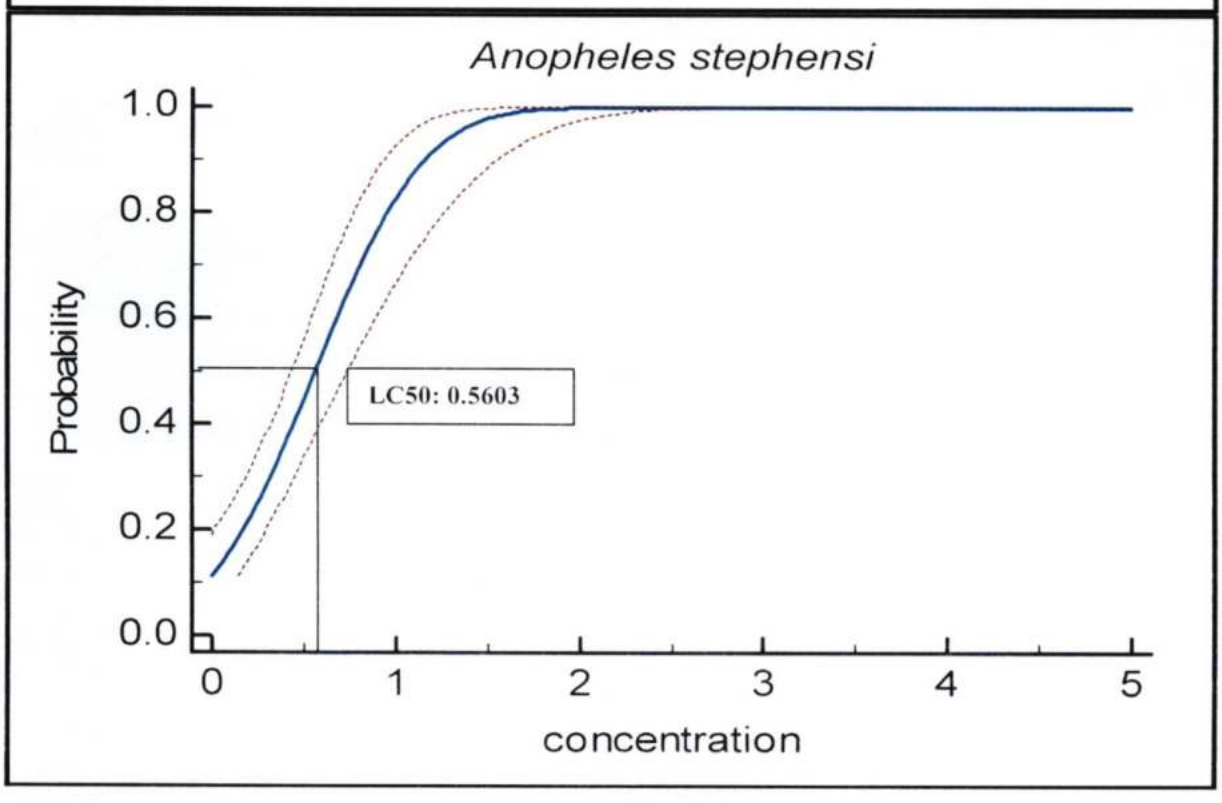
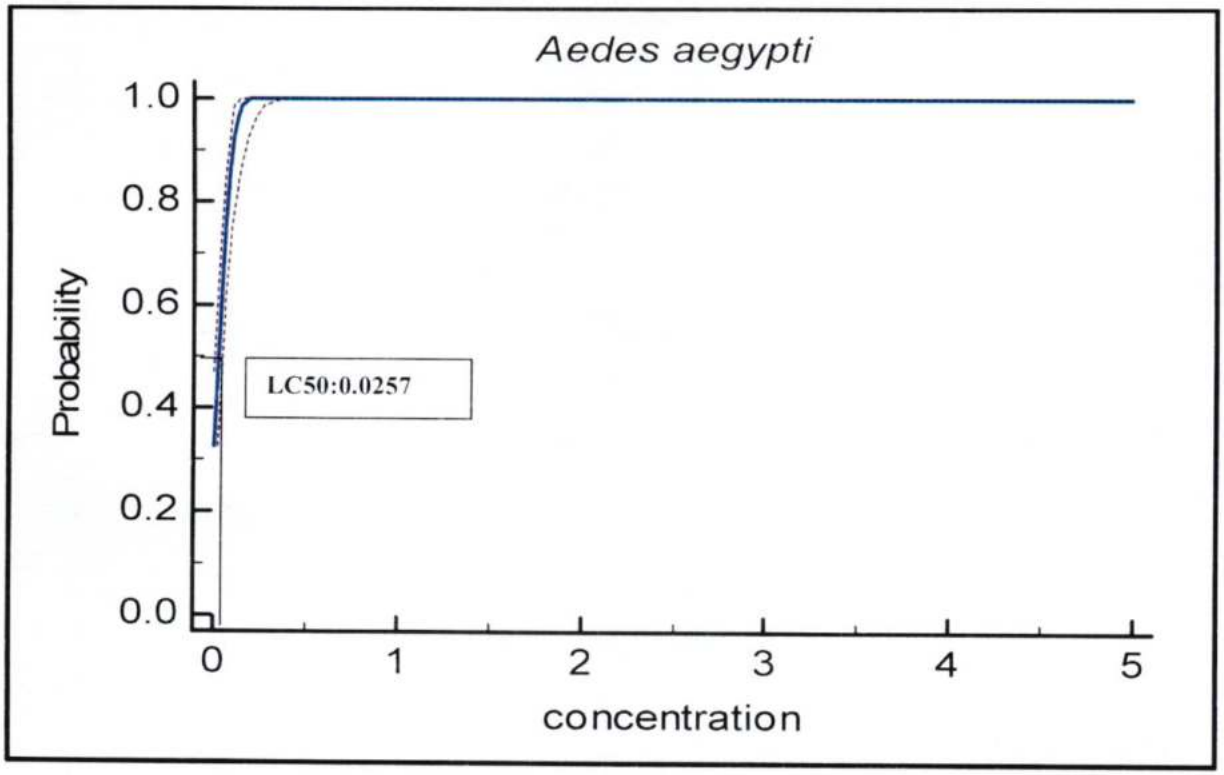
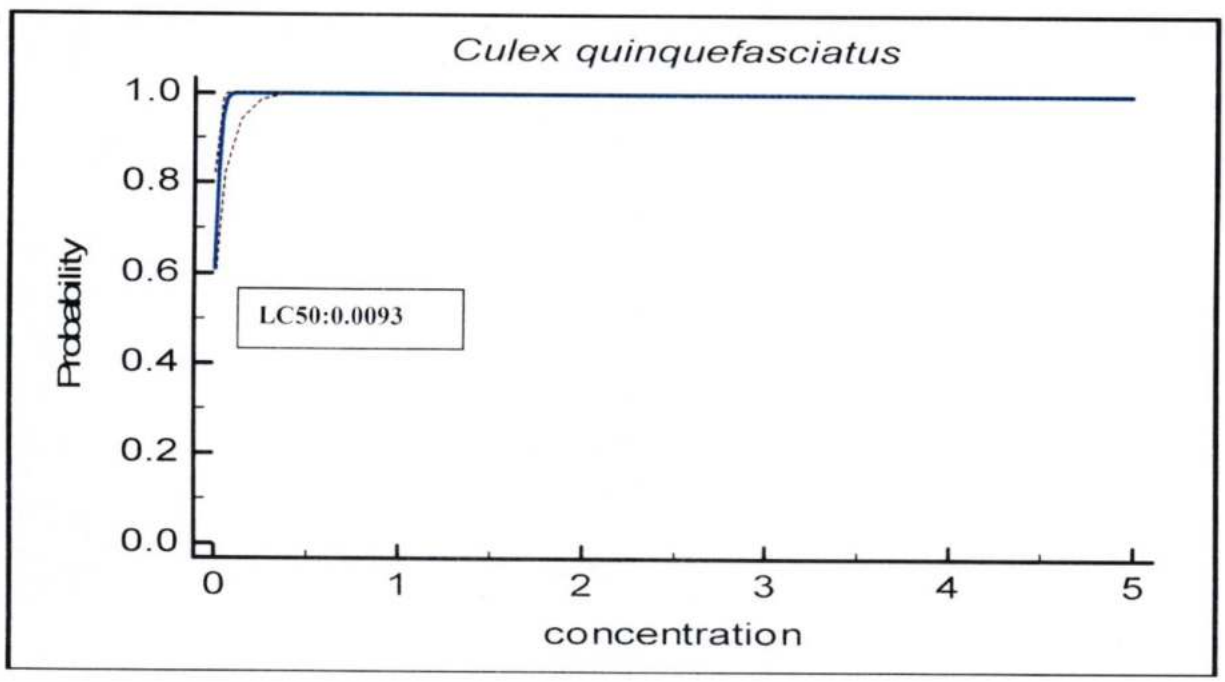
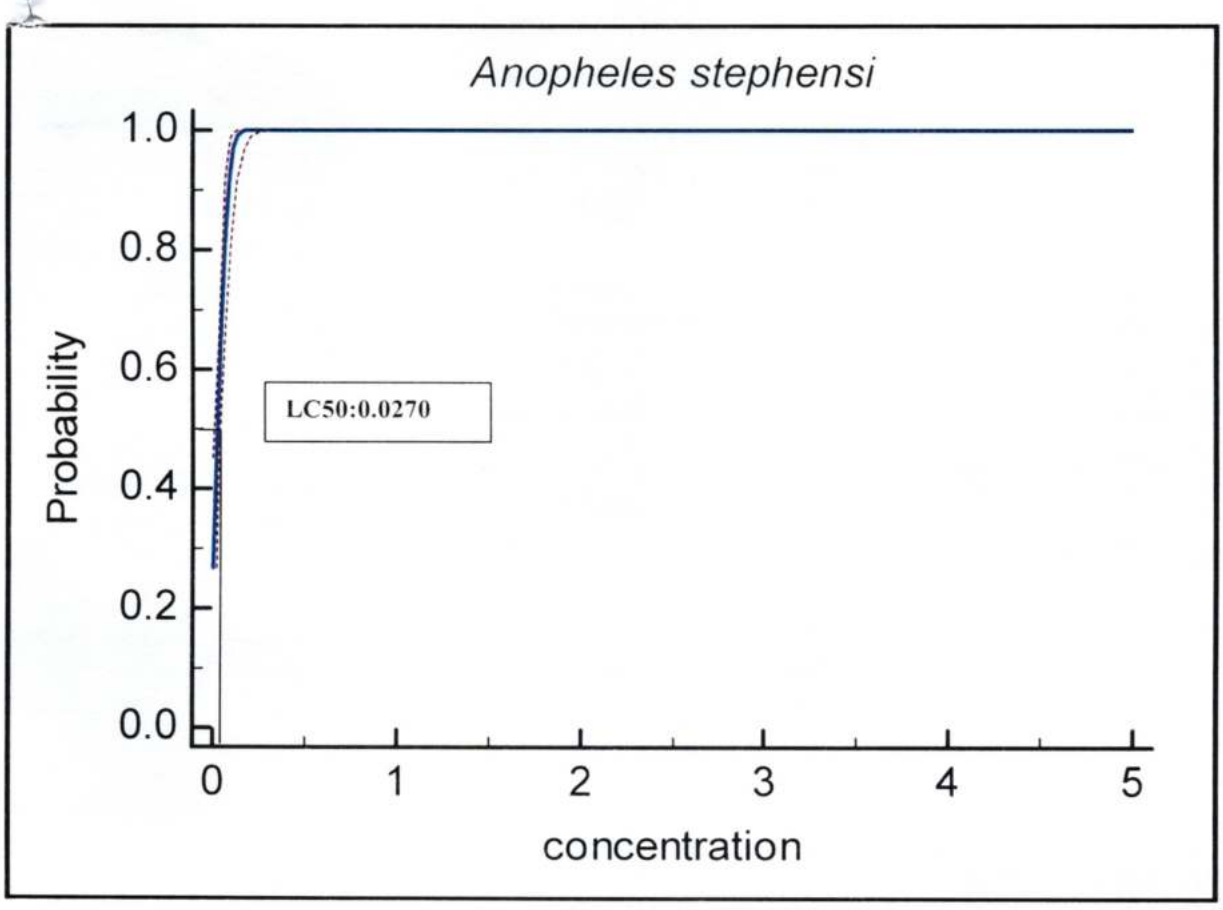

Anopheles stephensi
Probability
1.0
0.8
0.6
0.4
0.2
0.0
LC50: 0.5603
0
1
2
3
4
5
concentration

Figure 13. Bioassays after synthesis of 100mM $AgNO_3$ from *B.cereus* 24 hour cell lysate.







List of Publications:

1. **SubbiahPoopathi**, Manikandan Sivaprakasam, Ratima Jantima, Jiraporn Chumpol and Sineenat Siri (2019). Upsurge efficacy of mosquitocidal activity of bacterial lysate of *Bacillus cereus* VCRC- B641 after induced formation of silver nanoparticles. *Applied Microbiology and Biotechnology*, (MS-under preparation). *(First Draft copy enclosed).*

iii) Proposed utilization of the experience in India:

Mosquito control programs are facing critical challenges in both developing and developed countries, including the recent outbreaks of emerging arbovirus, the development of resistance in several *Culicidae* species, and the rapid spreading of highly invasive mosquitoes worldwide. Current control tools mainly rely on the employment of (1) synthetic or microbial pesticides, (2) insecticide-treated bed nets, (3) adult repellents, (4) biological control agents against mosquito young instars (mainly fishes, amphibians

and copepods) (5) Sterile Insect Technique (SIT), (6) "boosted SIT", (7) symbiont-based methods and (8) transgenic mosquitoes. Currently, none of these single strategies is fully successful. Therefore, novel eco-friendly strategies to manage mosquito vectors are urgently needed. The bacteria-mediated fabrication of nanoparticles is advantageous over chemical and physical methods, since it is eco-friendly, cheap, single-step, and does not require high pressure, energy, temperature, or the use of highly toxic chemicals. In the latest years, a growing number of bacteria-borne compounds have been proposed for efficient and rapid extracellular synthesis of metal nanoparticles effective against agricultural pests at very low doses (*i.e.* 1–30 ppm). Whereas against mosquitoes only scanty literatures available. Further, the increasing usage of silver nanoparticles (AgNPs) in all field of life sciences has raised environment concerns especially on the environmental toxicity. Whereas for mosquito control no such issues raised from past records.

Under these circumstances, the specialized training under gone in the host laboratory in Thailand will definitely pave way to acquire knowledge on improving the environment safety application of AgNPs synthesized from bacteria in mosquito control. Therefore this is the novelty of the present proposal.

Viability of application of the program envisaged to develop on return to India

Application of silver nano-particles in agriculture has been well accepted as it is cost-effective, non-toxic and more effective in controlling the agriculture pests. During the last decade, application of nano-technology has gained a lot of appreciation in drug administration especially in cancer therapy. Few institutes in India also are also attempting on developing nano-particle based pesticides for agriculture and public health applications. Being a novel approach it require improvisation for public health applications. This will open a new avenue to control mosquito vectors that are responsible for fatal diseases like malaria, JE and dengue. The host laboratory reported recently the ssDNA and dsDNA could function as stabilizing agents during the synthesis process, the ssDNA, generated by pre-heating the dsDNA at 100 °C, was more efficient to produce AgNPs with higher yield as determined by the intensity of the surface plasmon resonance (SPR) peak of silver at 475 nm. In addition to these, various hazard free, eco-friendly methods of synthesis of silver nanoparticles are in operation in the host

laboratory. Chemical reduction method involves the reduction of $AgNO_3$ in aqueous solution by an effective reducing agent in the presence of appropriate stabiliser, which is necessary in shielding the growth of silver particles through aggregation. During the formation of silver nanoparticles by the chemical reduction method, some of the parameters like the particle size and aggregation state of silver nanoparticles are affected by initial $AgNO_3$ concentration, reducing agent, $AgNO_3$ molar ratios and stabiliser concentrations. So many methods are evaluated for the chemical synthesis of silver nanoparticle formation; chemical reduction method, polyol method and radio lytic process have been developed for the synthesis of silver nanoparticles. The best and most easy method of yielding nanoparticles without aggregation, high yield and low preparation cost is chemical reduction method. Biological methods of synthesis of silver nanoparticles are also in operation in the host laboratory. Considering all these ongoing studies in the host laboratory, an in-depth training will be carried out to understand the method of overcoming environmental toxicity after application of AgNPs in the field. Outcome from this training program will have direct benefit to the parent organization as the technology will be imparted to the technical staff and the research scholars of the Department of Microbiology and immunology.

Relevance of work to ongoing/future program of parent Institute

The applicant has recently submitted a research project entitled, "Synthesis and characterization of silver nanoparticles using newly isolated bacterial strains (*Bacillus cereus* VCRC-B540 and *Bacillus subtilis* VCRC-B622) to control mosquito larvae of *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti*" to 40th Scientific Advisory Committee (SAC) of parent institute (20th December, 2018). The committee approved the proposal for extramural funding from DST. In this context, The training acquired from ICMR-DHR International Fellowship-2019 in the host laboratory, Thailand will be more useful to apply nanotechnology based synthesis of silver nanoparticles from bacteria in mosquito vector control program in the current scenario of development of resistance to bacterial bio-pesticides.

Manuscript under preparation for publication

Applied Microbiology and Biotechnology

DRAFT -1

Upsurge efficacy of mosquitocidal activity of bacterial lysate of *Bacillus cereus* VCRC- 641 after induced formation of silver nanoparticles

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Abstract

Highlight:

1.

2.

3.

1. Introduction

2. Materials and methods

2.1 Preparation of bacterial cell lysate

Single colony of B. cereus was cultured in Luria-Bertani (LB) media (5 mL) at 37 ℃ with shaking (200 rpm) for 6 h before transferring into 1L LB medium for a further growth of 24 h. Bacterial growth curve was determined by measuring the optical density (OD) at 600 nm at 0, 6, 12, 18, 24 h. To prepare cell lysate, bacterial cells cultured for 24 h were harvested by centrifugation at 8,000 ×g for 5 min. The cell pellet was suspended in 10 ml of deionized (DI) water and lysed by ultrasonication using the frequency at 60 kHz for 3 min on ice. The sample was centrifuged at 10,000 ×g for 20 min and the supernatant phase was collected as the bacterial cell lysate. The protein concentration of the bacterial cell lysate was determined by Bradford protein assay (Bio-Rad, Hercules, California, USA). The proteins of the bacterial cell lysate were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie brilliant blue R-250 dye (AppiChem, Darmstadt, Germany).

2.2. Induced formation of AgNPs by bacterial cell lysate

The eco-friendly production of AgNPs mediated by the bacterial cell lysate was carried out in the total reaction of 5 mL. The effect of protein concentration of bacterial cell lysate on the formation of AgNPs was investigated using silver nitrate (5 mM) and various protein concentrations of bacterial cell lysate (0, 0.5, 1, 2, and 4 mg/mL). The reaction was carried out under fluorescent light (15 W, 5,000 LUX) for 2 h, which the similar experiments without light exposure were also studied as the control reactions. The formation of AgNPs was monitored by the characteristic surface plasmon resonance (SPR) peak of AgNPs and the change of reaction colors from transparent to orange-brown. The effect of reaction times was also investigated using the reactions containing silver nitrate (5 mM) and bacterial cell lysate (2 mg/ml). After exposure to fluorescent light for 5, 10, 20, 30, 60, 90, 120, 180, and 240 min, the formation of AgNPs was determined by measuring the absorbance in the range of 300 900 nm using a UV-Vis spectrophotometer (Analytikjena Specord® 250 Plus, Jena, Germany). The effect of pH (6, 7, 8, and 9) on the formation of AgNPs was investigated using the reactions containing silver nitrate (12.5 mM) and bacterial cell lysate (2 mg/ml) under fluorescent light for 3 h. The formation of AgNPs was determined by the characteristic SPR peak of AgNPs and the reaction color.

2.3. Characterization of the produced AgNPs

The morphology of the produced AgNPs was observed using a Tecnai G2 S-Twin transmission electron microscope (TEM; FEI, Hillsboro, Oregon, USA) with an equipped Orius 200 CCD Camera and the accelerating voltage of 200 kV. To prepare samples for TEM analysis, the suspension of AgNPs was

dropped onto a carbon-coated copper grid and dried at room temperature in a desiccator. The crystalline structure of AgNPs was analyzed by TEM-selected area electron diffraction (SAED) operating at 200 kV with LaB6 filament. The images were recorded by an Orius 200 CCD Camera (Gatan, Pleasanton, CA, USA) equipped with TEM. The lattice structure of AgNPs was analyzed by high resolution (HR)-TEM using a Tecnai G2 S-Twin TEM operating at an accelerating electron source of 200 kV with LaB6 filament and provided with a Gatan Orius 200 CCD camera. The elemental composition of AgNPs was investigated through an energy-dispersive X-ray (EDX) using an EDAX r-TEM SUTW detector (FEI, Hillsboro, OR, USA) equipped with TEM. EDX spectrum was measured at 10 kV accelerating voltage.

2.4. Larvicidal activity of the production AgNPs

The larvae of Culex quinquefasciatus were purchased from the mosquito-larvae farming in Thailand. They were kept in aluminium trays with net covers containing dechlorinated tap water and fed with a dog feed twice a day. Mosquito larvicidal bioassay was carried out according to the standard method of the World Health Organization [1] using 25 larvae (late stage 3 or early stage 4) in 100 mL water containing AgNPs at various concentrations (360, 300, 240, 120, 60, 30, 6 ppm), which also containing the protein concentrations of bacterial cell lysate at 60, 50, 40, 20, 10, 5, 1 μg/ml. The negative control was the larvae in water without AgNPs nor bacterial cell lysate. In addition, the mortality activity of bacterial cell lysate was determined by using various concentrations of bacterial cell lysate (60, 50, 40, 20, 10, 5, 1 μg/ml.) to treat the mosquito larvae. After incubating for 24 h, the mortality of the larvae was determined. The lethal concentrations 50 and 90 (LC50 and LC90) were determined as the concentration of AgNPs killing 50% and 90% larvae, respectively. In each condition, five replicates were performed in each condition to obtain the average data.

2.5. Statistical analysis

All quantitative data were expressed as means ± standard deviations. Statistical comparisons were performed using one-way ANOVA with SPSS 11.5 for Windows software (SPSS, USA). P-values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1 Bacterial cell lysate of B. cereus

The growth curve of B. cereus at 37 C in a shaker incubator (200 rpm) in a time course of 24 h is shown in Fig 1a. The exponential phase of the bacterial growth was at 3– 12 h and the stationary phase was detected at 24 h of the growth curve. The bacterial doubling time was 69 min. The protein profile of the bacteria was visualized on a 12.5% SDS-PAGE gel as shown in Fig 1b. The prominent protein bands were at 15.2 and 33.7 kDa. Add discussion about toxin protein of B. cereus (ref).

Fig. 1

3.2 The synthesized AgNPs mediated by bacterial cell lysate

The bacterial cell lysate of B. cereus was used to mediated the formation of AgNPs, in which the reaction contained only silver nitrate and the bacterial cell lysate under the fluorescent light exposure. Fig 2a shows the effects of different protein concentrations of bacterial cell lysate and fluorescent light exposure on the formation of AgNPs. With the addition of bacterial cell lysate, the formation of AgNPs was detected as determined by the characteristic SPR peak of AgNPs at 460 nm. and the color of the reactions was changed from transparent to orange-brown. The increased formation of AgNPs were detected according to increased concentration of bacterial cell lysate as determined by the SPR peak intensity. It was noted that at the bacterial cell lysate concentrations of 0.5 and 1 mg/ml the precipitation of AgNPs was detected at the bottom of bottle, which likely due to insufficient complex molecules in bacterial cell lysate to stabilize the formed AgNPs in aqueous condition [2]. Based on the colloidal status and the highest SPR peak intensity, the optimal concentration of the bacterial cell lysate to mediate the formation of AgNPs was 2.0 mg/ml. Also, the similar reactions were carried out without the exposure of fluorescent light (Fig. 2 b), which no formation of AgNPs was detected as considered by the characteristic SPR peak of AgNPs. According to these results, the photocatalytic activity was a necessary factor for reduction of $AgNO_3$ to zero-valent Ag accompanied by proteins from cell lysate of B. cereus. Also, the aromatic amino acids (tryptophan, tyrosine, and phenylalanine) effectively

functioned as reducing agents in the bacterial cell lysate as they contained lone pair of electrons of the nitrogen and oxygen of indole ring and hydroxyl groups for electron donation in reduction reaction. Moreover, under light exposure the indole ring of these amino acids could be activated to form free radicals for catalysis. The production of the hydrated electron via the light activation also induced the silver ions to zero-valent silver and eventually AgNPs [3-5].

Fig. 2

Effects of reaction times and pH on the formation of AgNPs were also investigated. The formation of AgNPs was monitored in the reaction containing the optimal concentration of bacterial cell lysate and silver nitrate under fluorescent light exposure in a time course of 240 min (Fig. 3a). The formation of AgNPs was early detected at 20 min and the production of AgNPs was increasing according to the reaction times. The optimal reaction time was at 180 min as determined by the SPR peak intensity. Also, the synthesis of AgNPs was carried out in pH 5□9, in which pH 6 was the original pH of the reaction. The results showed that the colloidal AgNPs were obtained under the pH condition range from 6 to 9 (Fig. 3b), which the increased pH trended to cause the broader size distribution of AgNPs and the color was darker. However, at pH 5 the produced AgNPs were precipitated as seen at the bottom of bottle. It was hypothesized that at the acidic condition at pH 5 might cause damage to the stabilizing molecules in the bacterial cell lysate, resulting in the insufficient agent to stabilize the colloidal structure AgNPs in water [6].

Fig. 3

3.3 Characterization of the synthesized AgNPs

Morphology and size distribution of the synthesized AgNPs were determined from TEM images. The amorphous morphology of the synthesized AgNPs was detected (Fig. 4a). As determined from 100 particles, their diameters ranged from 1.22 to 55.23 nm and the average diameter of the synthesized AgNPs was 33.97 ± 8.26 nm (Fig. 4b).

Fig. 4

The identity of the synthesized AgNPs was confirmed by HR-TEM, SAED-TEM, and EDX analyses. The HR-TEM image of AgNPs (Fig. 5a) revealed the lattice spacing of 0.271 ± 0.030 nm, corresponding to the (111) plane of the face-centered cubic lattice of silver [7]. The SAED-TEM image (Fig. 5b) revealed the concentric rings of silver crystalline (2.34, 2.04, 1.46, 1.24, and 1.18 Å), corresponding to hlk planar of (111), (200), (220), (311), and (420), respectively (JCPDS No. 89-3722), suggesting the identity of the synthesized particles as AgNPs [8].

The EDX analysis (Fig. 5c) indicated that silver was the dominant element (26.68 % mass) of the synthesized AgNPs. The detection of carbon, oxygen, sodium, and chloride was likely contributed from the organic materials derived from bacterial cell lysate. In addition, the high level of copper was derived from a carbon-coated copper grid that was used to hold the sample [9].

Fig. 5

3.4 Larvicidal activity of bacterial lysate and AgNPs

Larvicidal activities of bacterial lysate before and after induced formation of AgNPs were investigated (Fig. 6). Waiting for the repeat experiments.

4. Conclusion

Acknowledgement:

The first author acknowledges Indian Council of Medical Research for the award of ICMR-DHR International Fellowship-2019 (Reference: INDO/FRC/452/(S-47)/2019-20-IHD dated 07/08/2019).

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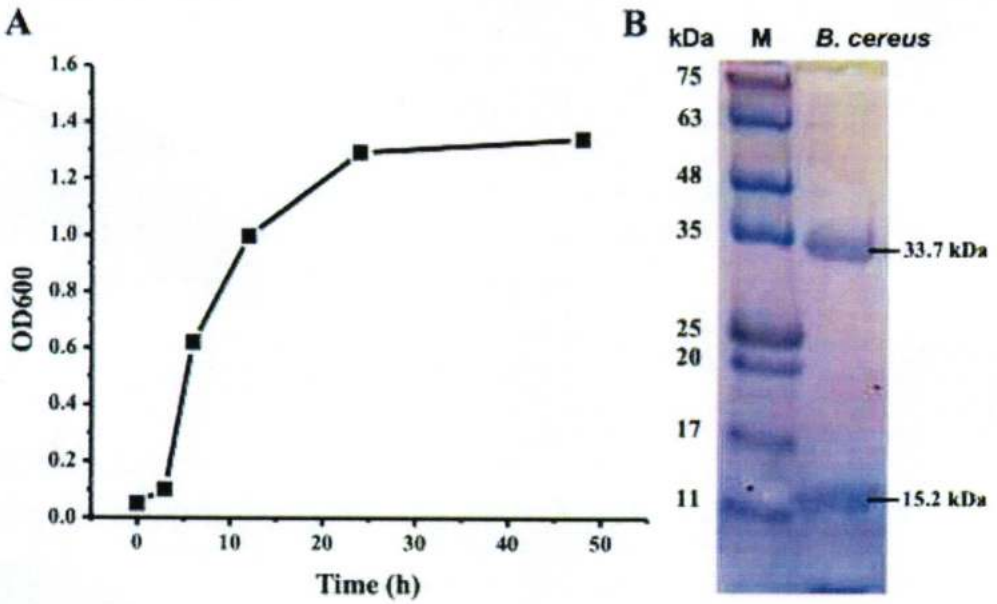

Figure 1. Growth curve and protein profile of bacterial cell lysate of B. cereus. (a) Growth curve of B. cereus in LB medium at 37 C in an incubator shaker. (b) The protein profile of B. cereus cell lysate in a 12.5% SDS-PAGE.

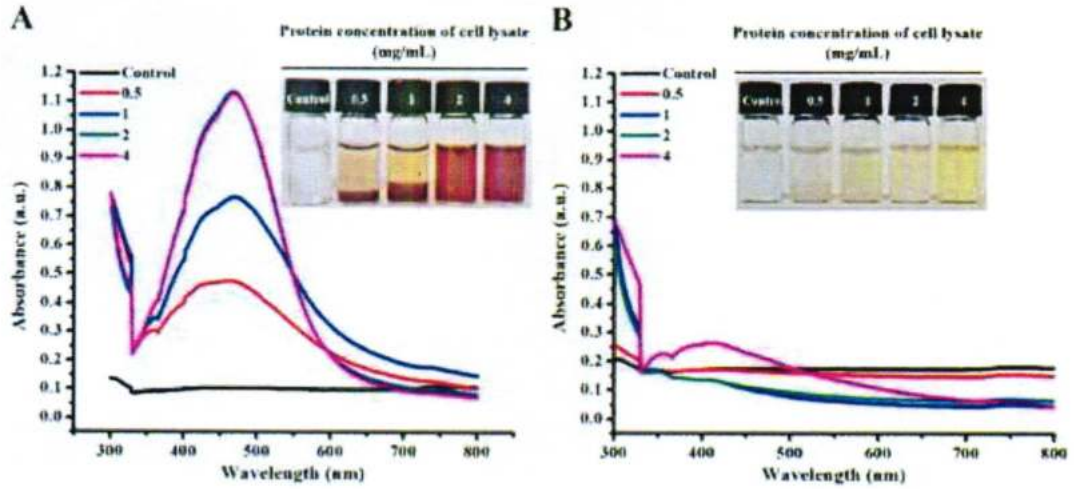

Figure 2. Effect of protein concentrations of bacterial cell lysate and photoactivation on the formation of AgNPs. (a) The UV-Vis spectra of the synthesis reaction using different protein concentrations of the bacterial cell lysate under fluorescent light exposure for 2 h. (b) The UV-Vis spectra of the synthesis reaction in the condition without light exposure for 2 h.

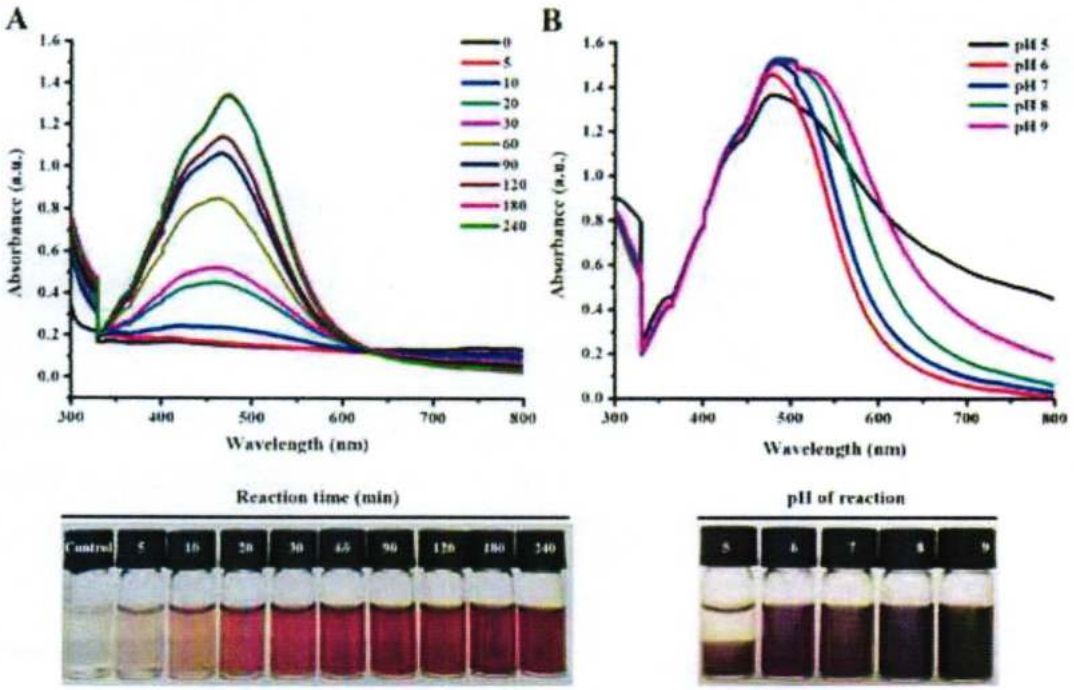

Figure 3. Effects of reaction time and pH on the formation of AgNPs. (a) The UV-Vis spectra of the synthesis reaction in the time course of 240 min. (b) The UV-Vis spectra of the synthesis reaction using

the pH 6, 7, 8, and 9.

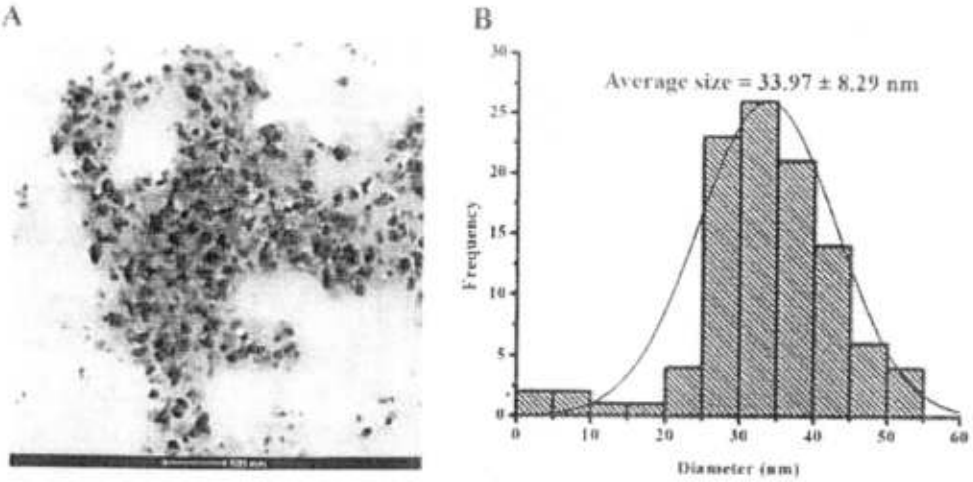

Figure 4. TEM image and histogram of the size distribution of the produced AgNPs. (a) TEM images showing the amorphous morphology of AgNPs. (b) Size distribution of the synthesized AgNPs as measured from 100 particles.

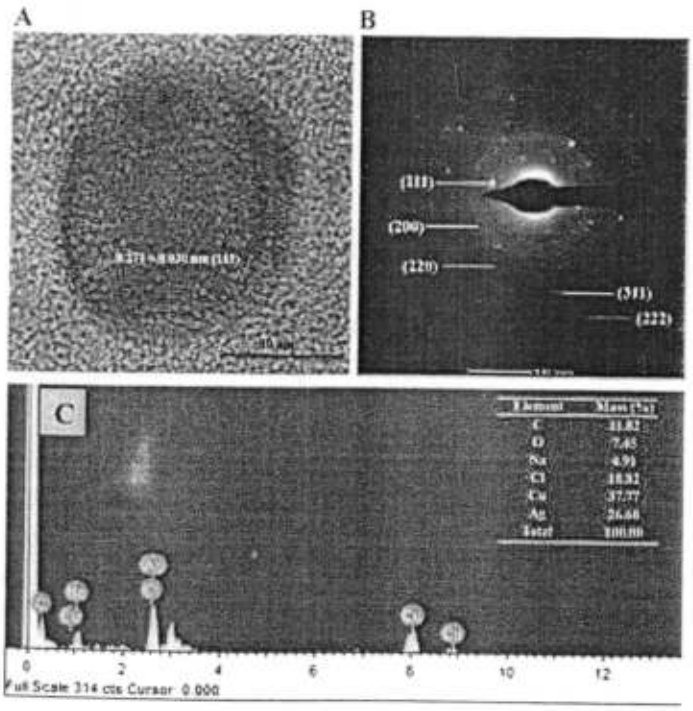

Figure 5. Characterization of the produced AgNPs by (a) HR-TEM, (b) SAED-TEM, and (c) EXD-TEM.

Figure 6. Larvicidal activity of AgNPs and bacterial cell lysate.