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Research Article

MYCOSYNTHESIS OF SILVER NANOPARTICLE BY *BEAUVERIA BASSISANA*

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ABSTRACT

In present study Entomopathogenic fungi *Beauveria bassiana* was used as biological agent of synthesizing silver nanoparticles that have tested for its antibacterial activity against different pathogenic bacterial strains .It showed maximum activity against *Escherichia coli (6mm)* followed by *Bacillus subtillis* (TCBS) (5mm),*Salmonella typhimurium* (XLD) (4mm) and *Klebsiella pneumoniae* (YC)(4mm) moderate activity. Characterization was done various techniques viz, UV-VIS spectroscopy, zeta potential and dynamic light scattering and relative values are obtained as follows 420 nm Surface absorbance peak ,-19.2 mV zeta potential value its assured the stability of nanoparticles and average size distribution calculated by DLS of 160 nm and SEM micrograph shows 25-35 nm sized irregular shapes nanoparticles.

Keywords: Nanoparticles, Entomopathogenic fungi, Antibacterial activity.

INTRODUCTION

Nanoscience has been recognized as an emerging field of science related to study of nano-size objects, nano materials and nanoparticles. The majority of researchers agreed that nanoparticles are clusters of atoms within size range of 1–100 nm (Nour *et al*. 2010). Among all metallic nanoparticles silver nanoparticles has gained attention because of their exceptional properties like good chemical stability, conductivity, catalytic activity depends on particle morphology, size distribution and coating of bio molecules. In recent times the point of interest antimicrobial activity has attracted researchers due to multi drug resistant problem. In this size range particles have high surface to volume ratio due to these nanoparticles gives antimicrobial activity (Khalil *et al*. 2013).

Synthesis of nanoparticles mainly involves conventional chemical and physical methods. It has some drawbacks like not ecofriendly, harmful effect and expensiveness. To aid this problem biosynthesis of nanoparticles are emerged as new way of fabrication method involving biological entities such as microorganisms like bacteria, fungi as well as plants (Bhattacharya and Rajinder, 2004).Among biological entities fungi is more advantageous as they can synthesize nanoparticles intracellular as well as extracellular and easy down streaming processing. Biosynthesis of nanoparticles has been reported by many researchers in recent times from fungi i.e. *Epicoccum nigrum* (Hafez *et al*. 2017), *Emericella nidulans* (Rajam *et al*. 2017),*Arthroderma fulvum* (Xue *et al*. 2016), *Metarhizium anisopliae* (Amerasan *et al*. 2016), *Neurosporacrassa* (Quester *et al*. 2016), *Aspergillus versicolor* (Netala *et al*. 2016).

MATERIALS AND METHODS

Maintenance of entomopathogenic fungal culture

Pure culture of entomopathogenic fungi *Beauveria bassiana* was kindly provided by Fungal Biotechnology and Invertebrate Pathology lab, Department of Biological Sciences, R.D. University, Jabalpur (M.P.). It was regularly sub cultured on Potato Dextrose Agar (PDA) (containing potato 200 gm, dextrose 20gm and agar 20 gm in 1 litre Distilled water) plates and slants and stored in refrigerator at 4ºC for further experimental use.

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Study of Microscopic Structure of *Beauveria bassiana* by Slide Culture Technique

Slide culture technique was used for study of morphological characteristics of the fungus on the basis of their microscopic characters. In this method a sterile moist chamber was prepared with thin cotton pad, wet filter paper and glass slide with cover slip inside a petriplate after assembly it was sterilized and along with PDA media was prepared and poured into the sterilized slide in the form of a thin film about 5 mm after solidification of film, film were cutted into small cubes. Cube was placed over slide after this fungi was inoculated in cube and covered with help of cover slip and incubated at 28±2 ºC. After the incubation period of 5 days slide and cover slip was placed individually on fresh slides each with drop of lacto phenol cotton blue. The stained fungal growth on slide and cover slip was observed under microscope (Chandrapaa *et al*. 2014).

Biosynthesis of Silver Nanoparticles

Biomass preparation

The fungus *Beauveria bassiana* was grown in 250 ml conical flasks containing 100 ml Potato dextrose broth (PDB) at 28°C for 7 days (Ramalingam *et al*. 2015). After 7 days of incubation 10 gms of wet fungal mycelium was isolated by filtration from PDB washed with sterile distilled water and used for synthesis of silver nanoparticles.

Extracellular synthesis of silver nanoparticles

7 days old harvested biomass was filtered through Whatmann filter paper No.1. The fungal mat was then washed with distilled water to remove media components and suspended in 100 ml distilled water for 96 hrs at 28±2ºC. After incubation, the cell filtrate was separated by filtration. Fungal cell filtrate also known as mycelia free water extract was collected and challenged with 1 m$MAgNO_3$ salt (Rehaman *et al*. 2011). This reaction mixture was then incubated at 28±2ºC for 72 hrs. After incubation this mixture was observed for a change in colour from colourless to brown.

Screening of Antibacterial activity of Synthesized Silver Nanoparticles

Silver nanoparticles synthesized by entomopathogenic fungi were tested for antibacterial activity *via.* , agar well diffusion method against pathogenic bacteria.

Agar Well Diffusion Assay

The silver nanoparticles (AgNPs) synthesized from *Beauveria bassiana* were tested for their antibacterial activity by well diffusion method against different pathogenic bacterial strains. The 20 µl suspensions of pathogenic bacterial cultures were seeded on Nutrient Agar Media (NAM) plates. Suspension of each bacterial strain was uniformly spread by spread plate method on separate plates. Wells of size 4 mm were made on NAM plates using sterile cork borer. Using micropipette, 80µl of the sample of nanoparticles solution was poured into wells on all plates. After incubation at 37ºC for 24hrs, the antibacterial activity was recorded in terms of diameter inhibition zone as measured by using Himedia Scale (Logeswari *et al*. 2012)**.**

Characterization of Biosynthesized silver nanoparticles

Nanoparticles have various physicochemical properties that are important for their behaviour, distribution, stability, and efficiency. Therefore silver nanoparticles sample synthesized by the entomopathogenic fungus *Beauveria bassiana* was characterized to evaluate their size, stability etc. which in turn determines their functionality. Characterization was performed using different techniques, including UV-VIS spectroscopy, Dynamic Light Scattering (DLS), Zeta Potential Analysis (Zhang *et al*. 2016).

UV-VIS Spectroscopic Analysis

This spectrophotometer analysis measures absorption (A) or optical density (O.D). Basically UV-VIS spectrophotometer is a device that based on Beer's law. According to this law the absorption is directly proportional to the number of molecules present in solution. Reduction of silver nitrate in the brown colloidal solution and the formation of silver nanoparticles (AgNPs) was preliminarily confirmed by visual observation of colour change from pale white to reddish brown and further confirmed by UV–Visible spectroscopy (UV 1800 spectrophotometer Schimadzu) at a resolution of 1 nm between 300 to 800 nm (which is a characteristic wavelength absorption range for silver nanoparticles). A small aliquot (2 ml) of the coloured suspended particles was taken in a quartz cuvette and observed for absorbance under wavelength ranging between 300-800 nm with distilled water as a reference (Hafez *et al*. 2017). The result is depicted in the form an absorption spectra showing an absorption peak at a specific wavelength.

Zeta Potential Analysis

The charge acquired by a particle or molecule in a given medium is its zeta potential and arises from the surface charge and the concentration and types of ions in the solution. Since particles of similar charge will repel each other, those with high charges will resist flocculation and aggregation for longer periods making such samples more stable. The charge or zeta potential of particles and molecules is determined by measuring their velocity while they are moving due to electrophoresis. Particles and molecules that have a zeta potential will migrate towards an electrode if a field is applied. The zeta potential is a key indicator of the stability of colloidal dispersion. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is small, attractive forces may exceed this

repulsion and the dispersion may break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate (Hanour *et al*. 2012). In the present research work the silver nanoparticles synthesized by *Beauveria bassiana* was analyzed laser Zeta Sizer (Malvern ZS 90, Malvern) to know the surface charge on the same which in turn determines its stability. For this the silver nanoparticles sample was diluted with double distilled water and place in sample holder for analysis. The end result of the analysis is exhibited in the form of a graph showing a peak at specific potential known as the zeta potential or surface charge of the particles.

Dynamic Light Scattering Analysis

The principle of dynamic light scattering is that fine particles and molecules that are in constant random thermal motion, called Brownian motion, diffuse at a speed related to their size, smaller particles diffusing faster than larger particles. The speed of Brownian motion is also determined by the temperature, therefore precision temperature control is essential for accurate size measurement. To measure the diffusion speed, the speckle pattern produced by illuminating the particles with a laser is observed. The scattering intensity at a specific angle will fluctuate with time, and this is detected using a sensitive avalanche photodiode detector (APD). The intensity changes are analysed with a digital autocorrelation which generates a correlation function. This curve can be analysed to give the size and the size distribution. To produce high quality data, the Zetasizer Nano series is designed to provide optimized components at every stage in the measurement chain from the laser and temperature control, through to the optical design and detector. The average size distribution of particles in the colloid was measured using a Malvern Zeta Sizer 90 instrument. Measurement parameters were as follows: a laser wavelength of 633 nm (He–Ne), a scattering angle of 173∘ (fixed without changing possibility), a measurement temperature of 25∘ C, a medium viscosity of 0.8872mPa·s and a medium refractive index of1.330, and material refractive index of 0.200. Before DLS measurement, the colloid was passed through a 0.2 μm polyvinylidene fluoride (PVDF) membrane. The sample was loaded into quartz microcuvette, and five measurements were performed, for which the mean result was recorded. DLS studies were carried out in two modes: general purpose mode (with normal resolution) and multiple narrow modes (with high resolution) **(**Tomaszewska *et al*. 2013). The outcome of this process was displayed in the form of a graph having two peaks. Average of both peaks was calculated and means size of synthesized silver nanoparticles was determined.

SEM analysis

SEM is type of electron microscopy that uses the secondary electron to form object images. For SEM analysis very minute amount of freeze dried was used in copper grid as specimen sample then it subjected to analysis at different magnifications and resolution. The scanning electron microscopy (SEM) analysis of freeze dried sample was performed by mounting nanoparticles on specimen stubs examined under ZEISS EVO 18SEM (Germany) at 15 kV. SEM analysis gives knowledge about nanoparticles topography and size distribution (Shaligram *et al*. 2009).

RESULTS AND DISCUSSION

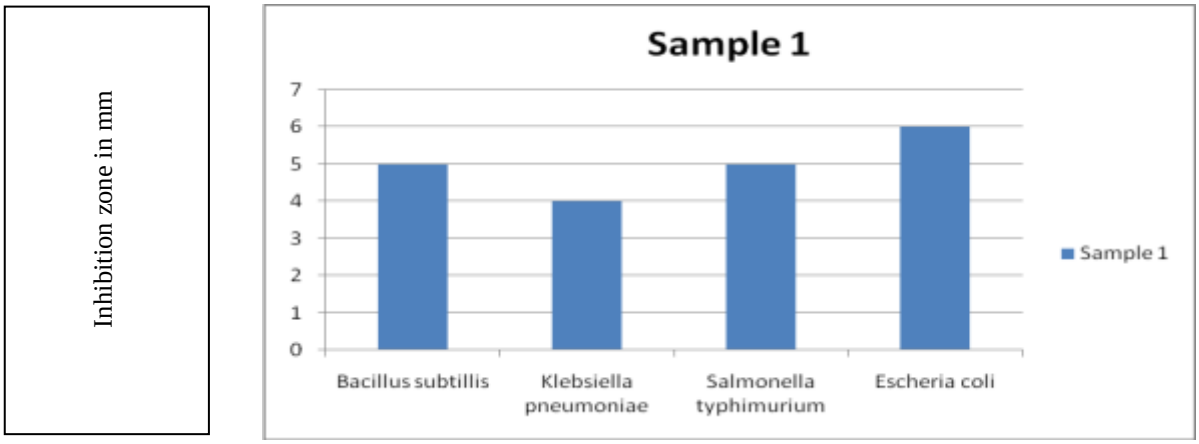
The pure culture of *Beauveria bassiana* was maintained on PDA slants at 4ºC by regular sub-culturing. The morphological appearance of pure culture of *Beauveria bassiana* showed following characteristics (Plate 1): According to visual appearance at PDA plates *B. bassiana* colonies were seen white in colour with a smooth powdery texture. Colony growth pattern was dispersed and not concentrical (Kulu *et al*. 2015). After incubation of 3 days when the slide of *Beauveria bassiana* was observed under the light microscope following characteristics were observed. It produced conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells. The conidiogenous cells of *B. bassiana* are short and ovoid, and terminate in a narrow apical extension called a rachis. The rachis elongates after each conidium is produced, resulting in a long zigzag extension. The conidia are single-celled and haploid. When the mixture of mycelia free water extract of *Beauveria bassiana* was subjected to 1 mM silver nitrate solution and incubated for 72 hrs at 28±2ºC the transparent colour of sample solution was converted into reddish brown (Plate 2). Amerasan *et al*, (2016) worked on an entomopathogenic fungus *Metarhizium anisopliae* based method of green synthesis of silver nanoparticles to control the rural malaria vector *Anopheles culicifacies*. Similarly, Ramalingam *et al*. (2015) studied the extracellular biosynthesis of AgNPs by an endophytic fungus, *C. lunata* on the basis of change in colour (dark brown) of the filtrate. Qian *et al*. (2013) also observed silver nanoparticles synthesis on the basis of colour change from colour less reaction mixture solution to yellowish brown. In the present study silver nanoparticles synthesized by *Beauveria bassiana* were screened for antibacterial activity by well diffusion method against 4 pathogenic bacteria i.e. *Bacillus subtillis (*TCBS*), Escherichia coli (*EC*), Salmonella typhimurium* (XLD), *Klebseilla pneumoniae* (YC). According to table 1 (Figure 1) highest inhibition zone was obtained against *Escheria coli* (6mm) followed by *Bacillus subtillis (*5mm), *Salmonella typhimurium* (5mm) and lowest against *Klebseilla pneumoniae (*4mm).

Devi and Joshi (2012) screened mycosynthesized silver nanoparticles from 53 fungal strains and reported significant inhibition *against S. aureus, Streptococcus pyogenes, Salmonella enterica* and *Enterococcus faecalis.* In a similar study done by Shukla and Sandhu, (2017) they tested antibacterial potential of silver nanoparticles synthesized by endophytic fungal strains *Alternaria* sp. against *Escherichia coli*, *Bacillus subtillis*, *Salmonella typhimurium*, *Enterococcus* sp. and *Klebsiella pneumoniae*. Synthesized nanoparticles primarily observed by changing

the colourless solution to reddish brown colour was further confirmed by UV-VIS spectroscopy while control solution has no colour change. The colour change obtained was due to reduction of silver ions into silver nanoparticles. Silver nanoparticles from fungus *Beauveria bassiana* showed maximum absorbance of 0.028 at 420 nm after 72hrs of incubation as a result of corresponding surface Plasmon resonance phenomenon (Figure 2). Soni and Prakash, (2012) synthesized silver nanoparticles by entomopathogenic fungus *Chrysosporium tropicum* and obtained maximum absorbance at 480 nm. Banu and Balasubramanian, (2014) obtained silver nanoparticles surface Plasmon absorption band at 420 nm from *Beauveria bassiana* isolated from infected *Hypothene mushampei* (coffee berry borer) Tamilnadu, India. Similarly Roy *et al*. (2013) reported 425 nm absorbance peak of biologically synthesized silver nanoparticles by *Aspergillus foetidus* that confirms reduction of silver ions into silver nanoparticles in their primary detection analysis of UV-VIS spectroscopy. Govindappa *et al*. (2016) worked on silver nanoparticles biofabricized by *Penicillium* species of *Glycosmis mauritiana.* They reported maximum absorption peak at 435 nm on their UV-VIS spectra during their studies. The zeta potential indicates stability of colloidal dispersions. This technique is used to determine the surface charge on nanoparticles. Previous studies showed that nanoparticles which have more negative or more positive zeta potential values are more stable. Zeta potential of mycosynthesized silver nanoparticles sample was -19.2 mV. This value confirmed the stability of nanoparticles at dispersed condition (Fig 3). In a similar work done by Ramalingam*et al*., (2015) the zeta potential of silver nanoparticles synthesized by fungus *Curvularia lunata* obtained -26.6 mV. Sonker *et al*. (2017) worked on *Nostac sp*.strainHKAR-2for the green synthesis of silver nanoparticles and they reported +1.80 mV zeta potential value due to capping of bio molecules at the surface of nanoparticles.

Table 1. Antibacterial analysis of mycosynthesized silver nanoparticles.

S.No	Name of sample	Antibacterial activity in terms of Inhibition zone in mm			
		Bacillus subtillis	*Klebsiella pneumoniae*	*Salmonella typhimurium*	*E. coli*
1.	Sample 1	5	4	5	6



Graphical representation of antibacterial activity of synthesized nanoparticles by *Beauveria bassiana*

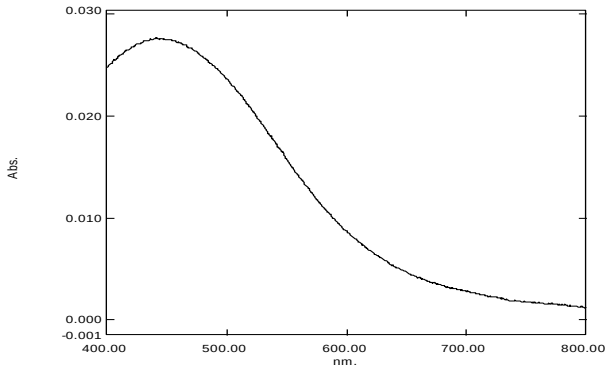


Figure 1. UV- Visible Spectroscopic Analysis of silver nanoparticles synthesized by *Beauveria bassiana*.

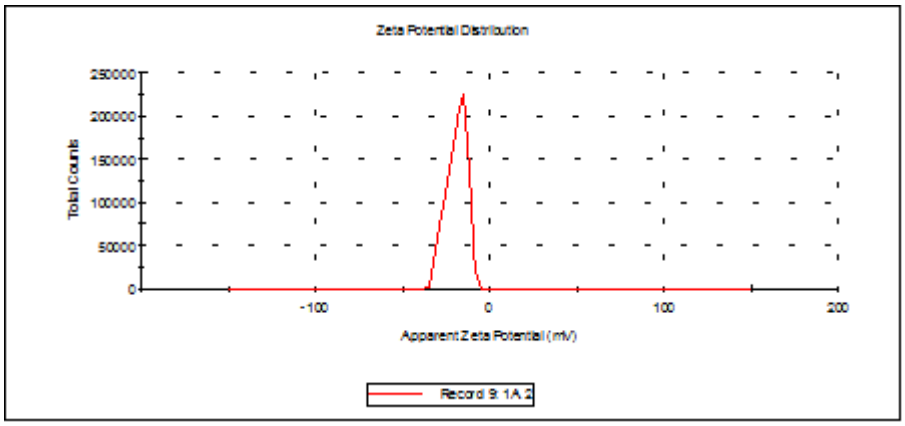


Figure 2. Zeta potential Analysis of silver nanoparticles synthesized by *Beauveria bassiana.*

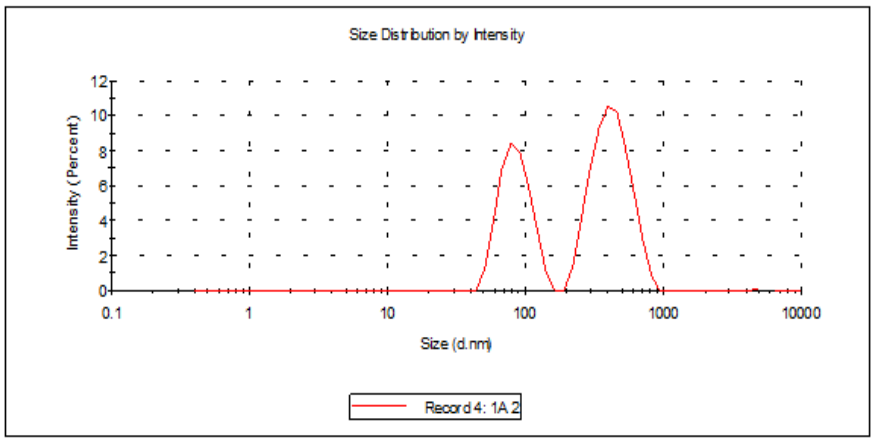
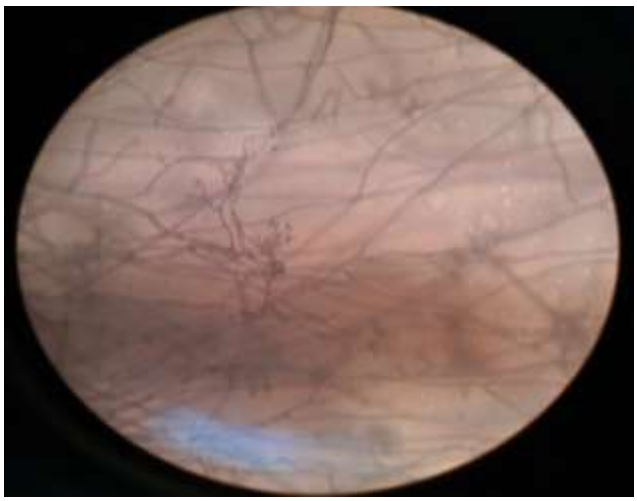
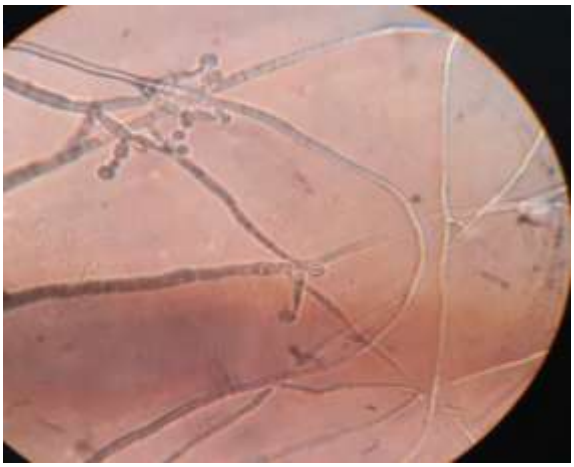


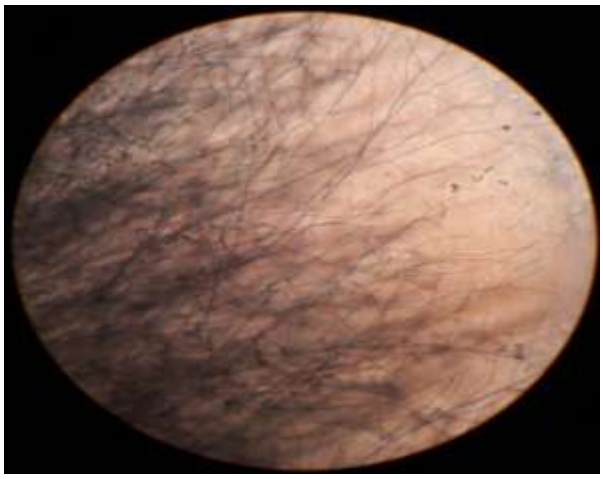
Figure 3. DLS Analysis of silver nanoparticles synthesized by *Beauveria bassiana.*



Pure culture of *Beauveria bassiana* on PDA plates. (A) 40X



(B)60X



(C) 100X

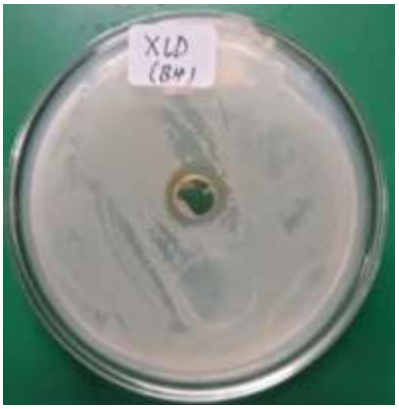
The purpose of applying this technique was determining the average size of biosynthesized silver nanoparticles. Malvern ZS 90 zeta sizer was used for this. As shown in fig 3, three peaks were found and the average size was calculated as 160 nm. Feng *et al* (2015) reported 35 nm average size determine by Dynamic light scattering obtained by extra cellularly synthesized silver nanoparticles using *Penicillium oxalicum*. Similarlily Jain *et al* (2011) worked on *Aspergillus flavus* for synthesis of silver nanoparticles and reported average particle size calculated using Dynamic Light Scattering measurements (DLS) to be 17±5.9 nm. According to SEM images obtained, silver nanoparticles synthesized in the present study were of irregular morphology with many shapes like square, rod and spherical. The SEM image also showed aggregation of silver nanoparticles. Their size was estimated to range between 25 to 35nm (Figure 5). Soni *et al* 2012 were repoted similar results of SEM micrographs of silver nanoparticles synthesized by pathogenic fungus *Chrysosporium tropicum* is a pathogenic fungus. The study confirmed the spherical shape and size between 20–50 nm of silver nanoparticles. Gopinath *et al* (2014) were reported similar results of SEM micrographs of silver nanoparticles synthesized by using fungus *Fusarium oxysporum* were roughly spherical to oval in nature with a little aggregation. The aggregation of AgNPs occurred during drying process (lyophilization) and size range between 16.5-70 nm.

Beauveria bassiana grown on PDB

Beauveria bassiana on DW

Brown Coloured Reaction Mixture of *Beauveria bassiana* obtained During Biosynthesis of Silver Nanoparticles.

Plate 2. Biosynthesis of silver nanoparticles by *Beauveria bassiana.*



Salmonella typhimurium

Bacillus subtillis

Escherichia coli

Klebeisella pneumoniae

Plate 3. Antibacterial activity of synthesized silver nanoparticles by *Beauveria bassiana.*

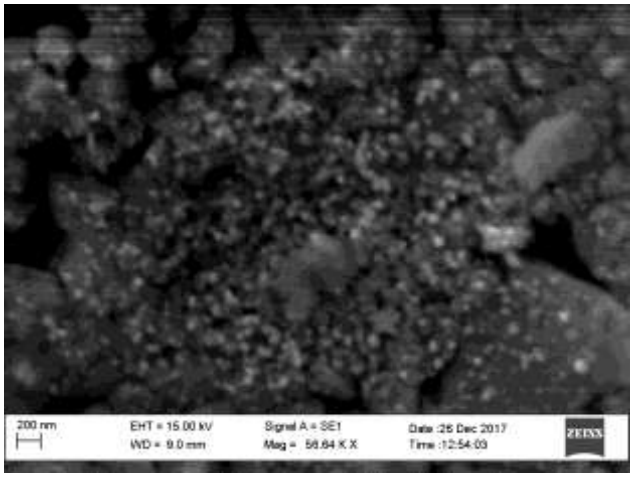


Plate 4. SEM Analysis.

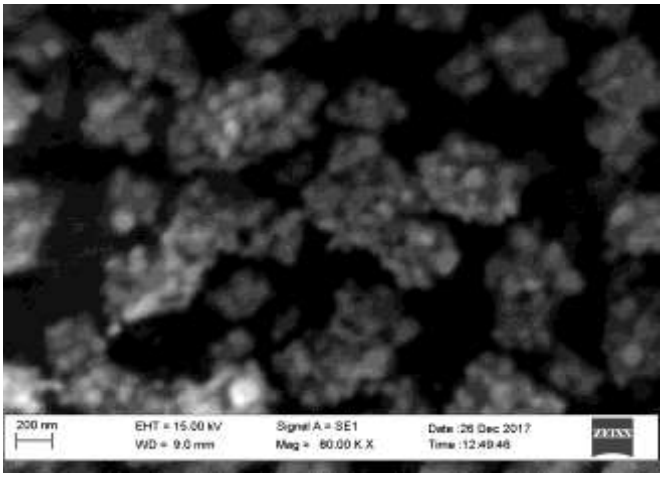


Figure 5. SEM image of silver nanoparticles synthesized using fungal extract of *Beauveria bassiana*.

CONCLUSION

Nanoscience is the new emerging field of science deals with study of nano sized objects i.e. nanomaterials like nanoparticles. Metallic nanoparticles are currently point of interest as potential reliable source of biomedical products substitute due to multi drug resistance problems. Nanoparticles are synthesized by both chemical and physical methods but they are not ecofriendly. This has resulted in the emergence of the need for development of reliable, eco-friendly processes for synthesis of nanomaterials. One approach that shows immense potential is based on the biosynthesis of nanoparticles using biological agents such as bacteria, fungi, plants etc. In all microorganism fungi are more advantageous to synthesize nanoparticles as compared to other microorganism. Fungi secrete enzymes and metabolites extracellular as well as intracellularly. Extracellular production is more beneficial as it is followed by an easy downstream processing and cost effective that attracts the industries. From above study it could be concluded that the Entomopathogenic fungus *Beauveria bassiana* has great potential to synthesize silver nanoparticles. The biosynthesized silver nanoparticles possess considerable antibacterial activity thus resulting in the inhibition of all pathogenic bacterial strains. Therefore, these particles could be developed as broad spectrum antibiotics in future and used as nanomedicine. Besides the Entomopathogenic fungus *Beauveria bassiana* could be subjected to strain improvement techniques for better yield of silver nanoparticles. Further these nanoparticles would be characterized by advanced techniques for illucidating details about their shape, size and functional groups attached.

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