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Biosynthesis and Properties of Silver Nanoparticles of Fungus *Beauveria bassiana*

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Abstract: Objective: The objective of this study was biosynthesis of silver nanoparticles (AgNPs) using *Beauveria bassiana* biomass and characterize them as fungi is very effective secretaries of extracellular enzymes, as culturing and keeping it in the laboratory is very simple . Extracellular secretion of enzymes offers the advantage of obtaining great quantities in a relatively pure state, free from other cellular proteins associated with the organism, and can be simply processed by filtering of the cells and isolating the enzyme for nanoparticles synthesis from cell-free.

Methods: The fungus was cultured in sterile conditions in the laboratory to obtain pure strain and reacted with aqueous Silver nitrate (AgNO3) for a period from January to march 2016 in order to convert the metal silver particles to silver nanoparticles, a surface Plasmon resonance band was observed at 234 nm in UV-vis spectrophotometer. The morphology and structure of synthesized AgNPs were analyzed by using UV-Visible spectroscopy, scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD).

The results: From the FTIR analyses showed that the AgNPs have large amount of C-C and O-H bonds. The average crystallite size of the calculated by Scherer's equation, it was 49 nm.

Conclusion: From the present study, it can be concluded that *Beauveria bassania* mycelia extract with neutral pH and appropriate temperature is an effective method in the synthesis of silver nanoparticles. Also, it is able to produce metal nanoparticles and nanostructure via reducing enzyme intracellular or extracellular. The current approach suggests that rapid synthesis of nanoparticles of silver nitrate would be appropriate for developing a biological process for mass scale production of formulations.

Keywords: Silver nanoparticles*; Beauveria bassiana*; green synthesis; FTIR ;(SEM).

INTRODUCTION

Nanoparticles are particles having one or more dimensions of the order of 100 mm or less, nanoparticles synthesize by a biogenic enzymatic process are far superior, compare to those particles produced by chemical methods. Despite that the latter methods are able to produce large quantities of nanoparticles with a defined size and shape in a relatively short time, they are complicated, outdated, very cost, inefficient and produce harmful toxic wastes that are dangerous, not only to the environment but also to human health. With an enzymatic method, the use of expensive chemicals is eliminated, and the more useful "green" route is not as energy intensive as the chemical method and is also environment friendly [1]. 'Green synthesis' is a procedure of synthesis and assembly of nanoparticles and has been used for a series of special production procedures. This

process, defined as the development of clean, non-toxic and environmentally acceptable ways which include organisms ranging from bacteria to fungi and even plants [2]. An important character of metal nanoparticles synthesis is their ability to remain dispersed in liquids without clusters due to the proteins in fungus filtrate play role as stabilizing and capping agents[3]. Microorganisms such as bacteria, actinomycetes, and fungi play an important role in the treatment of toxic metal through reduction of metal ions and are considered as potential Nano factories. Filamentous fungi are ideal candidates for environmental friendly synthesis of AgNPs [4]. A number of researchers have been experimenting on the microbes ,especially fungi like *Cladosporium tropicum, Aspergillus niger, Pencillium* sp. But few experiments have been informed using bacteria like *Bacillus thuringiensis*, to synthesize silver nanoparticles [5, 6]. The anamorphic entomopathogenic fungi *Beauveria bassiana* belong to the order Hypocreales (Ascomycota) which have a worldwide distribution as members of the natural soil flora [7]. *B. bassiana* is a cosmopolitan ascomycete fungus that is able to inhabiting a wide range of environments, including soil, insects, and plants. The fungus is able to live as a saprophyte in the soil, as an endophyte in plants, or as an entomopathogen affecting a wide range of arthropods [8].
In the present study, we have isolated, pure fungal strain for biosynthesis of silver nanoparticles and the synthesized AgNPs were characterized by using UV-Visible spectroscopy spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscope, (SEM) hot stage microscopy and Atomic force microscopic analysis(AFM).

MATERIALS AND METHODS

Chemicals

PDA was manufacturered in United Kingdom , Silver nitrate was manufacturered in Germany, Hydroxide sodium was manufacturered in Iraq, PDB was manufacturered in India. All the chemicals purchased were of analytical grade.

Preparation of broth and culture of *Beauveria bassiana*

The fungal *Beauveria bassiana* was obtained from the Directorate of Agriculture and Research / Ministry of Science and Technology. It was cultured on potato dextrose agar (PDA). The media was sterilized in the autoclave at 121ºC for 15min. After cooling it was supplemented with chloramphenicol 250mg/L as bacteriostatic agent. The fungal spores were taken from original cultures and inoculated in PDA and incubated for 7 days at 25ºC [9]. PDB broth was prepared in1000 ml Erlenmeyer flask and sterilized in the autoclave. It was allowed to cool broth and provided with chloramphenicol 250 mg/L then inoculated aseptically with a 5Mm actively grown culture disc of the fungus (5 discs per 250ml) and incubated for 2 weeks at 25ºC [10].

Synthesis of silver nanoparticles

The biomass were harvested after 2 weeks of the growth by sieving through Whatman No.1 filter paper followed by 3 times extensive wash with distilled water to remove any medium components from the biomass. Twenty grams of fresh and clean biomass were taken into an Erlenmeyer flask containing 500 ml of deionized water. The flask was incubated at 25ºC for120h. After incubation, the cell filtrates were obtained by passing it through Whatman No. 1 filter paper and later by Millipore filter 0.2 µm. Then it was brought in contact with 50mM of 100ml (AgNO3) (Fig.1).

Fig (1) Biomass of *Beauveria bassiana*

Molecular weight of $AgNo_3$

Ag=107.87, N=14, O=16 = 107.87 + 14 + 16 × 3 = 169.87. So the molar mass of AgNo3 is 169.87, therefor 1000 ml of 50 mM contains 169.87 × 50 /1000 = 8.4935 gm of $AgNo_3$ (Was added to 1000 ml of deionized water ,800 ml of this solution was added 200 ml of cell filters and heated at 60 c for 10 min and was shacked with magnetic hot plate stirrer and adjust PH to 7 by adding drops of NaOH solution, the flasks were covered with aluminium foil to prevent photoreaction of silver nitrate[11]. the flask was incubated at 25°C in a dark condition for 120 h, AgNPs turned brownish yellow color solution[12] .The control without adding AgNO3 was maintained under the same conditions, separately. The protein, enzyme and other compound existing in the fungal liquid work as reducing agents and are responsible for conversion of silver nitrate to silver nanoparticles (Fig 2). The reaction may be written as:

B. bassiana (fungal liquid) + silver nitrate solution $\frac{\text{Enzyme/protien}}{\text{reducing agent}}$ =silver nanoparticles.

The culture filterate was efficaciously used as straight bio-reductant to convert Ag+into Ag ° [13].

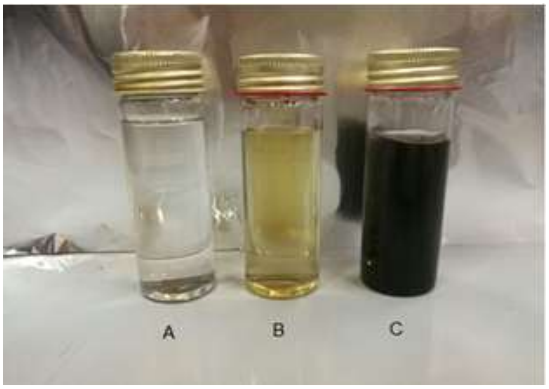

Fig (2) comparison between three types of solution.

A- 50mM AgNo3 solution. B-Fungal cell filtrate. C- AgNPs solution

RESULTS

(Fig.3) shows the 3D image and granulation characterization biosynthesized nanoparticles characterized using atomic absorption spectroscopy (AA-3000, Angstroms Advanced Inc. USA AFM contact mode) in the Lab of Baghdad University /Iraq [14]. The figure displays the 3D image and distribution chart for AgNP3 which placed on glass the average size is found to be around 89.99 nm and the roughness and the root mean square are 4.08nm, 4.97nm respectively. Also, the figure refers that the shape of this particle is semi rode distribution as matrix on the vertical axis, which means that the thin film is homogenous and uniform.

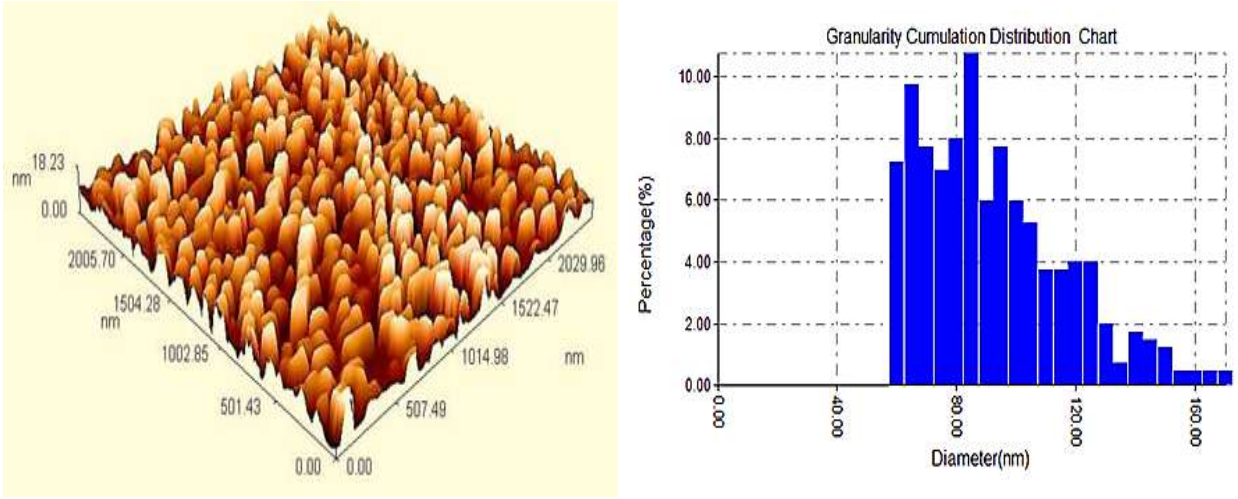

Fig (3): 3D AFM images of AgNPs

Fig (4) shows the solution of the developed nanoparticles of silver, which was centrifuged at 4000rpm for 60 min. The solid residues of AgNPs were mixed with deionized water and centrifuged at 4000 rpm for 60 min. After that, the drops of residue deposited on a slide and heated at 60ºC for 30 min. The phase variety and grain size of synthesized Silver nanoparticles was determined by Shimadzu X-Ray diffractometer (XRD 6000).The silver nanoparticles were studied with CUKα radiation at a voltage of 40 KV and current of 30 (MA) with a scan speed in 5.0000 (deg/min). The particle size of the prepared samples was determined by using the Scherrer′s equation as follows [15].

$$D = \frac{0.9\,\lambda}{\beta\ COS\ (\theta)} \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where D is the crystal size, λ is the wavelength of X-ray, θ is the Braggs angle in radians and β is the full width at half maximum of the peak in radians, the microstrain value ‘ε’ and the dislocation density ‘σ’ was evaluated by using the following relations[16].

$$\varepsilon = \frac{\beta\ cos}{4} \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

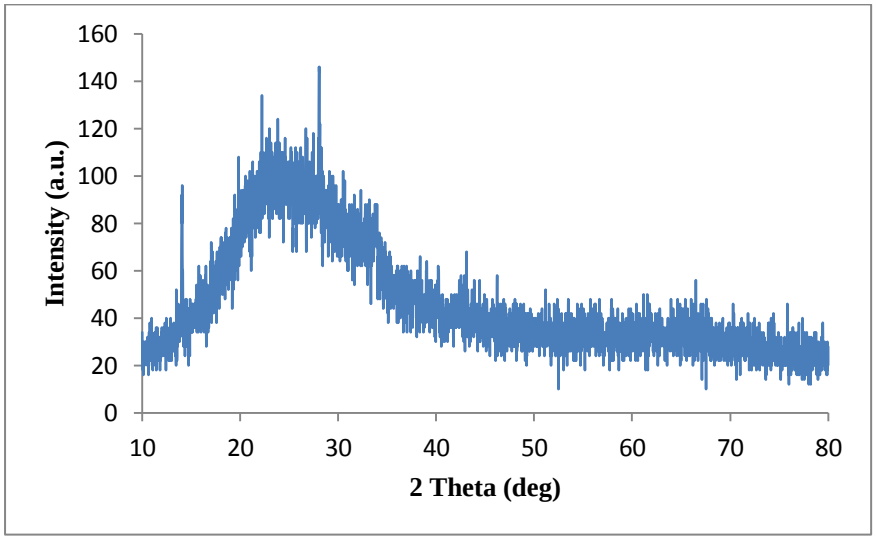

Fig (4): XRD pattern of (AgNPs) thin

Table 1: powder X-ray diffraction data of (AgNPs) thin film

2Theta (deg)	β (deg)	D (nm)	ε x10^{-4} lines^{-2}.m^{-4}	σ x 10^{14} lines/m^{2}
28.05	0.35	23.27295	14.88853	18.46279
14.03	0.33	24.19577	14.32068	17.0813
32.67	0.10	82.36097	4.20709	1.474202

The color intensity of synthesized AgNPs increased with duration of incubation, the color of the solution changed to dark brown after 120 h of incubation for the synthesis of nanoparticles. The bioreduction of silver ions in the solution was observed periodically by measuring the UV-Vis spectroscopy of the solutions. The reaction was rapid as the yellowish- brown color appeared within 120 h and the reaction confirmed the formation of AgNPs and there was no color change further. The optimum time required for the completion of reaction from our study was 120 h; It was observed that the reduction of silver ions reaches saturation within 48 h of incubation. (Fig. 5) Shows the UV-Vis spectra using Shimadzu UV-1800 spectrophotometer from 190nm to 1100 nm at regular intervals. Vis spectroscopy is used for the characterization of colloidal particles. noble metal particles possess strong Surface plasmon resonance (SPR) absorption in the visible region and are highly sensitive to the surface modification[17]. The AgNPs solution was exposed to ultra-sonication at room temperature and their surface plasmon resonance was recorded at 234nm.

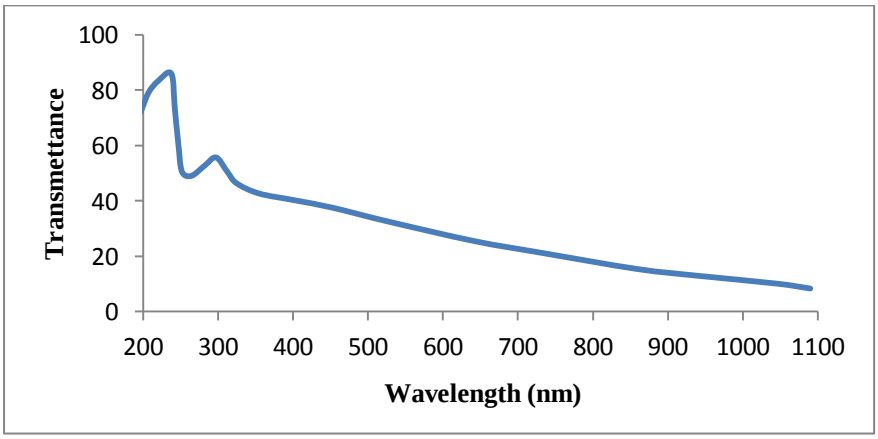

Fig (5): Transmittance spectrum of (AgNPs) thin film

For using the fundamental relation of photon transmission and absorbance, the absorbance (A) is defended as the logarithm (base 10) of the reciprocal of the transmittance

$$A=\log_{10}\frac{1}{T} \ldots\ldots\ldots\ldots (3)$$

T is the transmittance and A is the absorbance of the (Fe_3O_4). A thin film was prepared by chemical method and deposited by drop casting technique on glass. The reflection of the film has been found by using the relationship: (Fig. 6)

$$R+T+A=1\ldots\ldots\ldots\ldots (4)$$

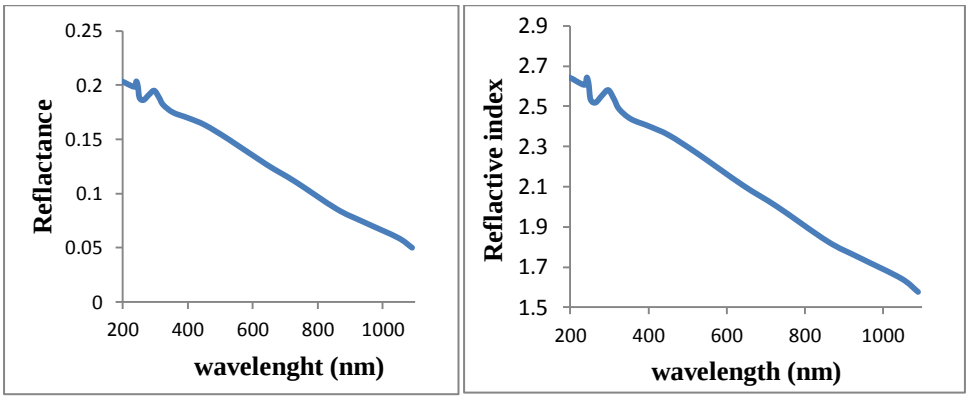

Fig (6): Reflectance index spectrum of (AgNPs) thin.

From the reflection *R* of the thin film, the refraction index can be calculated from the following relationship [18]: $n=\frac{1+\sqrt{R}}{1-\sqrt{R}} \ldots\ldots\ldots\ldots(5)$

The maximum value is 2.5. The optical absorption coefficient α was evaluated by tauc relation$\alpha hv= (hv-Eg)^n$ when$\alpha=2.303\frac{A}{t}$ Where *t* is the film thickness , *hv* is the photon energy, $Eg=\frac{1240}{\lambda\,(nm)}$ And *no*= 0.5 for allowing direct transition. Plotting the graph between *(αhv)2* versus photon energy *(hv)* provides the value of the direct band gap. The extrapolation of the straight line to *(αhv) 2* = 0, provides the value of the band gap s [19], shown in (fig. 7). The optical band gab is 4.95 eV.

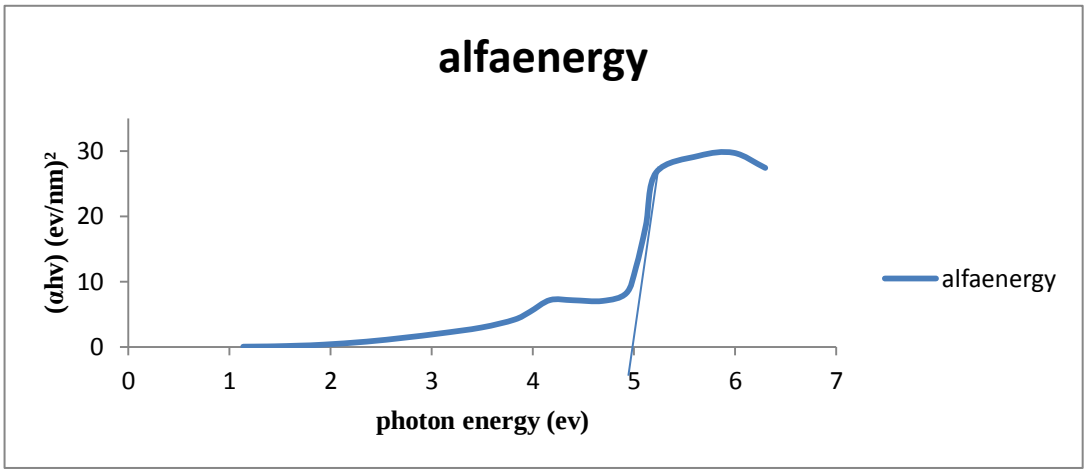

Fig (7): (αhv) versus photon energy plot of (AgNPs) thin film

FTIR analysis was carried out to identify the possible interactions between silver and bioactive molecules, (Fig. 8) Which may play role in the synthesis and steadiness (capping) of silver nanoparticles, FTIR spectroscopy was used to identify the functional groups of the active compounds depend on the peak value in the infrared region [20]. FTIR spectra obviously show that the biomolecules especially proteins prevailing in the filtrate are responsible for the synthesis and stabilization of silver nanoparticles [21].

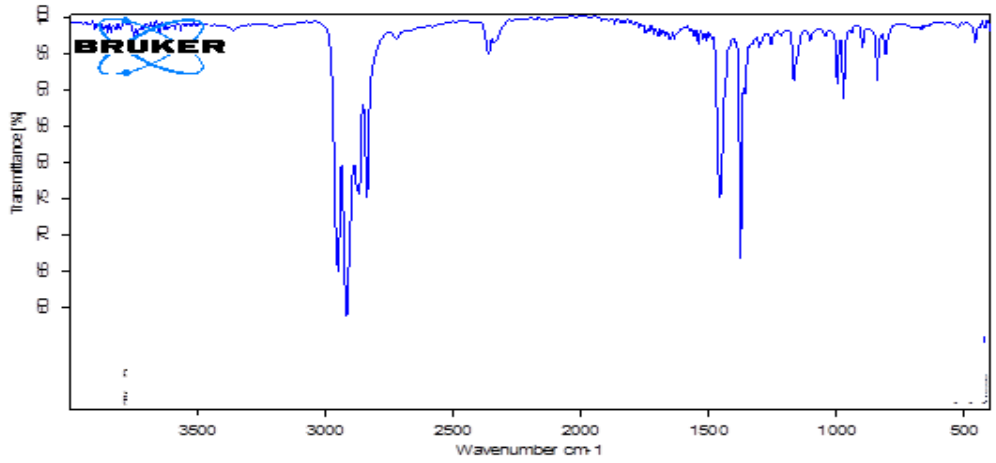

Fig (8): FTIR spectra of *Beauveria bassiana*

Fig (9) shows the FTIR spectra of (AgNPs) the peak at around 3300 cm^{-1} is from C-H SP^3 stretching modes, 3000 cm^{-1} is from C-H SP^2 stretching modes , 2300 cm^{-1} that corresponds to the bending vibrations of the O-H and 1600 cm^{-1} that corresponds to the bending vibrations of the C-C cm^{-1} which depend on the oxidation degree of (AgNPs) .

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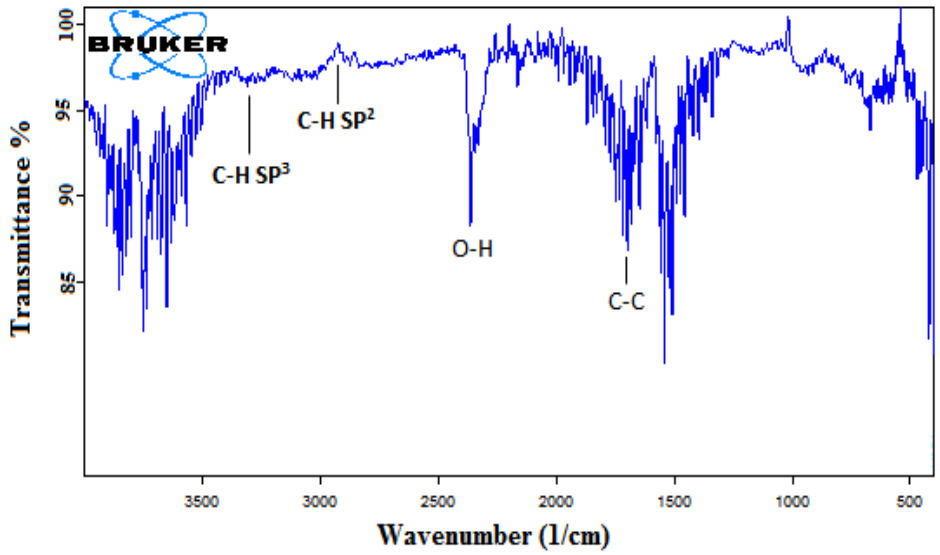

Fig (9) FTIR spectra of (AgNPs)

Scanning electron microscopy images offer further understanding about the morphology and size details of AgNPs. The silver nanoparticles (freeze-dried) were measured under a scanning electron microscope AIS2300C (Oxford instruments scanning electron micrographs enabled visualization and shape of the silver nanoparticles [22] (Fig.10). SEM analyses of the synthesized AgNPs clearly showed clustered and irregular shapes which magnified at magnification: 5x to 100,000x.

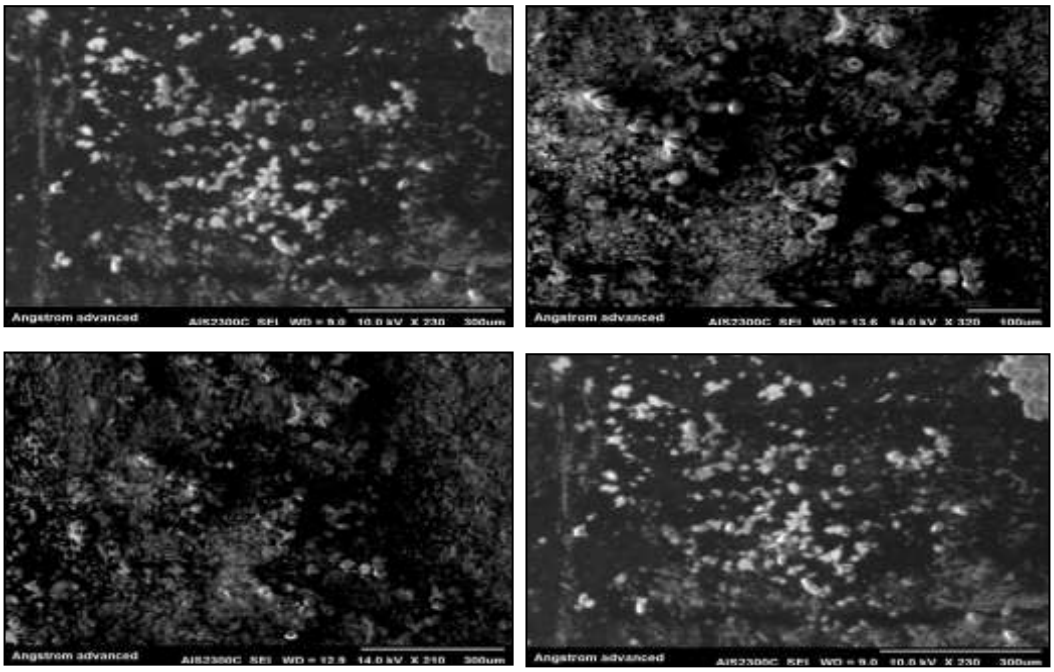

Fig (10): SEM image of AgNps (440 x)

Microscopy and thermal analysis of AgNPs was determined by using Lecia DM 2500 hot stage microscopy in the Central Service Laboratory, College of Education for Pure Science Ibn –Al-Haitham, as shown in (fig. 11).

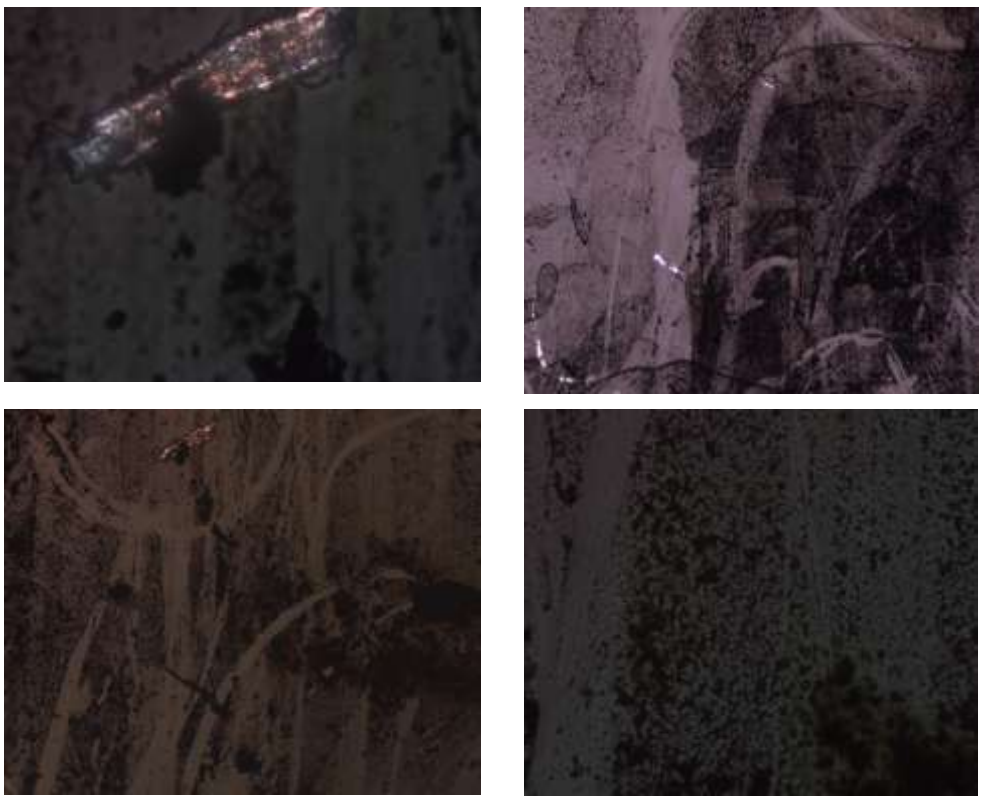

Fig (11) : Hot stage images of *Beauveria bassiana* silver nanoparticles 500x

DISCUSSION

In this study, AgNPs were synthesized extracellularly by *Beauveria bassiana* at room temperature. The AgNPs were quite stable without using any toxic chemicals as capping agents.The filtrated was treated with AgNO3; the reaction started after 24 h of incubation in dark condition,with a change in color of filtrate from pale yellow to brownish yellow, indicating the formation of silverbionanoparticles which correlated with the results obtained by[23] [24][25].In addition, they have given a characteristic band at 240 nm. Thus, it indicates the complete reduction of silver ions to turn yellowish brown color in aqueous solution due to excitation of surface plasmon vibration in silver nanoparticles [26]. the UV-Vis spectrum study on silver nanoparticles produced by the fungus,*A. fumigatus*. They found two absorbance peaks in the UV range corresponding to 220 nm (may be due to absorption by amide bond) and 280 nm (may be attributed to the tryptophan and tyrosine residues in the proteins) which indicated the secretion of some proteinic components into the medium by *A. fumigatus* [27]. In another study, investigated the extracellular biosynthesis of silver nanoparticles by *Fusarium oxysporum*. They reported that *F. oxysporum* secreted NADH-dependent reductase which was probably responsible for the reduction of silver ions and the formation of silver nanoparticles [28].However, the bioreduction of the Ag+ could be related with metabolic processes utilizing nitrate by reducing nitrate to nitrite and ammonium [29]. The reduction of silver ions by *F. oxysporum* strains has been attributed to anitrate-dependent reductase and a shuttle quinine extracellularprocess. The extracellular biosynthesis of silver nanoparticlesusing the filamentous fungus *Aspergillus fumigatus* has been explored [30] [31]. The pH was found to be an important parameter affecting AgNPs synthesis in *Beauveria bassiana*. At lower pH, protein structure gets affected and the protein becomes denatured and loses its activity so big size of nanoparticles NPs was observed [32]. The enzyme reductase catalyzing the synthesis is probably deactivated gradually as the conditions become alkaline, and this may be the reason for reduced synthesis and increase in size which is noticed at higher pH values. A similar conclusion is informed on AgNPs production by *Penicillium fellutanum* [33]. In the study of AFM, the morphology of synthesized sliver nanoparticles was found to be spherical shape In summary, *B. bassiana* has the ability to synthesize AgNPs. The biosynthesized AgNPs showed appropriate uniformity and stability when the substrate concentration50mM incubated at 25ºC with biomass weight 20 g at pH 7 with young biomass culture with 14 days for 5 days.

CONCLUSION

We have carried out production of silver nanoparticles as safe and economically viable by successfully synthesized using culture filtrates of *Beauveria bassania* with high stability, “green” method for nanoparticle synthesis, which is fast replacing traditional chemical syntheses, is of great concern because of eco-friendliness, economic visions, feasibility and varied range of many applications. Currently, various sorts of biological units which serve a dual role as both the reducing and stabilizing agents have been used. In the synthesis of bioactive nanoparticles, the mycosynthesis of silver nanoparticles is an effective protocol and completely safe. From the present study, it can be concluded that *Beauveria bassania* mycelia extract with neutral pH and appropriate temperature is an effective method in the synthesis of silver nanoparticles. *B. bassiana* is highly promising for the green, sustainable production of nano-metals, and also enhances its widespread application as an important strategy.

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