IJCRT Publish Paper record is available at DOI: http://doi.one/10.1727/IJCRT.17173

Production of Metal Nanoparticles By Microbial Fermentation

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ABSTRACT: The development of rapid and reliable processes for the synthesis of nanosized materials is of great importance in the field of nanotechnology. In this paper, we describe a novel synthesis approach which is rapid, simple and "green" for the synthesis of metallic nanostructures of noble metals such as silver (Ag), by using culture supernatanant of *Bacillus subtilis, Aspergillus flavus, and Fusarium oxysporum*. We have worked with different strains and have derived Important conclusions about the most efficient, rapid and reliable strain for Nanoparticle synthesis. The nanoparticles were examined using UV-Visible Spectroscopy, and Transmission Electron Microscopy (TEM) analyses. The formation of nanoparticles by this method is extremely rapid, requires no toxic chemicals and the nanoparticles are stable for several months. The main conclusion is that the bio-reduction method to produce nanoparticles is a good alternative to the electrochemical methods.

KEYWORDS: Silver Nanoparticle, Culture supernatant, UV-Spectroscopy and Bioreduction

Introduction:

Nanotechnology, shortened to "**nanotech**", is the study of the controlling of matter on an atomic and molecular scale. Generally nanotechnology deals with structures sized between 1 to 100 nanometer in at least one dimension, and involve developing materials or devices within that size. Nanoparticles were used by artisans as far back as the 9th century in Mesopotamia for generating a glittering effect on the surface of pots. [1] The development of reliable processes for the synthesis of silver nanomaterials is an important aspect of nanotechnology today.

Nanoparticles are viewed as the fundamental building blocks of nanotechnology.

Nanobiotechnology is that branch of one, which deals with the study and application of biological and biochemical activities from elements of nature to fabricate new devices like biosensors points for preparing many nanostructured materials and devices. Their synthesis is an important component of the rapidly growing research efforts in nanoscience and nanoengineering. Nanoparticles from a wide range of materials can be prepared by a number of methods. Precursors from liquids, solid or gas phase are used for synthesis and assembly of nanoparticles or nanomaterials. Metal nanoparticles are typically produced on a small laboratory scale using methods such as chemical vapour deposition, irradiation or chemical reduction of metal salts. However, there is a growing need to prepare environmentally friendly nanoparticles that do not produce toxic wastes in their process synthesis protocol. To achieve this, scientists in the field of synthesis and assembly of nanoparticles are inclined to shift to benign synthesis processes, which happen to be mostly of a biological nature. Biological entities like microorganisms and living cells possess operating parts at the nanoscale level and may perform a number of jobs ranging from generation of energy to extraction of targeted materials at a very high efficiency.

Here, we are using novel methods for the synthesis of different metal nanoparticles by using various biological entities (micro-organism).

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Nanomaterials have played a vital role in various fields, which includes catalysis, paints, coatings and pigments, drug delivery, photonic crystals and electronics. Nanotechnology is one of the key technology having the ability to solve accompanying problems arises in various fields to which cannot be solved by commercial material. Besides that, they are in high demand for various applications such as surface treatment, pigments for nutritional or pharmaceutical use, in transmittance of UV light and polymer composites that block efficiently transmittance of UV light and polymer composites for gas transport, some environmental protections, flammability reduction.

Metal nanoparticles are widely used in Electronics and Material science Industry. Nanocrystals are used in light emitting devices (LEDs), in solar cells and in telecommunication amplifiers. Silver nanoparticles are used in cancer treatment. It is used in drug delivery.

Methodology:

Chemicals:

1. Silver Nitrate ($AgNO_3$) (HiMedia)
2. Silver Sulphate (Ag_2SO_4) (HiMedia)
3. Ethanol (Qualigens Ltd.)

Media and Strains:

Media:

1. Nutrient Broth (HiMedia)
2. MGYP media (Lab Prepared)
3. Luria bertini Broth (Lab Prepared)

Strains:

All strains were brought from National chemical Laboratory (NCL), Pune

1. *Bacillus subtilis* (2724)
2. *Fusarium oxysporum* (1043)
3. *Aspergillus flavus* (555)

Experimental procedure for silver nanoparticle synthesis by $AgNO_3$

By Using *Bacillus subtilis:*

Bacillus subtilis stock cultures were maintained by subculturing at monthly intervals. Growth medium used was Luria Broth (LB) medium (1% tryptone, 0.5%yeast extract, 1% NaCl, pH 7.5). 250 mL of Luria broth (LB) was prepared, sterilized, and inoculated with fresh batch of
the bacteria, *Bacillus subtilis*. The culture flasks were incubated for at 40ºC. After incubation period, the cultures were centrifuged at 5,000 rpm for 10 minutes and the supernatant and pellets were collected.

Extracellular biosynthesis of Ag nanoparticles using culture supernatant of *Bacillus subtilis:*

In a typical synthesis of silver nanoparticle extracellularly, 50 mL aqueous solution of 1 mM silver nitrate (AgNO3) and 3.5mM silver nitrate (test) was treated with 50 mL of *Bacillus subtilis* supernatant solution in a 250 mL Erlenmeyer flask (pH adjusted to 8.5). The whole mixture was put into a orbital shaker at50°C (160 rpm) for 8 days and maintained in the dark. Control experiments were conducted with aqueous solution of silver nitrate, to check for the role of bacteria in the synthesis of nanoparticles. The reduction of Ag+ ions was monitored by sampling an aliquot (2 mL) of the solution at intervals of 24 h. and measuring the UV-Vis spectra

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of the solution. Absorption measurements were carried out on a UV-VIS spectrophotometer. The spectra were recorded at room temperature using a one-centimetre quartz cuvette.
The transmission electron microscopy (TEM) analysis of extracellular synthesize silver nanoparticles were prepared by drop-coating biosynthesized silver nanoparticles solution on carbon-coated copper TEM grids. Samples were dried and kept under vacuum in IR lamp before loading them onto a specimen holder. TEM measurements were performed on a Transmission electron microscope operated at an accelerating voltage at 120 kV.

Results and Discussion:

Extracellular biosynthesis of Ag nanoparticles using culture supernatant of *Bacillus subtilis:*

Extracellular production of silver nanoparticles by the culture supernatants of *Bacillus subtilis* with aqueous silver nitrate solution, 1mM and 3.5mM was investigated.

- Change in colour:
  The test tubes of silver nitrate solution after exposure to culture supernatant of *Bacillus subtilis*. The appearance brown colour clearly indicates the formation of silver nanoparticles in the reaction mixture containing 1mM but there is no colour change in the reaction mixture containing 3.5mM. The characteristics brown colour of colloidal silver solution is due to the excitation of surface plasmon vibrations in the nanoparticle. Thus, it was evident that electron shuttle or others reducing agents released by *Bacillus subtilis* are capable of reducing silver ions to silver nanoparticles. In Standard solution (Aq. Solution of silver nitrate), there is no colour change. On the other hands, the reduction of silver ions did not occur in the absence of bacterial cells. This clearly indicates that the reducing agents that are released into the cultures of the aforementioned *Bacillus subtilis* are involved in the reduction process.

Fig.2 Supernatant of Bacillus subtilis before Incubation

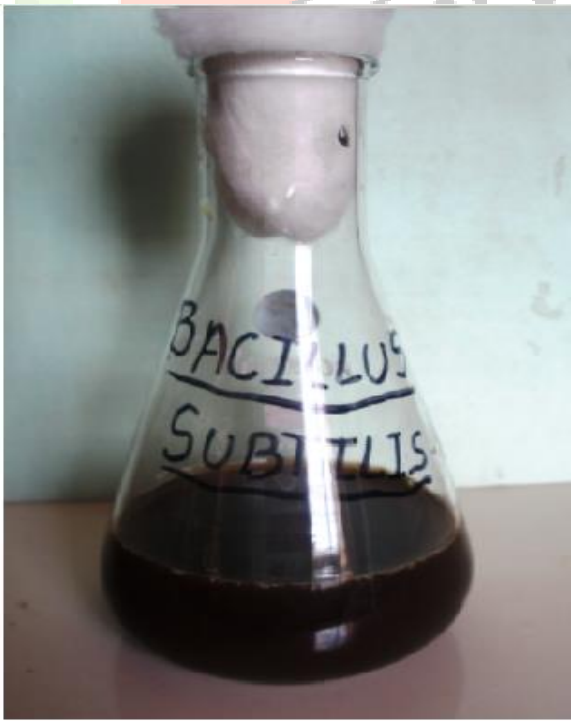

Fig.3 Supernatant of Bacillus subtilis after Incubation turns to brown

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- UV-Visible spectroscopy analysis:

  From the UV-Vis spectra recorded for the aqueous silver nitrate - *B. subtilis* reaction medium as a function of time it can be observed that the silver surface Plasmon band[7] occur at around 480 nm and steadily increase in intensity as a function of time of reaction. The surface plasmon band in the silver nanoparticles solution remains close to 480 nm throughout the reaction period, suggesting that the particles are dispersed in the aqueous solution with no evidence for aggregation.

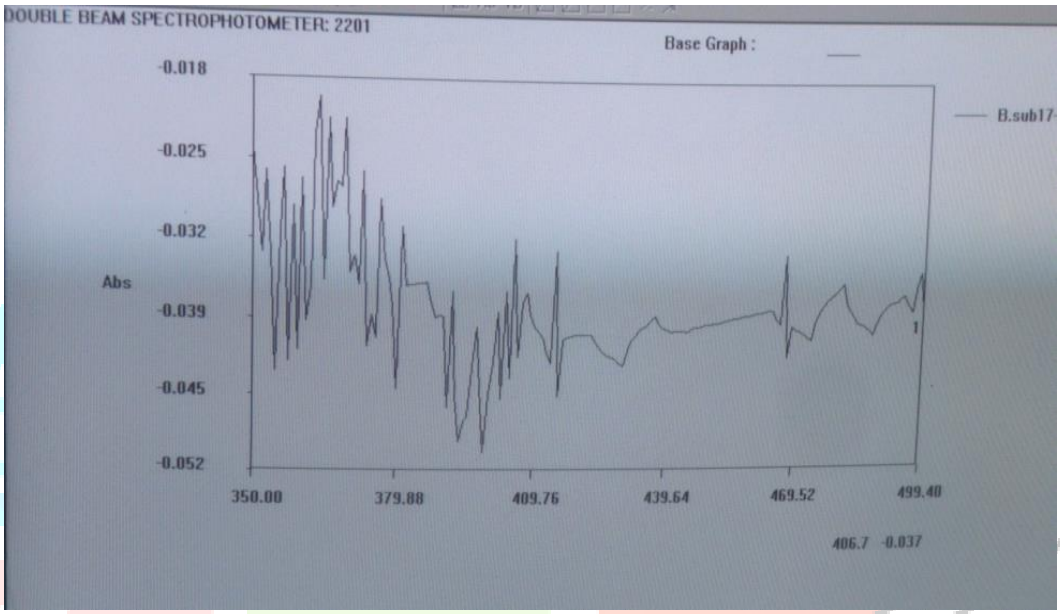

Fig.4 Graph- Abs vs wavelength after 1 day

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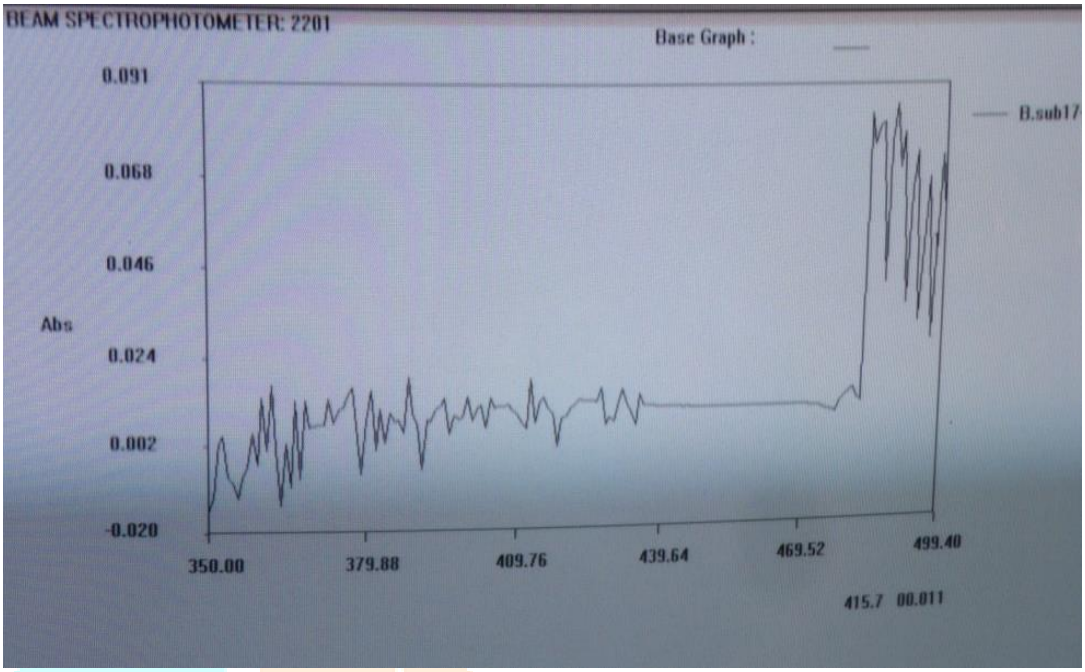

Fig. 5 Graph – Abs vs Wavelength after 6 days

- **TEM Analysis:**

A representative TEM picture recorded from the silver nanoparticle film deposited on a carbon coated copper TEM grid .This picture shows individual silver particles as well as a number of aggregates [8]. The morphology of the nanoparticles is highly variable, with spherical and occasionally triangular nanoparticles observed in the micrograph. Under observation silver nanoparticles in the size 16-50 nm.

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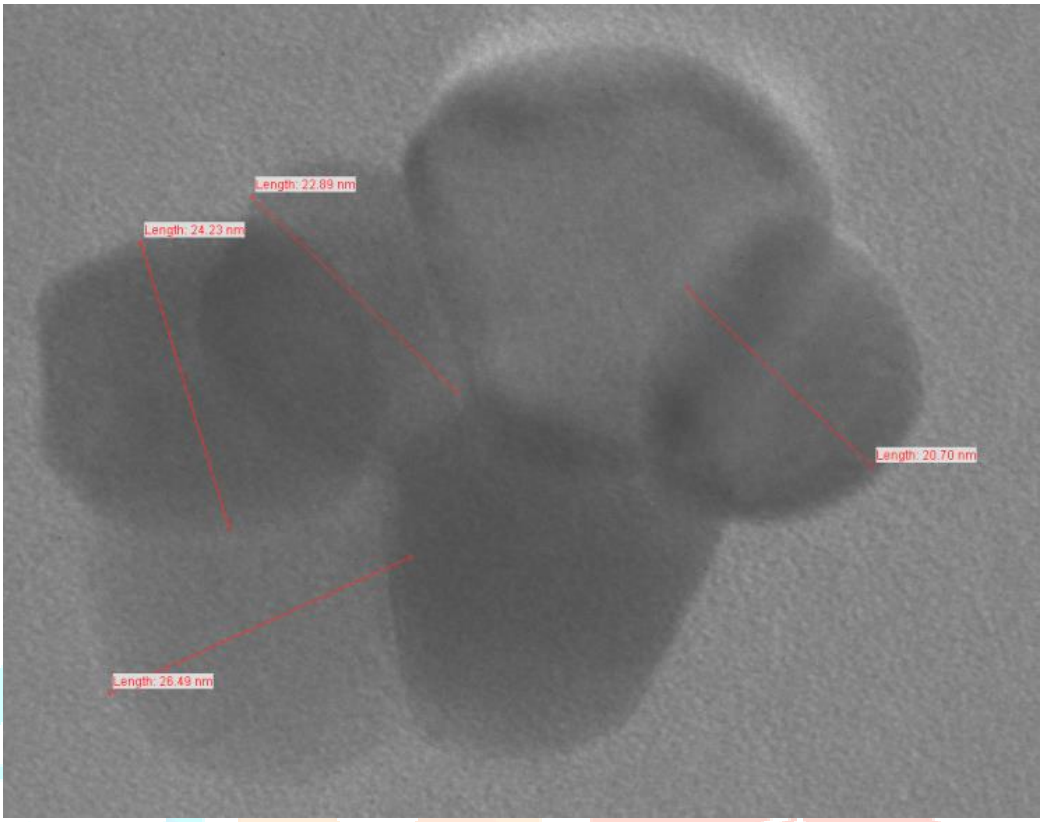

Fig. 6 TEM image of Silver Nanoparticles formed by Bacillus subtilis (Avg. size -20 nm, Shape-Spherical)

By Using *Fusarium oxysporum:*

Fusarum oxysporum stock cultures were maintained by subculturing at monthly intervals. The medium composed of malt extract 3 g, glucose 10 g, yeast extract 3 g and peptone 5g per in one litter of distilled water. The medium was designated as MGYP and autoclaved at 121±1ºC for 15 minutes. The fungus was grown in 500-ml Erlenmeyer flasks each containing 100 ml MGYP medium at 25±1ºC and180 rpm for 96 h. After 96 h of growth, mycelia were separated from the culture broth by centrifugation (3500 rpm) at 10ºC for 20 min and the settled mycelia were washed three times with sterile distilled water before use.

Extracellular biosynthesis of Ag nanoparticles using culture supernatant of *Fusarum oxysporum:*

In a typical synthesis of silver nanoparticle extracellular, 50 mL aqueous solution of 1 mM silver nitrate (AgNO3) and 3.5mM silver nitrate(test) was treated with 50 ml *Fusarum oxysporum* supernatant solution in a250 mL Erlenmeyer flask (pH adjusted to 6.0). The whole mixture was put into a orbital shaker at50°C (160 rpm) for 8days and maintained in the dark. Control experiments were conducted with aqueous solution of silver nitrate, to check for the role of bacteria in the synthesis of nanoparticles. The reduction of Ag+ ions was monitored by sampling an aliquot (2 mL) of the solution at intervals of 24 h. and measuring the UV-Vis spectra of the solution. Absorption measurements were carried out on a UV-VIS spectrophotometer. The spectra were recorded at room temperature using a one-centimetre quartz cuvette.
The transmission electron microscopy (TEM) analysis of extracellular synthesize silver nanoparticles were prepared by drop-coating biosynthesized silver nanoparticles solution on carbon-coated copper TEM grids.

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Samples were dried and kept under vacuum in IR lamp before loading them onto a specimen holder.TEM measurements were performed on a Transmission electron microscope operated at an accelerating voltage at 120 kV.

Results and Discussion:

Extracellular biosynthesis of Ag nanoparticles using culture supernatant of *Fusarium oxysporum*

Fig. shows Erlenmeyer flasks containing the cell-free filtrate of *Fusarium oxysporum* without (A) and with silver nitrate (B) after completion of reaction at 72 h.

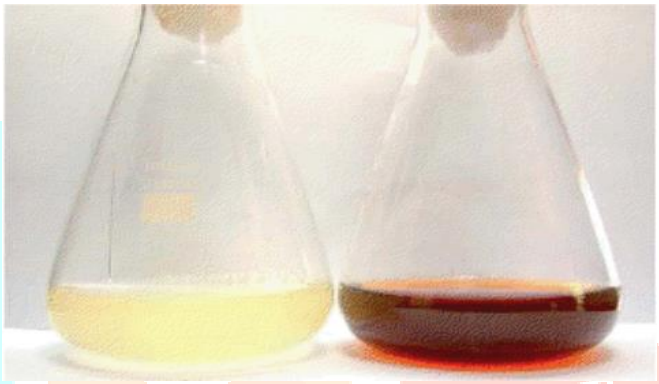

Fig.8 Supernatant of *Fusarium oxysporum* colour change from yellow to brown

- **Change in colour:**
  The flask containing silver nitrate solution showed gradual change in color of reaction mixture from colorless to brown with intensity increasing during the incubation period. The negative control (pure silver nitrate solution without cell-free filtrate) did not show the characteristic change in color indicating that the synthesis is not a thermal and temporal process.

- **UV-Visible spectroscopy analysis.**
  The bio-transformed products were simultaneously characterized by UV-Vis. Spectroscopy [7] measurements performed at different time intervals to study the change in light absorption profile of the solution and increase in intensity.

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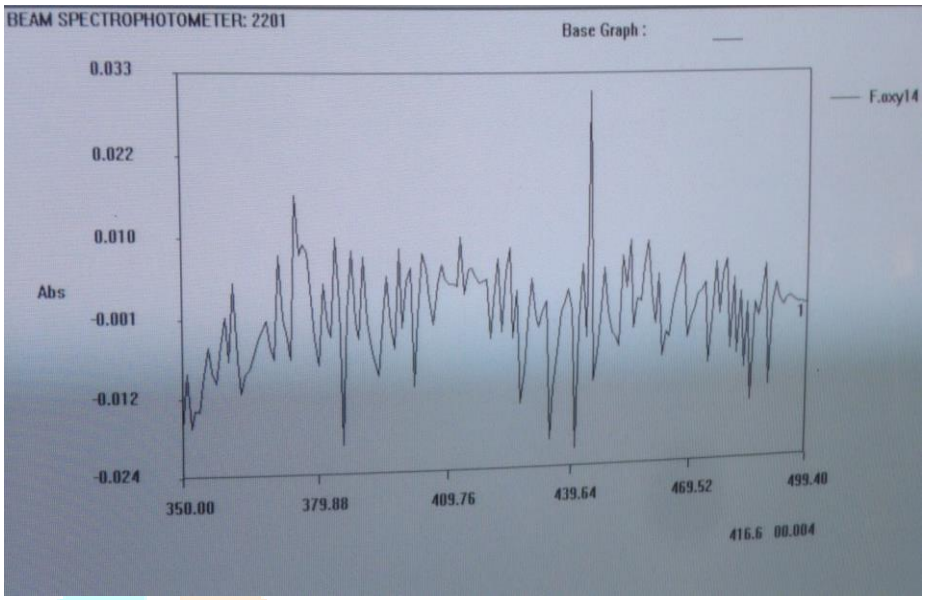

Fig.9 Graph – Abs Vs Wavelength after 1 day

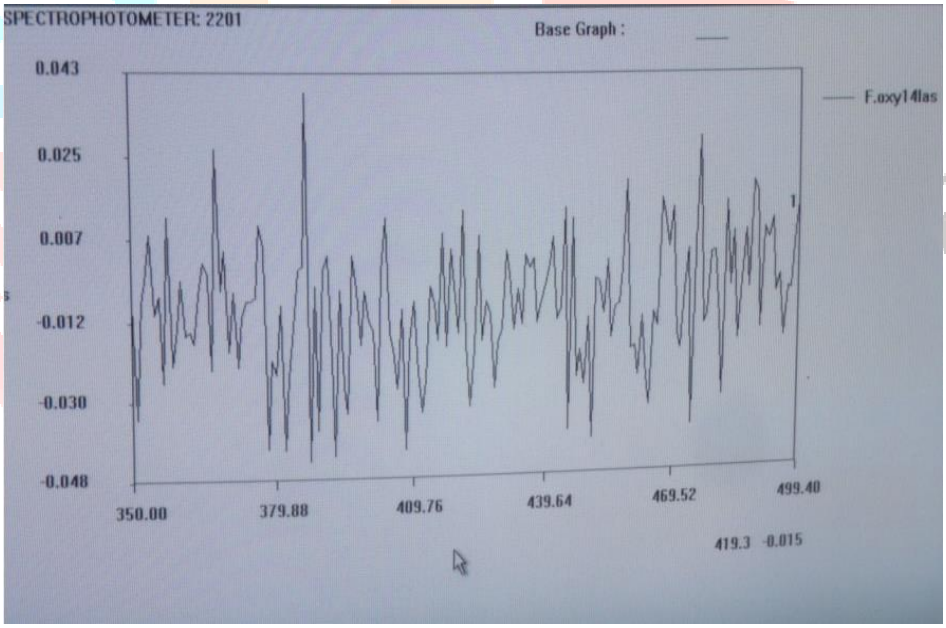

Fig.10 Graph – Abs vs Wavelength after 4 days

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- **TEM analysis**

  TEM measurements were used to determine the morphology and shape of nanoparticles.TEM micrographs (Fig. 6a) revealed that the particles are spherical in shape and uniformly distributed (monodispersed) without significant agglomeration [10].

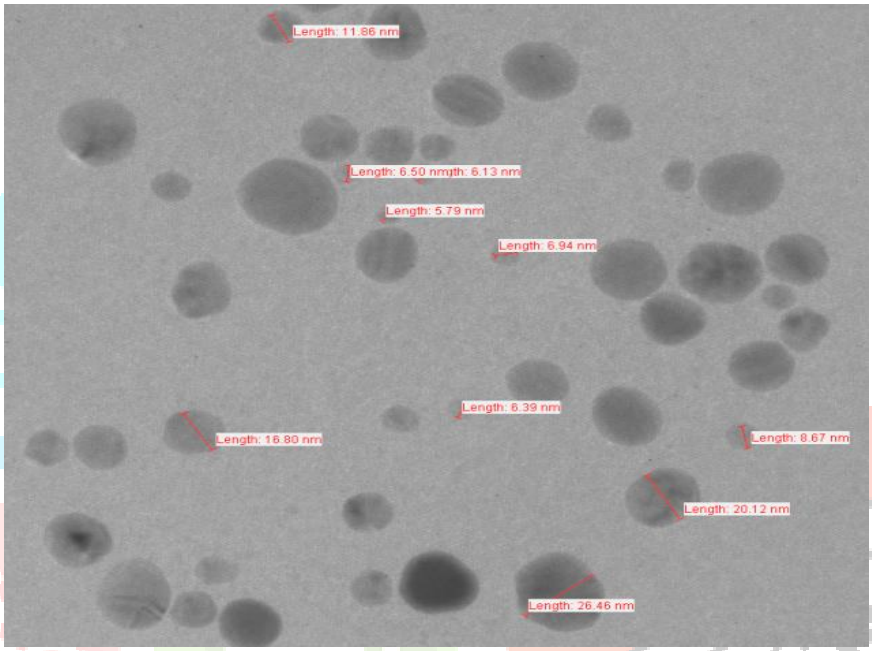

Fig.11 TEM Image of Silver Nanoparticles formed from *Fusarium oxysporum* (Avg. size-20nm, Shape-Spherical)

By Using *Aspergillus flavus*

Extracellular biosynthesis of silver nanoparticles using culture supernatant of *Aspergillus flavus*

The stock Culture of *Aspergillus flavus* (4 days old) was inoculated in 100ml of MGYP medium (0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5%peptone; pH 7.0) in 250 ml Erlenmeyer flasks. The inoculated flasks were then incubated at 28 C for 4 days on a rotary shaker (150 rpm). Fungal mycelium were separated from the culture medium by centrifugation (8000 rpm, 10 min, and 4 ^{o}C) and washed thrice with sterile water. Typically, 10 g of biomass (fresh weight) was resuspended in 100 ml of sterile water and further incubated for 72 h in an Erlenmeyer flask and agitated in similar conditions as described earlier. After incubation, biomass was separated by filtration using Whatman filter paper and the cell-free filtrate was obtained. For synthesis of silver nanoparticles, aqueous silver nitrate solution at a final concentration of 3.5 mM was added to the reaction vessels containing cell-free filtrate and incubated at 28 C on a rotary shaker (150 rpm) without light. Controls containing cell-free filtrate (without silver nitrate) as positive and pure silver nitrate

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Solution (without cell-free filtrate) as negative control was also run simultaneously along with the experimental.

Results and Discussion:

Extracellular biosynthesis of Ag nanoparticles by using culture supernatant of *Aspergillus Flavus:*

The picture of test tubes of silver nitrate solution after exposure to culture supernatant of *Aspergillus Flavus* is shown in the inset of Figure.

Fig.13 supernatant of *Aspergillus flavus* after Incubation changes to from yellow to brown

- **Change in colour:**
  The appearance brown colour clearly indicates the formation of silver nanoparticles in the reaction mixture containing 3.5mM. The characteristics brown colour of colloidal silver solution is due to the excitation of surface plasmon vibrations in the nanoparticles. Thus, it was evident that electron shuttle [4] or others reducing agents released by *Aspergillus Flavus* are capable of reducing silver ions to silver nanoparticles. In Standard solution (Aq. Solution of silver nitrate), there is no colour change. On the other hands, the reduction of silver ions did not occur in the absence of bacterial cells. This clearly indicates that the reducing agents that are released into the cultures of *Aspergillus Flavus* are involved in the reduction process.

- **UV-Visible spectroscopy analysis:**
  Change in color was visually observed in the silver nitrate solution incubated with *Aspergillus flavus*. The bioreduction of precursor silver ions was monitored. Absorption measurements were carried out on UV-Visible Spectrophotometer [7] at a resolution of 2 nm.

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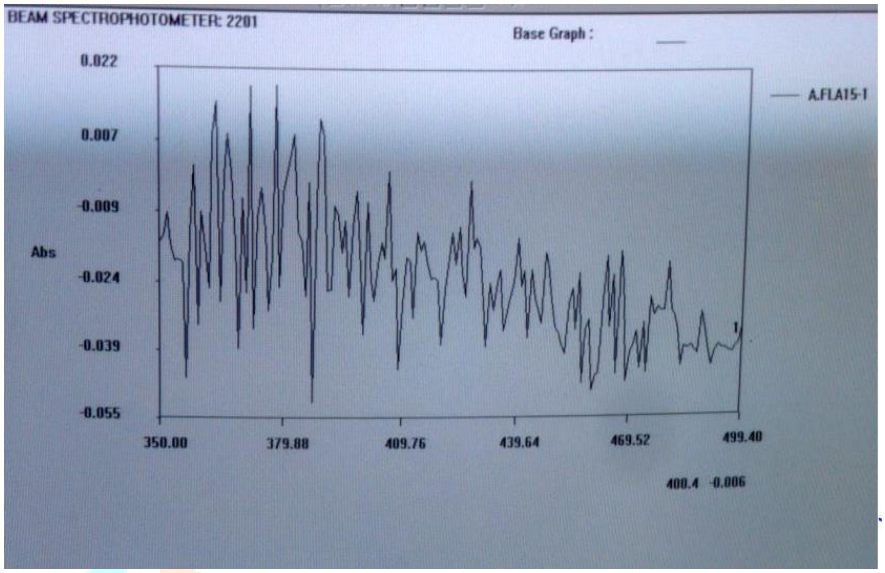

Fig.15 Graph Abs vs Wavelength after 1 day

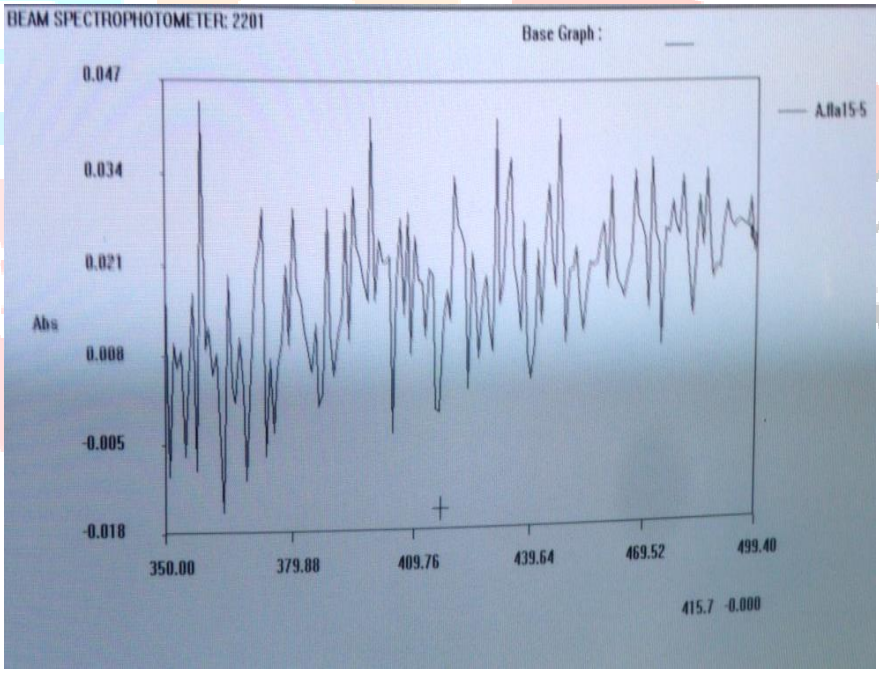

Fig.16 Graph – Abs vs Wavelength after 5 days

- **TEM analysis.**

For transmission electron microscope (**TEM**) measurements, a drop of solution containing synthesized silver nanoparticles was placed on the carbon coated copper grids and kept for air drying before loading them onto a specimen holder. **TEM** micrographs were taken by analyzing the prepared grids on **TEM** instrument using low voltage (100 kV). Transmission electron microscope (**TEM**) micrographs of the sample were taken using the JEOL-2100 **TEM** [9] instrument having selected area electron diffraction (SAED) attachment. The instrument was operated at an accelerating voltage of 200 kV.

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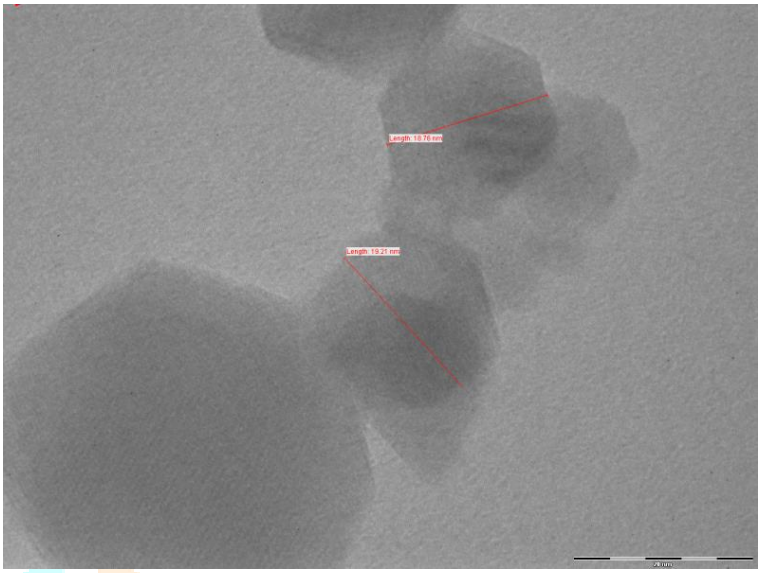

Fig.17 TEM image of Silver Nanoparticles from *Aspergillus flavus* (Avg.size-20nm, Shape-Hexagonal)

CONCLUSION:

1. Silver Nanoparticles were formed extracellularly in all the three species of micro-organisms used found after characterization under TEM with average size 20nm.
2. *Bacillus subtilis* and *Fusarium oxysporum* formed spherical shaped nanoparticles and *Aspergillus flavus* formed hexagonal shaped nanoparticles.
3. Both Bacteria and Fungus were used and it was found that there is rapid production of nanoparticles in fungus giving colour change from pale yellow to brown in 4 days while Bacteria giving the change in 8 days.
4. Fungus gave results in 3.5mM $AgNO_3$ and Bacteria gave results in 1mM $AgNO_3$ solution.

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