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Biogenic Synthesis of Nanoparticles Using Secondary Metabolites from *Pseudomonas fluorescence* and its Antimicrobial Activity.

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Abstract: Biogenic synthesis of Silver nanoparticles (AgNPs) using extracellular metabolites from the bacterium *Pseudomonas fluorescence* offers an eco-friendly and sustainable way of metal nanoparticle synthesis. The present work highlights the extracellular secondary metabolite, siderophore, produced by *P. fluorescence* mediated biotransformation of silver nitrate solution (1mM) into AgNP. AgNPs synthesized by Green synthesis using siderophore, were characterized by X-ray diffraction (XRD), UV- spectroscopy (UV), Fourier Transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The characterization techniques determined the size, morphology and distribution of size of NPs. The effect of temperature as well as incubation period on synthesis of NPs was studied. The biologically synthesized nanoparticles were found to have antimicrobial activity against some Gram positive and Gram negative bacteria and fungi as indicated by the zone of inhibition. By studying physicochemical properties of siderophore and using it as a reducing agent in synthesis of AgNPs will enable us to study its antimicrobial activity against phytopathogens and hence can be used as a biocontrol agent. It will also have plant growth promoting activity exhibited by siderophore.

Keywords: *Pseudomonas fluorescence*, silver nanoparticle synthesis, antimicrobial, *Aspergillus flavus*, *Pseudomonas aeruginosa.*

Introduction

Metal nanoparticles are increasingly being used in various fields of the economy, due to its significant application in bioscience and biomedicine as biological tagging agent, biomarker, antimicrobial agents, pharmaceutical ingredients, pollution control, cosmetic industry, drug delivery and safety control, cancer therapy, cryogenic superconducting material, solar cell technology, and biosensors. Among various types of nanoparticles, silver nanoparticle (AgNP or nanosilver) has attracted increasing interest because of its unique properties (e.g., size and shape; optical, electrical, and magnetic properties; chemical stability; and catalytic action. These characteristics make AgNPs nowadays superior and indispensable. In recent years, AgNPs has also attracted much attention due to its novel application in energy sector where stability and good catalytic performance off silver-based nano-compounds make it a promising alternative to platinum (Pt) in dye-sensitized solar cell technology.

Along with various applications of silver nanoparticles, synthesis of silver nanoparticles is of fundamental importance in the recent nanotech researches. There are many ways reported in literatures for synthesis of silver nanoparticles such as physical, chemical, and biological methods. The shape and size of silver NPs can be controlled by the reaction conditions such as reducing agent, stabilizer, or capping agents in different synthetic methods.

Though numerous physical and chemical methods are reported, many of these methods are expensive or use toxic substances which are not preferred due to non-eco-friendly nature of the process. The biogenic synthesis of nanoparticles is considered to be non-toxic, cheaper, and more eco-friendly as compared to physical and chemical procedures. The biological method of nanoparticle synthesis relies on the systematic use of plant extracts and microorganisms like bacteria, yeast, and fungi with low risk of eco-hazards. Various bacterial strains such as *Pseudomonas aeruginosa, Escherichia coli, Bacillus megaterium, Bacillus subtilis, Bacillus licheniformis, Klebsiella pneumonia, Streptococcus aureus,* and *Streptococcus thermophilus* are reported to show biogenic synthesis of AgNPs. Culture supernatants of *S. aureus, Staphylococcus epidermidis*, and *Streptococcus pyogenes* were found to form silver nanoparticles rapidly. But in order to avoid destruction of the biological resources for synthesis of nanoparticles, a revolutionary change in the biogenic synthesis of nanoparticles is envisaged by the use of exogenously produced secondary metabolites with little interference of toxic nature of the nanoparticles on the biological system.

Pseudomonas fluorescence is a gram-negative bacterium which is widely exploited for various commercial and biotechnological applications. It is well known to produce metal-chelating siderophores like pyocyanin (blue-green), pyoverdine (yellow, green fluorescent), pyomelanin (light brown), and pyorubrin (red-brown). Pyoverdine component of secondary metabolite is a yellow-green, water-soluble, fluorescent pigment and is considered to be a powerful iron scavenger and transporter. Metal ion redox potential of these pigments might play an important role in the synthesis of metal nanoparticles.

The use of bacterial secondary metabolite pyoverdine for biogenic synthesis of silver nanoparticles in the present study is likely to provide broad spectrum benefits such as the following:(i) Growth and productivity of the bacterium would never be influenced by the presence or toxicity of silver metal ions, (ii) segregation of nanoparticles will not be subjected to trivial purification procedure where bacterial siderophore will tend to chelate the nanoparticles, and (iii) the process of NP's synthesis does not make application of toxic chemicals. The same metabolite can be produced in an unhindered way from the batch or continuous culture of bacterium for largescale production of nanoparticles.

EXPERIMENTAL DETAILS

Material and method

Production of siderophore:

- Active and pure culture of *Pseudomonas fluorescence*
- Succinate media.

Media composition:

Components	Quantity (g/L)
Potassium Phosphate Dibasic Anhydrous (K_2HPO_4)	6.0
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	3.0
Ammonium Sulphate [$(NH_4)_2SO_4$]	1.0
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.2
Succinic Acid ($C_4H_6O_4$)	4.0
pH	7.0

Medium and growth conditions for bacterial culture preparation:

The bacterial culture *Pseudomonas fluorescence* was inoculated in succinate medium and grown at 25^0C for 48hr at 100 rpm on orbital shaker. The pH of the culture was maintained in the range of 7.0–7.5 by periodical addition of HCl. Culture medium was then centrifuged at 8000 rpm to obtain cell-free supernatant.

Extraction and purification of siderophore:

Extraction of siderophore was carried out by following the method of Meyer and Abdallah (1978). In cell-free supernatant containing secondary metabolites, equal amount of chloroform was added. The green-colored siderophore in the aqueous extract was separated.

Determination of siderophore type:

The type of siderophore was determined by using specific assays viz. Arnow's assay for catecholate type and Csaky assay for hydroxymate type of siderophore.

Csaky Assay:

• **Reagents:**

i. 6N sulphuric acid- 0.165ml in 10ml D/W
ii. Sulphanilic acid- 100ml D/W + 30ml acetic acid + 1g sulphanilic acid
iii. Iodine solution- 1.3g in 100ml glacial acetic acid
iv. Sodium arsenite solution- 2g in 100ml D/W
v. Naphthalamine solution- 100mlD/W + 3ml acetic acid + 0.3g naphthalamine
vi. 3.5% Sodium acetate solution- 3.5g in 100ml D/W

• **Methodology:**

i. 1ml supernatant and 1ml 6N sulphuric acid was placed in a test tube and was kept in boiling water bath at 110^0C for 25-30min. ii. The excess of sulphuric acid was buffered with 3ml 3.5% Sodium acetate and 1ml sulphanilc acid was added

iii. After 3-5min, 0.5ml iodine solution was added. iv. 1ml Sodium arsenite solution and 1ml□-Naphthalamine was added and volume was made up to 10ml by adding D/W.

v. All the tubes were kept undisturbed for 20-30min.
vi. Absorbance was taken at 526nm.

Arnow's Assay:

• **Reagents:**

i. 0.5N HCl- 4.41ml in 100ml D/W
ii. 1N Sodium hydroxide- 4g in 100ml D/W
iii. Nitrite molybdate reagent- 10g Sodium nitrite + 10g sodium molybdate in 100ml D/W

• **Methodology:**

i. To 1ml supernatant, 1ml 0.5N HCl and 1ml nitrite molybdate reagent was added (yellow coloration at this point)
ii. To this 1ml of sodium hydroxide was added.
iii. Presence of catecholate group is indicated by pink or red colour formation.
iv. Absorbance was measured at 510nm

Bioreduction of silver nitrate to silver nanoparticles by siderophore:

i. Siderophore was mixed with1 mM silver nitrate.
ii. The reaction mixture was incubated up to 24 h in the dark at room temperature.
iii. In the control treatment, Distilled water was added to siderophore and kept under similar conditions. Experiments were carried out in triplicates.
iv. Visual observation was done periodically examining color change for nanoparticle formation. After completion of the reaction time, again it was centrifuged to obtain cellfree supernatant.
v. A visible change in the color of solution was recorded continuously.
vi. The color change in solution from pale yellow to dark brown confirmed the reduction of the silver ion (Ag+) and formation of AgNPs.

Synthesis and optimization of Ag nanoparticles:

• **Concentration:**

i. 1ml, 3ml and 5ml of 1 mM silver nitrate was taken in 3 test tubes, respectively. ii. 1ml siderophore was added to each test tube and incubated for 24h in the dark at room temperature. iii. In the control treatment, siderophore solution without silver nitrate solution was kept under similar condition.
iv. Visual observation was done periodically examining color change for nanoparticle formation. After completion of the reaction time, again it was centrifuged to obtain supernatant.
v. A visible change in the color of solution was recorded continuously.
vi. The color change in solution from pale yellow to dark brown confirmed the reduction of the silver ion (Ag+) and formation of AgNPs.

• **Temperature:**

i. 1ml siderophore was mixed with 1mM silver nitrate.
ii. The reaction mixture was incubated up to 24 h in the dark at different temperatures viz. at room temperature, 50^{0}C, 37^{0}C and 4^{0}C to determine optimum temperature required for the synthesis of silver nanoparticles.
iii. In the control treatment, siderophore solution without silver nitrate solution was kept under similar condition.
iv. Visual observation was done periodically examining color change for nanoparticle formation. After completion of the reaction time, again it was centrifuged to obtain cellfree supernatant.

v. A visible change in the color of solution was recorded continuously.
vi. The color change in solution from pale yellow to dark brown confirmed the reduction of the silver ion (Ag+) and formation of AgNPs.

• **Effect of Incubation Period on synthesis of silver nanoparticles:**

The effect of incubation time on the synthesis of nanoparticles was monitored over the period of 30h. Synthesis of nanoparticles was measured spectroscopically after every 2hr for a period of 30hr

Confirmation by UV–visible spectroscopy:

UV–visible spectroscopy is one of the important techniques toverify the formation of metal nanoparticles. Absorption measurements were carried out by using UV–visible spectrophotometer (PerkinElmer double beam spectrophotometer; Lambda 35, PerkinElmer, USA) between absorption range 300 and 550 nm. The bio reduction of silver ions was monitored by recording the spectral change in the absorbance of reaction mixture at different time intervals and the appearance of the characteristic absorption peak (415 nm) of silver nanoparticle.

Characterization of silver nanoparticles

FTIR analysis: FTIR (Nicole 6700) was used to determine the nature of associated biomolecules of bacterial extracts with nanoparticles. The dried silver nanoparticles were compressed with KBr at1:100 ratio by using hydraulic press at a pressure of 10 t, and spectra measured at the wavelength
range from 4000 to400 cm^{-1}. Spectra were monitored against a KBr background And manipulated using the OMNIC software “automatic base-line correct” function.

XRD analysis: The XRD (Shimadzu, Japan) technique is used to establish the metallic nature of particles. The energetic X-rays can penetrate deep into the materials and provide information about the bulk structure. X-ray diffractometer operated at a voltage of 40 kVand current of 30 mA using CuKa (k = 1.54 A°) radiation in the scan range 2θ = 5°–100°.

Scanning electron microscopy (SEM): Scanning electron microscopy was used to examine the size and morphology of the silver nanoparticles (JEOL JSM6490LV, Tokyo). Thin film of the silver nanoparticle was prepared on a carbon-coated copper grid by just dropping a very small amount of the solution on the grid; extra solution was removed using a blotting paper, and then, the film on the SEM grid was allowed to dry by putting it under a mercury lamp for5min and examined under SEM at 10 kV accelerating voltage.

Anti-microbial activity of silver nanoparticles:

Anti-bacterial activity:
Test organisms: *Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus.*

Bacterial growth and experimental conditions:

The bacterial cultures were obtained from Modern College, Shivajinagar, India. The bacteria, *Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus* were grown in sterile Muller- Hinton broth and incubated at 37^0C for 24hr. The anti-microbial activity of AgNP was tested on Muller-Hinton agar plates, using agar Well Diffusion method. The growth inhibition zones formed around wells on culture plates upon different temperatures viz room temperature and 37^0C keeping same concentration of AgNP were measured after 2-day incubation period and expressed in millimeter.

Well diffusion method:

i. The pure cultures of *Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus* were subcultured on nutrient agar medium in petri dishes (20 ml volume).
ii. 0.1ml Bacterial culture was spread plated on Muller Hinton agar plates. iii. Wells of 0.5 mm diameter were bored on Muller-Hinton agar plate using gel puncture. iv. Using micropipette, 10µl AgNP was added to each well.
v. Three replicates of experiments were carried out.

Anti-fungal activity:
Test organism: *Aspergillus flavus*

Fungal growth and experimental conditions:

The fungal culture was obtained from Modern College, Shivajinagar, India. The fungus, *Aspergillus flavus* was grown in sterile Potato Dextrose Broth and incubated under optimum growth condition at 37^0C for 24hr. The anti-microbial activity of AgNP was tested on Sterile Potato Dextrose agar plates, using agar Well Diffusion method. The growth inhibition zones formed around wells on culture plates at different temperatures viz room temperature and 37^0C keeping same concentration of AgNP were measured after 2-days incubation period and expressed in millimeter.

Well diffusion method:

i. The pure culture of *Aspergillus flavus* were subcultured on nutrient agar medium in petri dishes. ii. 0.1ml fungal culture was poured homogeneously onto the individual plates and was spread plated.
iii. Wells of 0.5 mm diameter were bored on Potato Dextrose agar plate using gel puncture. iv. Using micropipette, 10µl AgNP was added to each well.
v. Three replicates of experiments were carried out.

Studying plant growth promoting activity:

- **Seeds used**- *Triticum aestivum* (wheat)

Triticum aestivum (wheat) variety used was Ankur Kedar, class;Truthful, Lot number 272-456291 was selected from local market.

- **Three variations with control were selected for Tray Assay as follows:**

a. Seeds soaked in nanoparticles.
b. Seeds soaked in siderophore.
c. Seeds soaked in silver nitrate solution(Positive control).
d. Seeds soaked in distilled water(Negative control).

• **Tray Assay:**

i. Seeds were soaked in respective solution as described above for 30min.
ii. Soaked seeds were then sowed in tray containing sterile cocco pit. iii. Plants were watered regularly.
iv. Plants were then observed for the length of root and shoot and their ramification after 2 weeks.
v. Observations were recorded and results were interpreted using graphs.

RESULTS:

Production, extraction and purification of siderophore:

Green-colored, Siderophore was produced in the Succinate Media and extracted by Meyer and Abdallah's method, successfully.

Determination of siderophore type:

Assay	Observation
Csaky Assay	No colour change.
Arnow Assay	Appearance of light pink colour.

Table1: Determination of siderophore type.

Fig1: Determination of siderophore type; Arnow's Assay

From the above observations, a slight pink coloration was seen after performing Arnow Assay. Hence, Catecholate type of Siderophore was successfully detected.

Bioreduction of silver nitrate to silver nanoparticle.

i. The siderophore was extracted from the cell-free secondary metabolites of *P. fluorescens* strain, and it was quantified using the absorbance maxima at 362 nm at room temperature.
ii. Addition of 1.0 mM silver nitrate resulted into change in color of reaction mixture from pale yellow to reddish brown due to bioreduction of $AgNO_3$.
iii. The change in color of reaction mixture was monitored continuously during the next 24-h incubation period, using silver nitrate solution as control. iv. The results on absorbance spectra of reaction mixture showed characteristic absorbance maxima at 394 nm as reported for silver nanoparticles.
v. Thus, these results indicated that bioreduction of silver nitrate into AgNPs by siderophore was found to be a time-dependent process, reaching to its maximum level after 24 h.

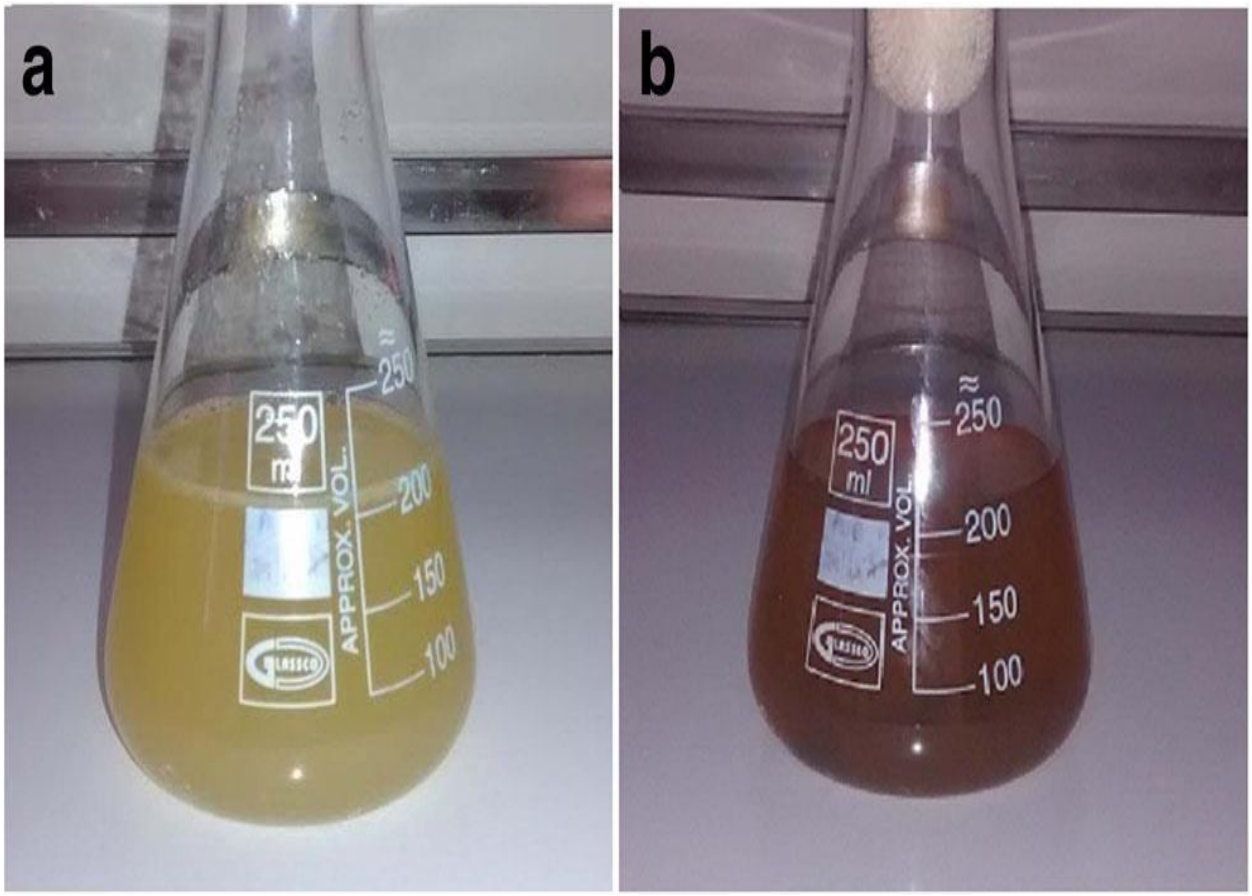


Fig. 2: Bacterial extract of *Pseudomonas fluorescence*(a) before bioreduction and (b) after synthesis to AgNP, 24-h incubation time after adding$AgNO_3$ solution (1 mM) **Optimization of silver nanoparticles:**

i. The optimum color change from pale yellow to dark brown was observed in solution of 1mM concentration.
ii. Similarly, the optimum colour change from pale yellow to dark brown was observed in solution incubated at room temperature.

- **Result of biogenic synthesis of silver nanoparticles at different temperature with different amount of mixture of silver nitrate and siderophore-**

A. Biogenic synthesis on AgNPs at room temperature-

Fig 2.1 Biogenic synthesis of silver nanoparticles at room temperature – Mixture containing constant amount of siderophore with different amount of 1mM silver nitrate solution incubated at room temperature . Synthesis on AgNPs was confirmed by changing the colour formation from pale yellow to dark brown.

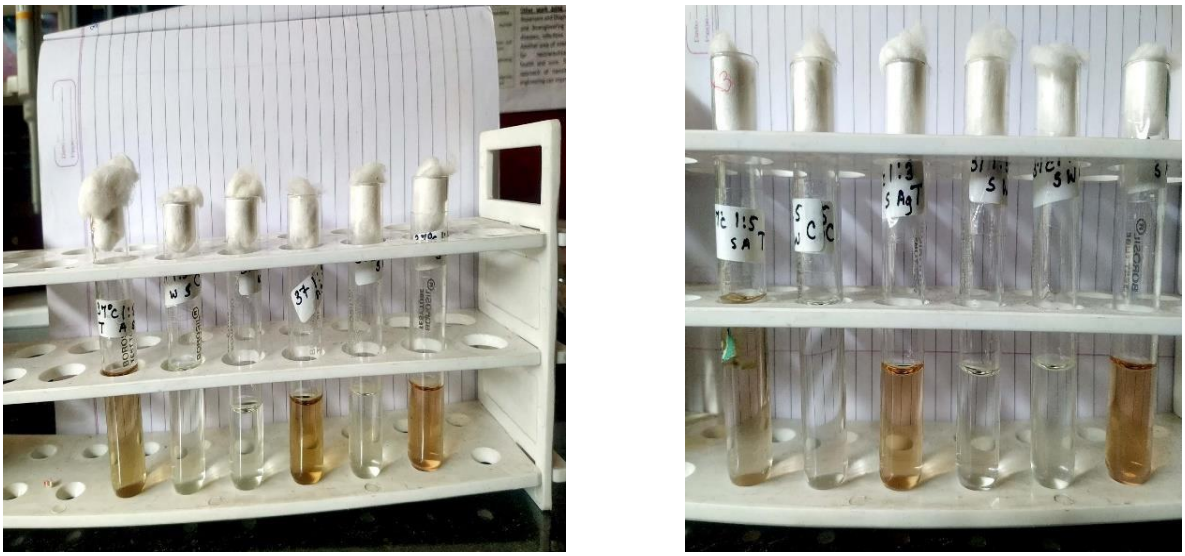
B. Biogenic synthesis of AgNPs at 4^0C

A B

Fig 2.2 Biogenic synthesis of silver nanoparticles at 4°C–(A) Mixture containing constant amount of 1mM silver nitrate solution with different amount of siderophore incubated at 4°c**. (B)** Mixture containing constant amount of siderophore with different amount of 1mM silver nitrate solution incubated at 4°c. Synthesis on AgNPs was confirmed by changing the colour

formation from pale yellow to dark brown.

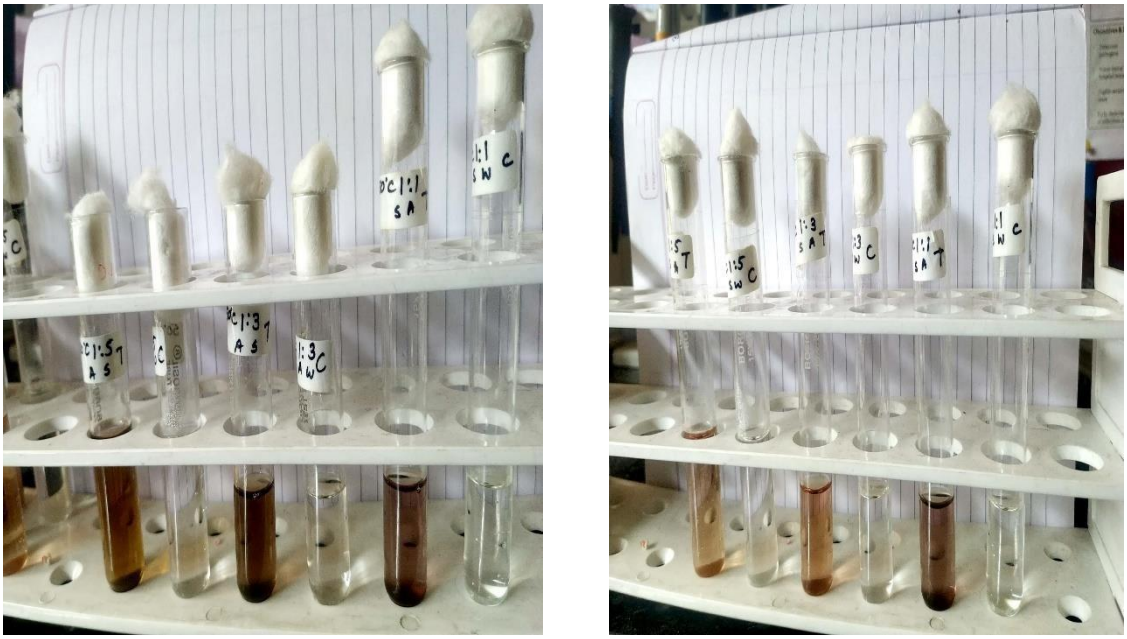
C. Biogenic synthesis of AgNPs at 37^0C-



A B

Fig 2. 3 Biogenic synthesis of silver nanoparticles at 37°C_(A) Mixture containing constant amount of 1mM silver nitrate solution with different amount of siderophore incubated at 37°c**. (B)** Mixture containing constant amount of siderophore with different amount of 1mM silver nitrate solution incubated at 37°c. Synthesis on AgNPs was confirmed by changing the colour formation from pale yellow to dark brown.

D. Biogenic synthesis of AgNPs at 50^0C-

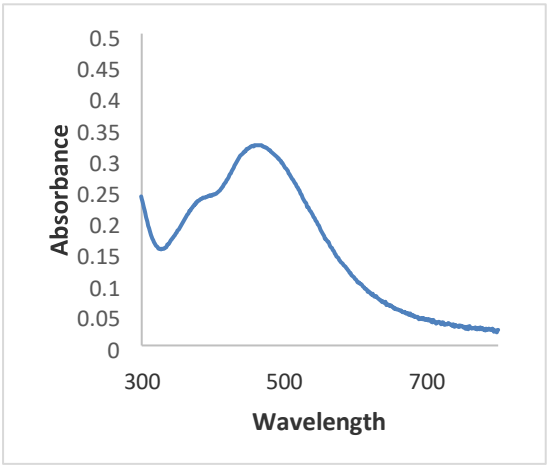


A B

Fig 2.4 Biogenic synthesis of silver nanoparticles at 50°C–(A) Mixture containing constant amount of 1mM silver nitrate solution with different amount of siderophore incubated at 50°c**.(B)** Mixture containing constant amount of siderophore with different amount of 1mM silver nitrate solution incubated at 50°c. Synthesis on AgNPs was confirmed by changing the colour formation from pale yellow to dark brown.

• **Result of optimization of silver nanoparticles at different temperature with different amount of mixture of silver nitrate and siderophore-**

1) Amount of siderophore kept constant at room temperature with changing the amount of silver nitrate –



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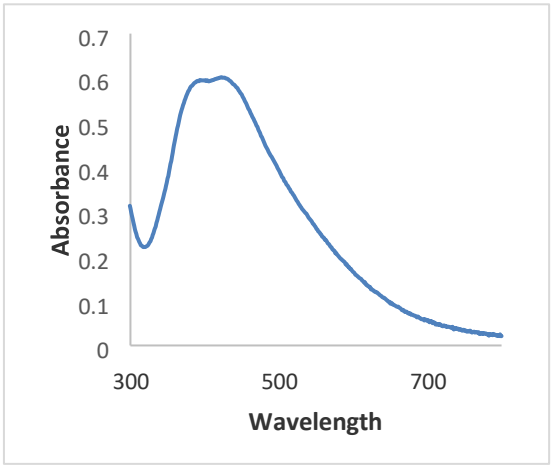

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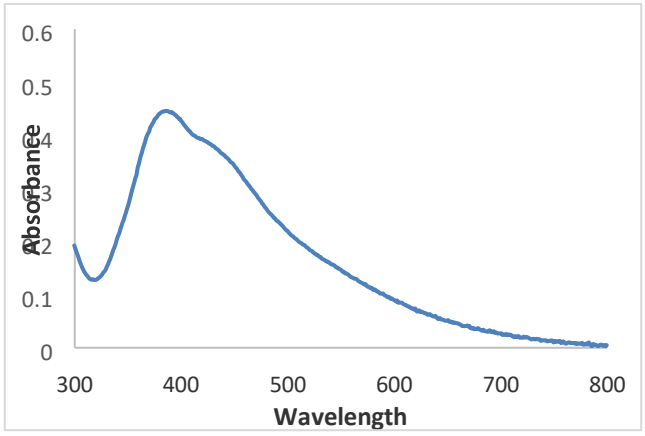


C

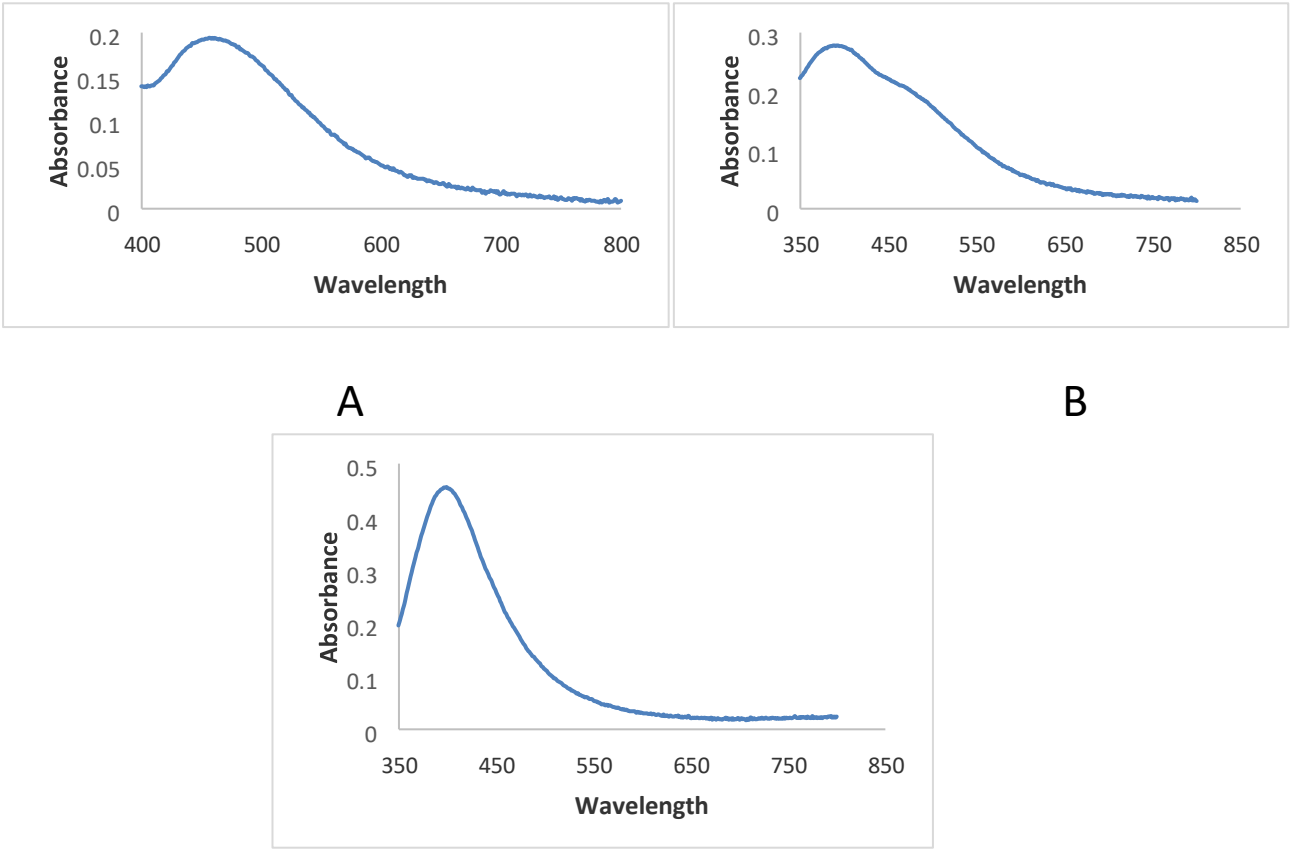
Fig 3.1 UV -Vis absorption spectra of – The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at room temperature. The synthesis of AgNPs was detected by the colour change from pale yellow

to dark brown (shown in fig 2.1). After 24 hr reaction mixtures were scanned using UV-Vis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for **(A)** RT (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$, **(B)**RT (1:3) i.e., mixture of 1 ml siderophore and 3 ml (1mM) $AgNO_3$, and**(C)** RT (1:5) i.e., mixture of 1 ml siderophore and 5 ml

(1mM) $AgNO_3$ was seen at 441 nm, 412 nm, and 400 nm respectively.

2) Amount of silver nitrate kept constant at 4^0Cwith changing the amount of siderophore-



– **The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at 4°C.** The synthesis of AgNP's was detected by the colour change from pale yellow to dark brown (shown in fig 2.2A). After 24 hr reaction mixtures were scanned using UV-

Fig 3.2 UV-Vis absorption spectra of

Vis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for**(A)**4°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$**, (B)** 4°C (1:3) i.e., mixture of 3 ml siderophore and 1 ml (1mM) $AgNO_3$, and **(C)** 4°C (1:5) i.e., mixture of 5 ml siderophore and 1 ml (1mM) $AgNO_3$ was seen at 460 nm,393 nm, and 400 nm respectively.

Amount of siderophore kept constant at 4^0Cwith changing amount of silver nitrate-

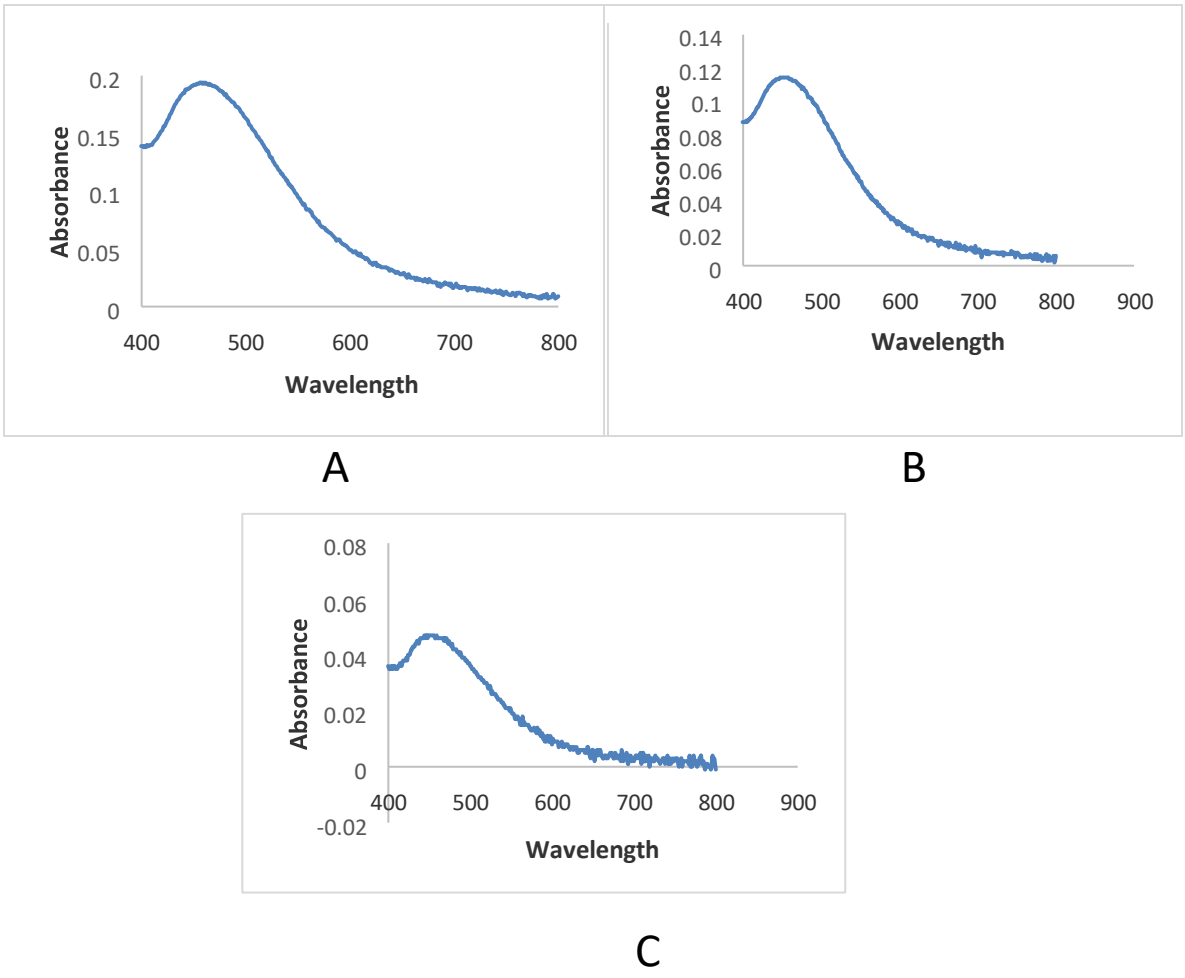
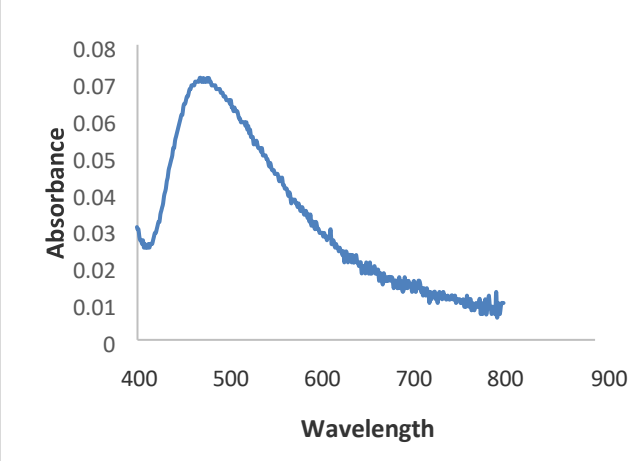


Fig 3.3 UV-Vis absorption spectra of – The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at 4°C. The synthesis of AgNPs was detected by the colour change from pale yellow to dark brown (shown in fig 2.2B). After 24 hr reaction mixtures were scanned using UV-Vis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for **(A)** 4°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$, **(B)** 4°C (1:3) i.e., mixture of 1 ml siderophore and 3 ml (1mM) $AgNO_3$, and **(C)** 4°C (1:5) i.e., mixture of 1 ml siderophore and 5 ml (1mM) $AgNO_3$ was seen at 460 nm,393 nm, and 400 nm respectively.

Amount of silver nitrate kept constant at 37^0Cwith changing the amount of siderophore-



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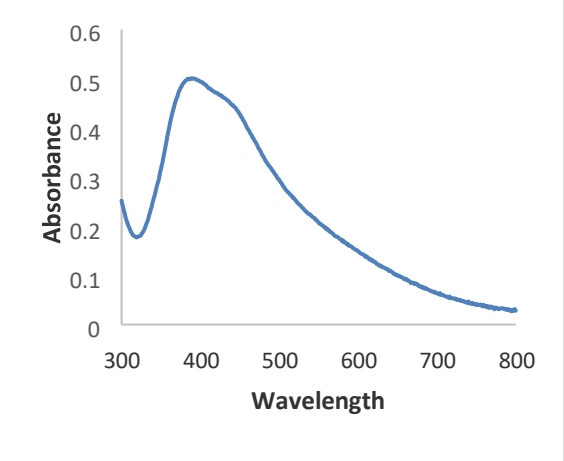

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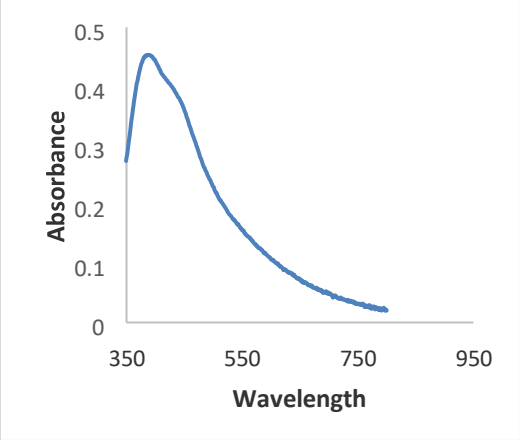
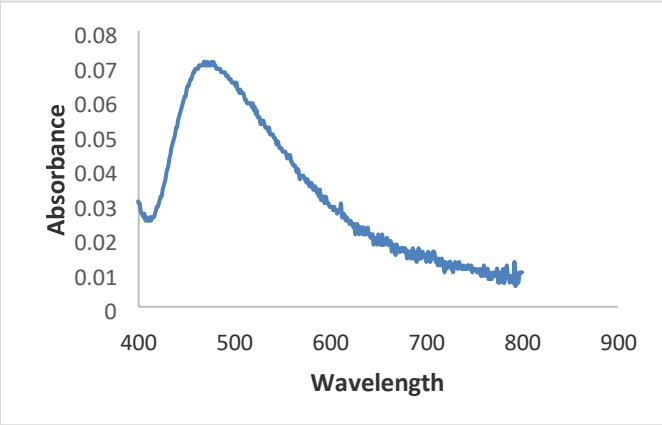


C

Fig 3.4 UV-Vis absorption spectra of – The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at 37°C. The synthesis of AgNPs was detected by the colour change from pale yellow to dark brown (shown in fig 2.3A). After 24 hr reaction mixtures were scanned using UV Vis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for **(A)** 37°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$**, (B)** 37°C (1:3) i.e., mixture of 3 ml siderophore and 1 ml (1mM) $AgNO_3$, and **(C)**37°C (1:5) i.e., mixture of 5 ml siderophore and 1 ml (1mM) $AgNO_3$ was seen at 456 nm,387 nm, and 388 nm respectively.

Amount of siderophore kept constant at 37^0Cwith changing the concentration of silver nitrate-



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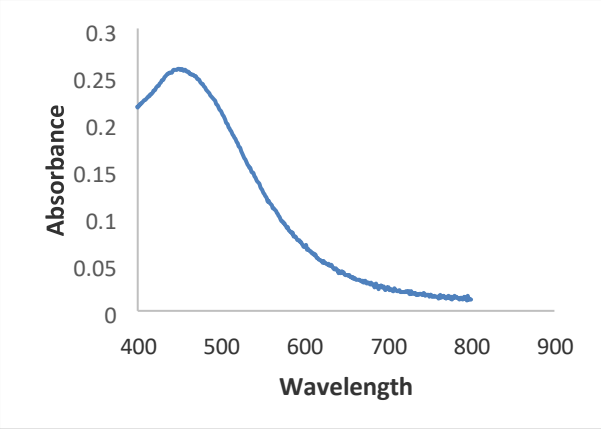

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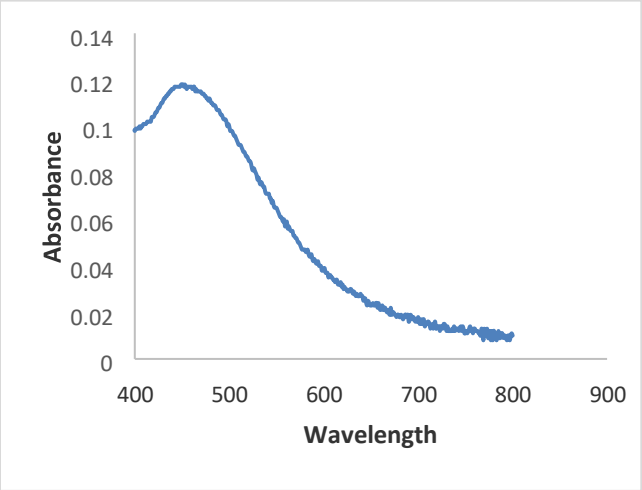
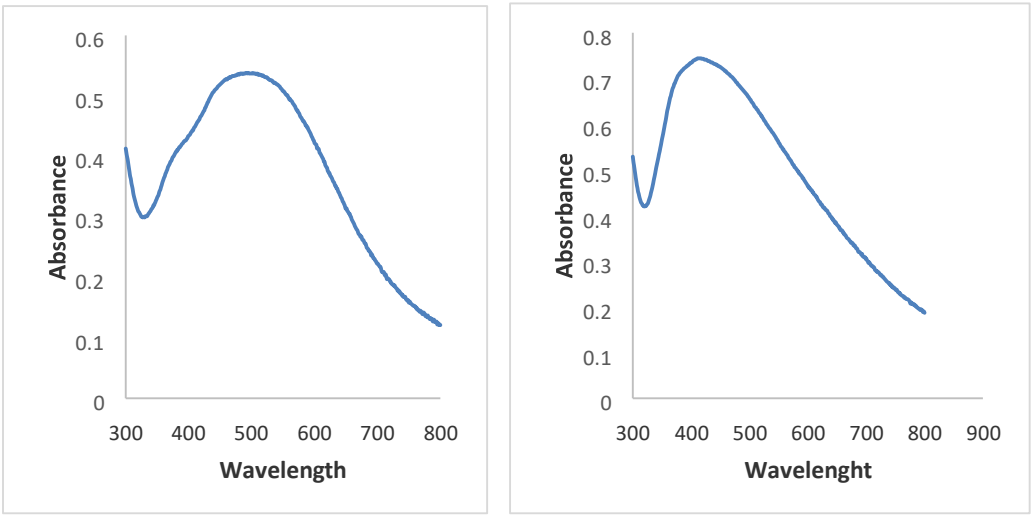


C

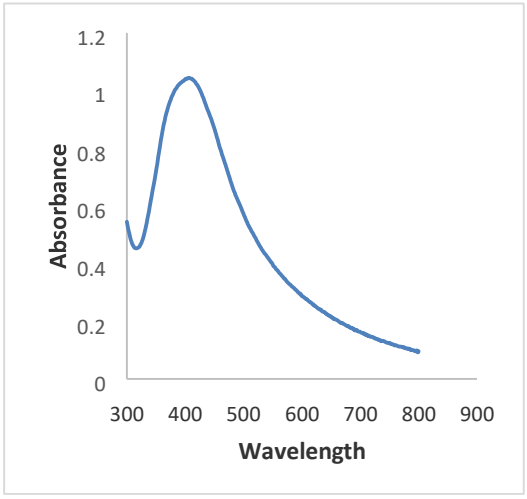
Fig 3.5 UV-Vis absorption spectra of – The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at 37 °C. The synthesis of AgNPs was detected by the colour change from pale yellow to dark brown (shown in fig 2.3B).After 24 hr reaction mixtures were scanned using UVVis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for **(A)** 37°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$**, (B)** 37°C (1:3) i.e., mixture of 1 ml siderophore and 3 ml (1mM) $AgNO_3$, and **(C)** 37°C (1:5) i.e., mixture of 1 ml siderophore and 5 ml (1mM) $AgNO_3$ was seen at456 nm,454 nm, and 449 nm respectively.

6) Amount of silver nitrate kept constant at 50^0Cwith changing the amount of siderophore-



A B



C

Fig 3.6 UV-Vis absorption spectra ot - The mixture of siderophore and (1 mM) $AgNO_3$ were kept for 24 hr at 50°C. the synthesis of AgNP's was detected by the colour change from pale yellow to dark brown (shown in fig 2.4 A). After 24 hr reaction mixtures were scanned using UV-Vis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for**(A)** 50°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$**, (B)**50°C (1:3) i.e., mixture of 3 ml siderophore and 1

ml (1mM) $AgNO_3$, and**(C)** 50°C (1:5) i.e., mixture of 5 ml siderophore and 1 ml (1mM) $AgNO_3$ was seen at 473 nm, 417 nm, and 413 nm respectively.

7) Amount of siderophore kept constant at 50^0Cwith changing the amount of silver nitrate:

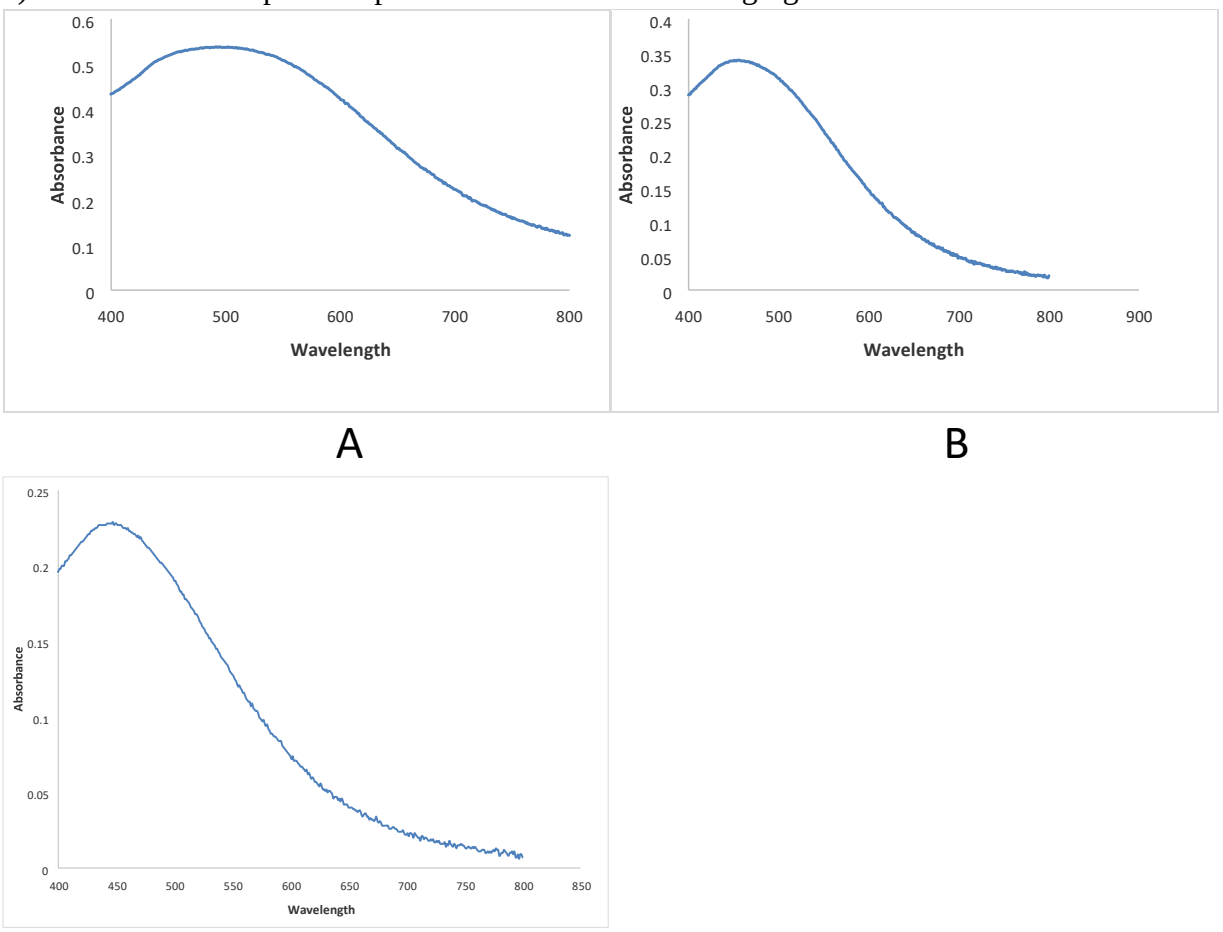


Fig 3.7 UV-Vis absorption spectra of – The mixture of siderophore and (1mM) $AgNO_3$ were kept for 24 hr at 50°C. The synthesis of AgNPs was detected by the colour change from pale yellow to dark brown (shown in fig 2.4B). After 24 hr reaction mixtures were scanned using UVVis spectrophotometer.

Spectrophotometric analysis gives a prominent peak, the maximum absorption for **(A)** 50°C (1:1) i.e., mixture of 1 ml siderophore and1ml (1mM) $AgNO_3$, **(B)** 50°C (1:3) i.e., mixture of 1 ml siderophore and 3

ml (1mM) $AgNO_3$, and **(C)** 50°C (1:5) i.e., mixture of 1 ml siderophore and 5 ml (1mM) $AgNO_3$ was seen at473 nm,458 nm, and 448 nm respectively.

Effect of incubation period on synthesis of silver nanoparticles-

I. Effect of incubation period on synthesis of AgNPs at room temperature

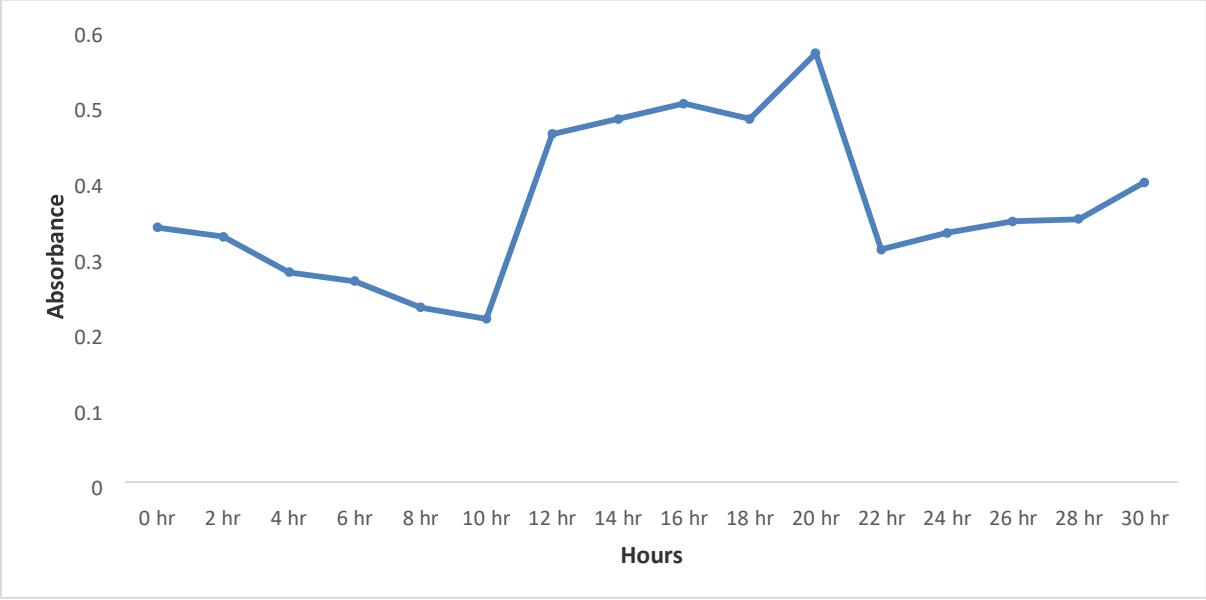


Fig 4.2 Effect of incubation period on synthesis of AgNPs at 37^0C, λ_{max} is 456 nm

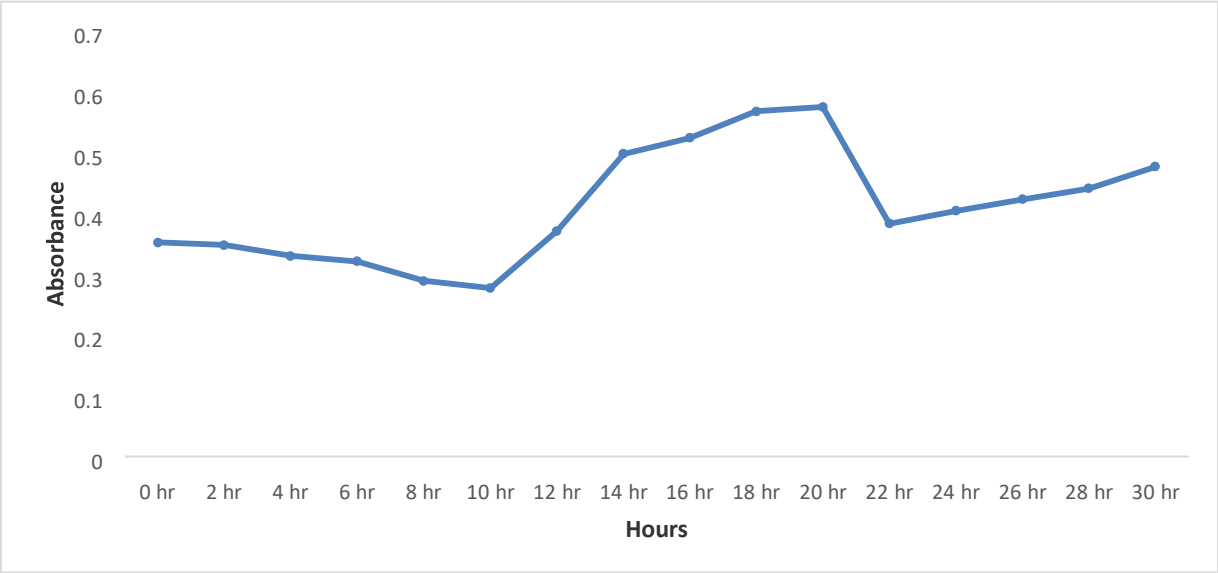
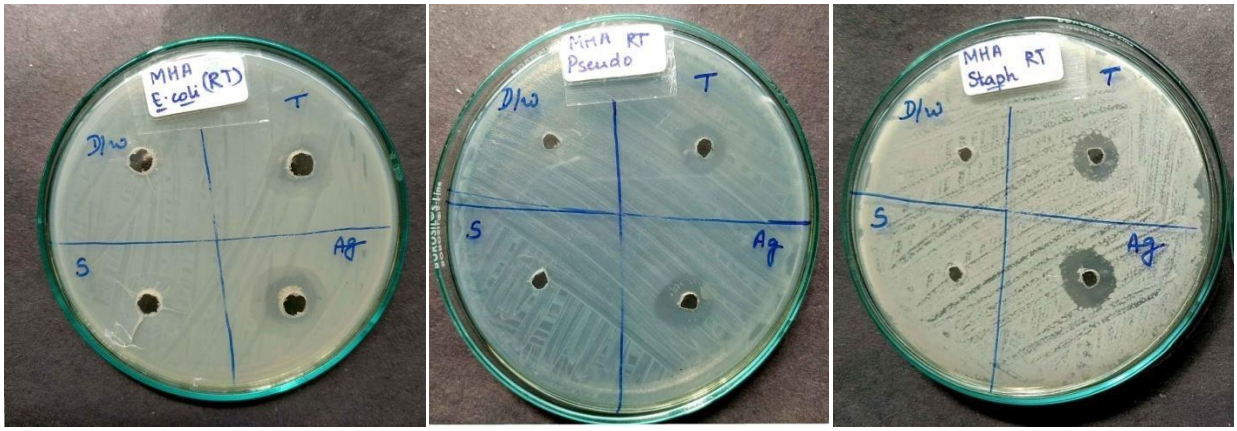


Fig 4.1 Effect of incubation period on synthesis of AgNP's at room temperature, λ_{max} is 412 nm.

II. Effect of incubation period on synthesis of AgNP's at 37^0C.

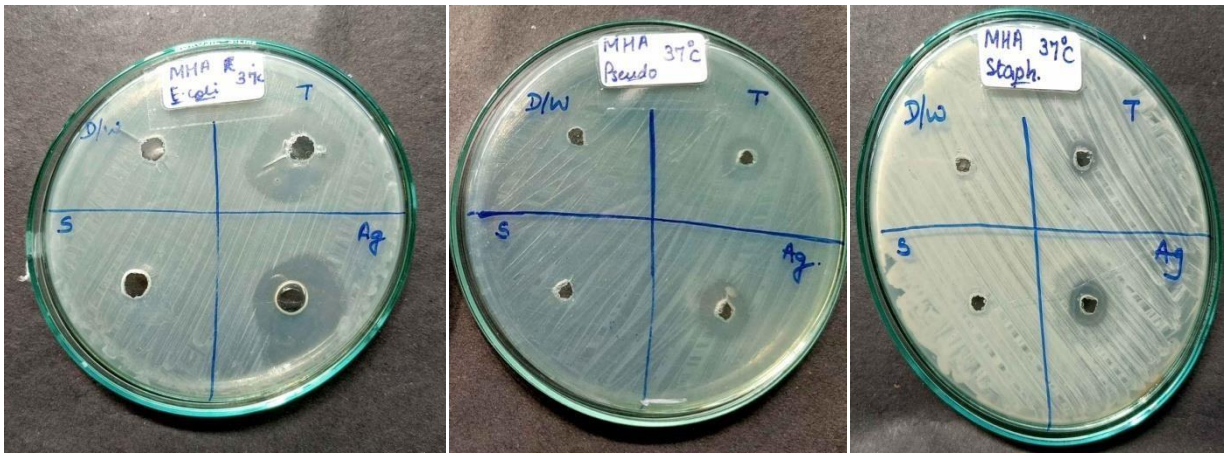
Anti-microbial activity of silver nanoparticles:

- **Anti-bacterial activity:**



(a) (b) (c)

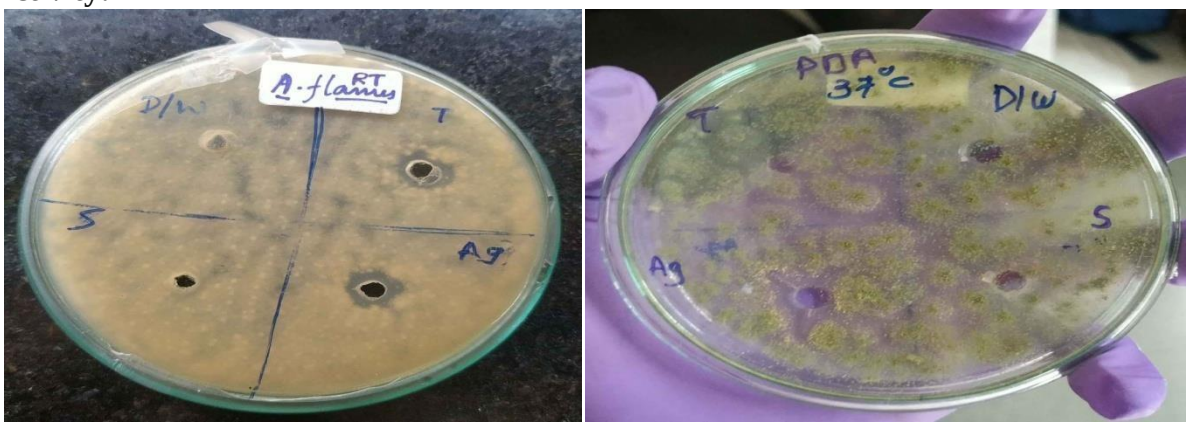
Fig5: Zone of inhibition (a) *E.coli*,(b)*P.aeruginosa and* (c)*S. aureus at* **room temperature.**



(a) (b) (c)

Fig6: Zone of inhibition (a) *E.coli*,(b) *P.aeroginosa and* (c) *S. aureus at* **37^{0}C.**

- **Anti-fungal Activity:**



(a) (b)

Fig7: Zone of inhibition of *A.flavus at* (a) room temperature (b) 37^{0}C.

Organism	neter (mm) of the zone o inhibition at **room temperatur**	neter(mm) of the zone o inhibition at **37^{0}C**
Escherichia coli	7	12
Pseudomonas aeruginosa	4	5
Staphylococcus aureus	5	3
Aspergillus flavus	4	3

Table: 2 Zone of inhibition in *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Aspergillus flavus* culture plates against different incubation temperatures

The effect of AgNPs on the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Aspergillus flavus* was observed by measuring the zone of inhibition. For organisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Aspergillus flavus*) zone of inhibition was found in the range of 3-5 mm at both the temperatures. The highest zone of inhibition (12-mmdiameter) was observed in *E.coli* at 37^0C.

Plant growth promoting activity:

- **Tray Assay:**

The plant growth promoting activities of three variations as discussed in material and methods were checked by using the Tray Assay. The highest length of shoot and root of the three variations was observed in the plants whose seeds were prior soaked in nanoparticles.

(a) (b)

Fig 8: Tray assay (a) Day 1 (b) Day 12

a. Seeds soaked in Nanoparticles:

i. Synthesized at Room Temperature

Sr. No	Length of root (cm)	Length of shoot (cm)
I. Undiluted		
1.	9.0	20.0
2.	10.5	21.0

3.	12.0	22.0
4.	13.5	22.5
5.	8.7	22.0
6.	4.5	8.4
7.	5.9	11.4
8.	8.0	20.0
9.	12.0	22.5
10.	10.3	18.0
II.	**25%**(2.5ml AgNP+7.5ml D/W)	
1.	11.7	20.7
2.	10.0	20.5
3.	7.0	20.8
4.	11.8	18.0
5.	7.4	18.5
6.	7.7	17.0
7.	5.5	16.8
8.	4.7	7.0
9.	5.8	19.8
10.	6.0	18.0
III.	**50%** (5ml AgNP + 5ml D/W)	
1.	9.0	21.0
2.	7.5	13.0
3.	9.0	17.0
4.	12.5	22.0
5.	11.0	19.0
6.	11.5	23.0

7.	10.0	22.5
8.	10.5	27.5
9.	7.0	20.0
10.	8.5	18.5
IV. **75%** (7.5 ml AgNP + 2.5 ml D/W)		
1.	12.0	23.8
2.	9.0	23.0
3.	10.6	17.0
4.	8.4	20.0
5.	12.0	22.5
6.	13.0	21.0
7.	11.0	25.0
8.	9.2	17.0
9.	8.7	23.0
10.	11.6	21.6

ii. Synthesized at 37°C

Sr. No	Length of root (cm)	Length of shoot (cm)
I. Undiluted		
1.	7.0	14.3
2.	2.8	6.3
3.	9.0	15.3
4.	7.6	16.0
5.	4.8	16.6
6.	8.0	17.0
7.	7.5	16.5
8.	5.2	11.5

9.	9.7	18.4
10.	6.3	16.3
II. 25%(2.5ml AgNP+7.5ml D/W)		
1.	7.0	21.0
2.	9.5	17.0
3.	7.0	16.0
4.	7.5	19.5
5.	10.0	19.5
6.	7.0	22.0
7.	6.0	15.0
8.	9.5	18.7
9.	11.0	19.0
10.	9.0	20.0
III. 50% (5ml AgNP + 5ml D/W)		
1.	5.5	10.4
2.	12.0	23.0
3.	5.7	10.0
4.	7.0	18.5
5.	7.5	21.0
6.	9.0	19.5
7.	8.5	19.0
8.	6.0	20.0
9.	11.0	19.5
10.	9.5	21.0
IV. 75% (7.5 ml AgNP + 2.5 ml D/W)		
1.	6.0	18.0

2.	6.5	18.5
3.	11.0	21.5
4.	9.5	23.0
5.	10.5	20.0
6.	9.8	18.0
7.	7.5	20.0
8.	6.0	19.6
9.	7.0	20.0
10.	5.7	12.0

b. Seeds soaked in siderophore:

Sr. No	Length of root (cm)	Length of shoot (cm)
1.	9.5	18.0
2.	13.0	20.5
3.	9.0	20.5
4.	11.0	21.0
5.	8.0	20.5
6.	9.5	17.0
7.	8.0	19.5
8.	8.5	16.0
9.	7.0	18.5
10.	6.5	17.5

c. Seeds soaked in silver nitrate solution (Positive control):

Sr. No	Length of root (cm)	Length of shoot (cm)
1.	4.0	19.0
2.	5.3	12.3
3.	6.0	14.0
4.	5.8	16.0
5.	5.0	13.0
6.	10	17.0
7.	5.0	10.0
8.	8.5	17.5
9.	6.5	14.0
10.	5.5	17.0

d. Seeds soaked in distilled water (Negative control):

Sr. No	Length of root (cm)	Length of shoot (cm)
1.	7.0	16.0
2.	6.5	16.0
3.	5.5	13.0
4.	7.0	15.0
5.	7.5	17.0
6.	7.5	16.5
7.	6.0	18.5
8.	5.5	16.5
9.	6.5	16.5
10.	5.0	15.0

Seed soaked in	Average length of root (cm) (± SD)	Average length of shoot (cm) (± SD)
a) Nanoparticles: **I. Synthesized at room temperature:**		
I. **Undiluted**	9.4 ± 2.81	18.8 ± 4.93
II. **25%** (2.5 ml AgNP + 7.5 ml D/W)	7.8 ± 2.56	17.7 ± 4.04
III. **50%** (5ml AgNP + 5ml D/W)	9.6 ± 1.76	20.4 ± 3.89

IV. **75%** (7.5 ml AgNP + 2.5 ml D/W)	10.5 ± 1.62	21.4 ± 2.70
II. Synthesized at 37°c:		
I. **Undiluted**	6.8 ± 2.07	14.8 ± 3.51
II. **25%** (2.5mlAgNP+7.5ml D/W)	8.3 ± 2.74	18.8 ± 2.18
III. **50%** (5ml AgNP + 5ml D/W)	8.2 ± 2.23	18.2 ± 4.39
IV. **75%** (7.5 ml AgNP + 2.5 n D/W)	7.9 ± 2.04	19.1 ± 2.91
b) Siderophore	9.0 ± 1.91	18.9 ± 1.76
Silver nitrate solution (Positive control)	6.1 ± 1.79	14.9 ± 2.77
d) Distilled Wat (Negative control)	6.4 ± 0.87	16.0 ± 1.45

Table: 3 Plant growth promoting activity of AgNP was observed by tray assay where the average values of length of root and shoot of three variation were obtained in the plant whose seeds were prior soaked in: a. Nanoparticles at room temperature and 37°c with different concentration. b. Siderophore c. Silver nitrate solution. d. Distilled water.

DISCUSSION:

Several bacterial strains are found to show the ability of bioreduction of silver nitrate and synthesis of silver nanoparticles (Saravanan et al. 2011; Das et al. 2014). It has been proposed that mechanism of AgNP formation by the bacterial cell culture probably involved the enzyme nitrate reductase (Durán et al. 2005; Kalimuthu et al. 2008).Since the bacterial siderophore is an iron chelator and is reported to be involved in the iron sequestration into the bacterial cells(Voulhoux et al. 2006),the present investigation

was aimed at studying the role of siderophore on its metal transformation potential and mitigation of metal toxicity under in-vitro condition.

The siderophore thus produced was detected and confirmed by CAS Assay. The type of siderophore produced by *Pseudomonas fluorescence* was detected by Csaky and Arnow's Assay.

Catecholate type of siderophore was confirmed by Arnow's Assay of type detection.

The siderophore from the bacterium *Pseudomonas fluorescence* was used for the synthesis of AgNPs. This interaction between the silver ions and siderophore triggered a time dependent progressive change in color of the reaction mixture from pale yellow to brown, with absorption maxima at 412 nm, suggesting AgNP formation (Kalimuthu et al. 2008; Dane et al. 2016).

Further, antimicrobial activity of AgNPs was tested against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Aspergillus flavus*. The results on AgNP-dependent toxicity showed that *E.coli* was more sensitive to AgNP as compared to other strain.

This variable sensitivity of the individual microbial strain might be dependent on the physiology of individual organism. It was also demonstrated that nanoparticles produced through siderophore acts as plant growth promoter.

The plant growth promoting activity was checked by using the Tray Assay. The maximum growth was observed in the seeds prior soaked in silver nanoparticles. Thus, AgNPs acts as plant growth promoter.

As can be seen from **Figure 4.1**, **Figure 4.2** the synthesis of silver nanoparticles started during the first 24 h. The synthesis of AgNPs ceased after 24hrs but the production of AgNPs continued further reaching the final level of 30h of incubation. The synthesis of silver nanoparticles by *Pseduomonas fluorescence* thus, appeared to be time-dependent.

Overall, the study demonstrated biogenic synthesis of AgNPs by using siderophore exogenously produced by *Pseudomonas fluorescence*. The bacteria-based formation of AgNPs could be probably a self-protective mechanism of the bacterium against the toxic effects of silver. Thus, the biogenic synthesis of silver nanoparticles by using bacterial metabolite could be commercially exploited as an eco-friendly green method of metal nanoparticle synthesis.

CONCLUSION:

The preliminary investigation of the potential applications of silver nanoparticles produced using siderophore by bacterium *Pseudomonas fluorescence*was studied. The bacterium showed the production of siderophore in succinate media. Catecholate type of siderophore was detected. Biogenic synthesis of nanoparticles was done by the bioreduction of silver nitrate to silver nanoparticles by siderophore. Nanoparticles, thus possess antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Aspergillus flavus*. Furthermore, it was found that the nanoparticles can be used as a plant growth promoter.

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