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PRODUCTION AND CHARACTERIZATION OF SILVER NANOPARTICLES SYNTHESIZED BY *FUSARIUM OXYSPORUM*

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ABSTRACT

In this study, three different fungal isolates; Fusarium solani, Fusarium oxysporum f. sp pisi and Fusarium oxysporum f. sp lycopersici from infected tomato plants showing symptoms of wilt disease were isolated, identified and tested for the production of silver nanoparticles (SNPs). Fusarium oxysporum achieved the synthesis of SNPs by extracellular method after growth on MGYP induction medium and incubation the mixture of cell free culture filtrate with aqueous silver nitrate solution.

Reduction of silver ions to silver atoms were visualized through the change in the color of mixture from pink to dark brown color with a peak at 420 nm corresponding to the Surface Plasmonic Resonance (SPR) of SNPs by UV-Vis Spectroscopy. The formation of a typical crystalline structure of silver atoms was confirmed by Selected Area Electron Diffraction (SAED) when compared to the data base of crystal patterns. The synthesized SNPs were figured out as a high stable solution by Zeta potential analysis which recorded +99.75. Transmission Electron Microscopy (TEM) showed spherical shaped SNPs with a size range 0.5 to 50 nm, the spherical SNPs were homogenous according to Poly Dispersity Index (PDI) which showed 0 .27 value in the size distribution homogenous range 0-1. Finally, it could be concluded that: the Egyptian isolate Fusarium oxysporum f. sp lycopersici is a suitable biofactory for positive charged nanosilver along with other isolates all over the world.

Keywords: *Nano-silver particles, Myco-nanotechnology, Fusarium oxysporum, TEM, SPR, PDI.*

INTRODUCTION

In nanotechnology, nanoparticles (NPs) are small objects that have a nano-sized dimension less than100 nm. Generally, NPs either formed through transfer of macro-sized material (bulk material) to nano-sized particles by Top Down method (Klaine *et al.,* 2008) or by Bottom Up method in which ions or atoms initiate the nucleation and growth of atoms to form a characteristic crystal exhibiting nano-sized dimensions controlled by a capping agent (Sundarakani *et al.,* 2010).

Nanoparticles have an interesting chemical and physical properties differing considerably from its bulk phase (Dimas *et al.,* 2009), these unique properties come from its high surface area / volume ratio that related to its very

small size. The physical and chemical properties of NPs depend on their size, shape, charge and structure of NPs (Gopalakrishnan *et al.,* 2012).

Physical, chemical and biological synthesis are three lines applied to obtain variable classes of nanoparticles from different metals such as Cd, Au, Ag, Cu, Zn and Fe (Tran and Le, 2013). The biological synthesis of nanoparticles is considered as an ecofriendly method in which nanoparticles are synthesized either inside (Intracellular) or outside (Extracellular) the biological systems by reduction of metallic ions (Badawy *et al.,* 2010).

Plant extract, bacteria, algae and fungi are known for the ability to synthesize many NPs with different sizes and shapes. By myconanotechnology, fungi can be produced in a bulk biomass more than bacteria because they contain higher amount of extracellular enzymes that acting as reducing agents and facilitating the biosynthesis of NPs. Also the laboratory handling of fungi is easier than other biological systems (Bhainsa and D'souza, 2006). There are several fungi like verticillium spp. (Mukherjee *et al.,* 2001), *Aspergillus fumigatus* (Bhainsa and D'souza, 2006), *Cladosporium cladosporioides* (Balaji *et al.,* 2009) and *Penicillium fellutanum* (Kathiresan *et al.,* 2010) were used to synthesize SNPs by extracellular Bottom Up method.

Although the biosynthesis of SNPs by *Fusarium oxysporum* were achieved from different isolates (not including Egyptian isolates) at different conditions (type of media, temperature, PH ,time) all over the world, the synthesized SNPs showed differences in size, morphology and charge Ahmad *et al.* (2003), Durán *et al.* (2005), Ishida *et al.* (2014), Selvi and Sivakumar, (2012).

The aims of the present work were first to test the capability of the Egyptian isolate *Fusarium Oxysporum f.* sp *lycopersici* for the synthesis of SNPs. Second we have focused on the favorable conditions for the synthesis of SNPs. Third, we analyzed the synthesized SNPs physically by U.V-VIS Spectroscopy and morphologically by TEM. Finally, studying the crystalline structure by SAED and tested the stability and homogeneity by zeta potential analysis.

MATERIALS AND METHODS

Mycological study:

1.1 Isolation and culturing of Fungi

Endophytic fungi were isolated according to Merchant *et al.* (2007), from stems of infected tomato that exhibiting yellowing and wilting of leaves as well as browning of xylem vessels. Isolates were cultured and purified on PDA medium (potato extract 200 ml, Glucose 20gm, Agar 15 gm, distilled water to 1L) at 25^{o}C for 7 days.

1.2 Screening and identification of fungi:

The three endophytic fungal isolates (F*usarium* spp.) were tested for the production of nanosilver particles, then, the best nanosilver producing fungus was identified on the basis of morphological and biochemical characteristic by national center for fungi identification (El - Azhar University, Cairo).

1.3 Production of Nanosilver by Fusarium sp.

Biomass of *Fusarium* sp. was obtained by

culturing 6 mm fungal discs in 500 ml Erlenmeyer flasks, which contains 3 fungal discs in 100 ml of MGYP media (Malt extract 3gm, Glucose 10 gm, Yeast extract 3gm and Peptone 5gm) at 25±1°C and 180 rpm for 72 hr. The fungal mycelia were separated by filtration and extensively washed 3-5 times with sterilized distilled water. 20 gm of the fungal biomass in 100ml sterilized water was incubating at the same previous condition for 24 hr. after filtration with Whitman filter paper No1; the cell free filtrate is collected. Finally, 1mM $AgNO_3$ was added to cell free filtrate.

1.4. Ultra violet-Visible spectral study:

Samples from synthesized silver nanoparticles along with cell free filtrate as control to screen for nanosilver specific absorbance bands, two samples were measured on (Unicam UV-VIS. Spectrometer UV2) from 380-460 wave length range which including the nano-silver specific absorption range 400-450 nm (Maines *et al.,* 2009) .

1.5.Transmission Electron Microscopy (TEM):

Samples from synthesized nano-silver were characterized by Transmission Electron Microscope (TEM). Conventional carbon coated copper TEM grid (Type G 200, 3.05 μμ diameter, TAAP, U.S.A) was used. The sample was examined under TEM (Transmission Electron Microscope JEOL JEM-2100) working at 160 k.v. Several images were taken to obtain sufficient representations for shape and size of the synthesized nano-silver particles.

1.6. Zeta potential analysis and poly dispersity index (PDI):

The nanocolloidal solution stability is measured by zeta potential analyzer (Malvern Zeta size Nano-zs90), zeta potential magnitude refer to the stability degree of colloidal particles within solution, magnitude of zeta potential indicates the degree of electrostatic repulsion between adjacent similarly charged particles and the surface charge of SNPs. Poly dispersity index value refer to the size distribution homogeneity, the PDI value ranging from 0 and 1, as PDI value become higher than 1 as nanoparticle size distribution homogeneity decrease .

RESULTS

3.1. Identification of *Fusarium* spp.:

During the present work, three Fusarium spp. were tested for the production of SNPs, the best one was identified morphologically as *Fusarium oxysporum* f. sp *lycopersici*, this *Fusarium* sp. was isolated and cultured on PDA medium at 25°C for 7 days (Figure 1).

3.2. Production of Silver Nanoparticles:

The tested fungal sp was cultured in MGYP broth medium for production of fungal biomass; the fungal biomass has a reddish colored growth (Figure 2b) when compared with control flask (MGYP growth medium) which has a yellowish color (Figure 2a).

After incubation of *Fusarium oxysporum* f. sp *lycopersici* biomass with distilled water and further incubation of cell free filtrate with 1Mm silver nitrate, pink color raised (Figure 3b) then changed to brown color after 72 hrs (Figure 3a). This indicates the gradual reduction of silver ions into silver nanoparticles.

Fig. (1) : *Fusarium Oxysporum Lycopersici* growth after seven days incubation at 25°C on PDA medium, the aerial hyphal growth has a pinkish white colour.

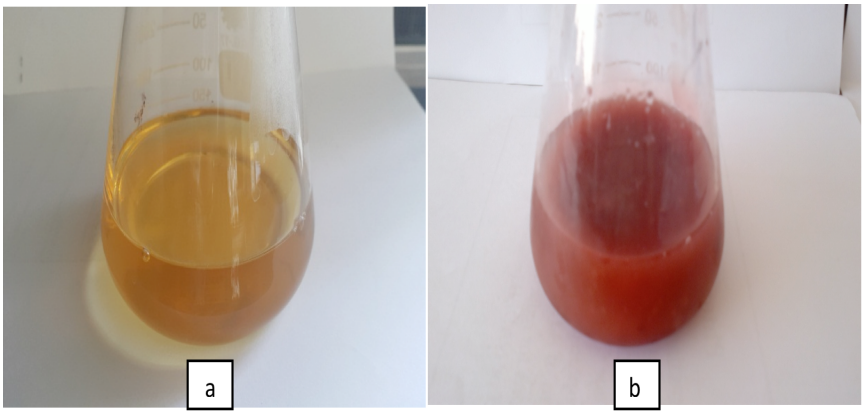

Fig. (2) : Mycelial mat formation in MGYP broth medium after incubation under agitated condition for 72 hours. (a) is a control flask (MGYP medium without inoculum), (b) is a biomass containing flask.

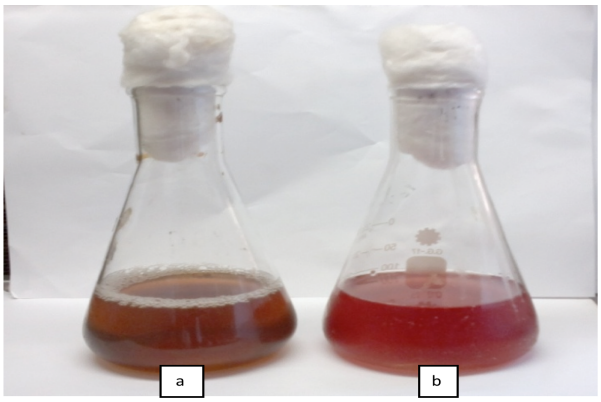

Fig. (3) : Fungal cell free filtrate (distilled water pre-incubated with biomass) has a pink color (b) and changed to brown colour after treatment with silver nitrate (a).

3.3 Characterization of Silver Nanoparticles:

3.3.1 Ultraviolet-Visible Spectral studies:

Nano solution was exposed to UV-Vis Spectral studies for screening to the best absorbance peak in nano silver specific range (400-450 nm), so the Plasmon peak was at 420 nm (Figure 4). At this characteristic wave length , the electrons of conduction band makes strong oscillation to give its characteristic brown color, that distinguish nanosized particles from macro sized particles.

3.3.2 TEM analysis:

The electron microscopy of silver nanoparticles showed spherical shaped nanoparticles exhibiting diameter ranging from 5 to 50 nm as illustrated in figure 5.

3.3.3 Zeta potential analysis:

The analyses of nanocolloidal solution by zeta potential analyzer is done for measuring its stability and surface charge, the measured zeta potential was +99.75 mv and the surface charge was positive, this indicated the higher stability of nanocolloidal solution over time because the value of zeta potential > 61 mv. PDI value was 0.270, this value fall in the homogenous size distribution range of 0-1, so size distribution of nanocolloidal particles was highly homogenous.

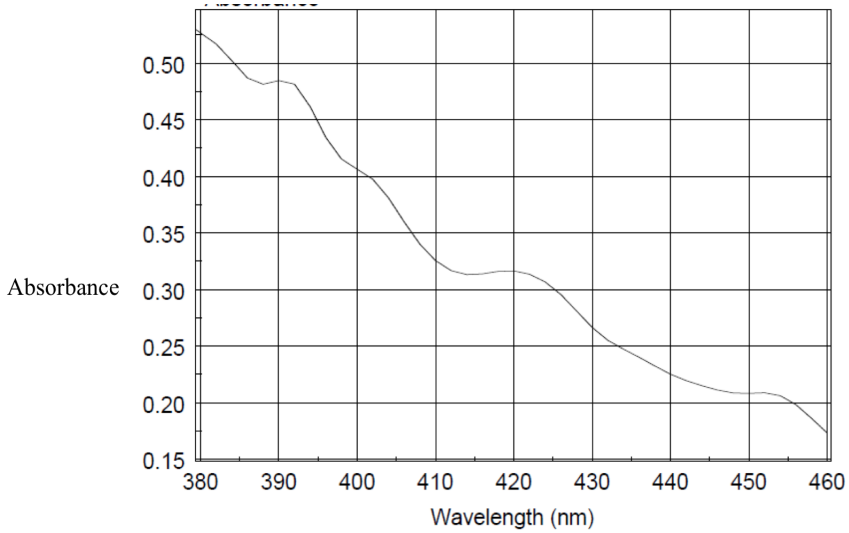

Fig. (4) : UV - Vis spectra of nano silver colloidal solution that is biosynthesized by *Fusarium oxysporum* after three days incubation from the figure the band was at 420 nm.

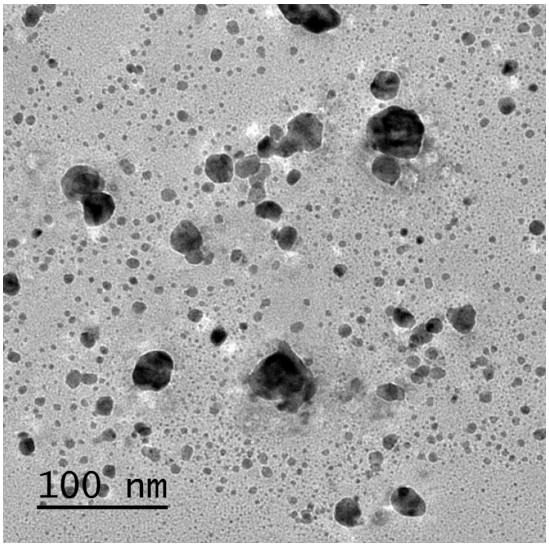

Fig. (5) : TEM micrograph show spherical shape of nano colloids and its distributing size is in this range (0.5-50) nm.

DISCUSSION

Silver nanoparticles (SNPs) are small objects exhibiting a size range from 1 to 100 nm and can be synthesized by three different methods which are chemical, physical and biological method. In the biological method, bacteria, fungi, yeasts and plant extract are the main sources for reducing agents in the biological synthesis of nanoparticles (NPs) from different metals such as Cd, Au, Ag, Cu, Zn and Fe. The biological synthesis of nanoparticles (NPs) starts with the reduction of ions to atoms by various reducing agents (Bottom Up method). Afterwards, the atoms were arranged in crystalline structure. Finally, the crystals were grown into nanoparticles. Fungi as a source of reducing agent in the biosynthesis of NPs is better than bacteria due to the easy handling and the high biomass of fungi besides the secretion of extracellular enzymes which facilitating the down-streaming process.

In this research , an attempt to replace the traditional chemical techniques by biological technique, as a result of some draw backs in physical and chemical methods so, the biological synthetic method has been developed to obtain size controlled nanoparticles, it is also an ecofriendly and inexpensive technique (Sadhasivam *et al.,* 2010).

The intracellular and extracellular enzymes secreted are responsible for the bio-reduction process (Prathna *et al.,* 2011), fungal spp. produce higher amount of protein than bacterial spp., thus resulting in a higher production of nanoparticles (Rai *et al.,* (2009) and Sastry *et al.* (2003).

Nanosilver is produced as a defensive method against the toxic effect of silver ions, silver ions at first are attached to cell surface and then penetrating the bacterial cell membrane, silver ions are rapidly reduced into sil-

ver metals by the action of redox enzymes as hydrogenase enzymes, nitrate reductase enzymes and NADPH dependent reductase enzymes, silver atoms are coalescence to give nanosilver crystals which are finally protected by capping agents that are responsible for their stabilizations, in extracellular method redox enzymes, naphthaquinones and anthraquinones derivatives are secreted into solution for reduction process (Mukherjee *et al.,* 2001).

Fusarium oxysporum is efficient in production of nanosilver particles due to the conjugation of nitrate reductase enzyme with electron shuttle mechanism with quinine; also it is characterized by production of anthraquinones and naphthaquinones derivatives.

Nanosilver shows visual changes in color of cell free filtrate from pink color to brown, this color related to Surface Plasmon Resonance (SPR), that distinguish nano-sized particles from macro-sized particles.

The confirmation for nano silver synthesis was done by UV-VIS Spectroscopy; nanosilver specific absorbance peak was centered at 420 nm, so this wave length confirmed the bio-synthesis of nanosilver as the peak fall in nanosilver specific range (400-450 nm).

TEM analyzed nanoparticles shape and size so the bio-synthesized particles were spherical-shaped and the size distribution range was from 0.5 to 50 nm, this size distribution range is highly homogenous as value of Poly Dispersity Index (PDI) is 0.270, this value fall in the standard size distributing homogenous range (0-1), silver nanoparticles stability are derived from capping agent that in turn derived directly from fungal proteins, this stability are confirmed by measuring of zeta potential value that was higher than 61 mv confirming the stability of nanocolloidal solution, nanosilver structure was studied by Selected Area Electron Diffraction Pattern (SAED), this analysis confirmed the bio-synthesis of silver nanoparticles. Also, this micrograph explained the crystal structure and the diffraction rings containing silver atoms as bright points.

Many studies discussed the bio-synthesis of SNPs by *Fusarium oxysporum* as, in one work silver nanoparticles were synthesized in size range of 20-50 nm, the SPR value was in the range (415-420) (Durán *et al.,* 2005), it is nearly the same value that obtained in my work also the obtained SNPs are spherical shaped. They confirmed the bio-synthesis process by energy dispersive spectroscopy which proved the presence of silver metals so silver ions are already reduced, the author also proved NPs stability by fluorescence emission spectroscopy, the appearance of a signal at 340 nm confirmed the attachment of silver crystals with capping proteins so this suspension is stable for several weeks, bio-synthesis process is relatively rapid as the bio-synthetic process is completed in 28 hr, this confirmed the higher amount of redox enzymes produced, (Ishida *et al.,* 2014) also synthesize SNPs using *Fusarium oxysporum* O7 SD that obtained from center for environmental science, university of Mogi das Cruzes, State of Sao Paulo, Brazil, this fungal strain synthesized SNPs in 60 days, the produced SNPs are varied in morphology and size, these particles are changed in morphology during the incu-

bation period from spherical shape to rod shape, also the size was varied during the incubation period, the synthesized SNPs are more homogenous in size and shape during the first five days than after 15 days. The rate of bio-synthesis is higher in the first three days and then decreased with time , the SNPs Surface Plasmonic Resonance was at 440 nm (Selvi and Sivakumar, 2012) are also synthesize SNPs with SPR 400-410 nm , the morphology and size were analyzed by TEM and SEM , the two technique proved the spherical shape of produced SNPs but differed in size, 5-50 nm in the first microscopy and 20-50 nm by SEM, FTIR analysis proved the presence of proteins in fungal filtrate. Other research was done by Pandiaraja (2010), in this work the produced SNPs had a variable shape but the size range was 20 to 70 nm, the produced SNPs have SPR peak at 440 nm, but in this work the size and morphology was analyzed by TEM, also the size distribution range homogeneity was studied by measuring PDI value, the NPs stability was confirmed by measuring zeta potential value and the crystalline structure was proved by SAED.

In conclusion *Fusarium oxysporum* is an excellent biofactory replacing the conventional chemical and physical techniques, so it can be used industrially at large scale for production of SNPs.

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الملخص العربى

انتاج وتوصيف جزيئات الفضة النانومترية المخلقة بالفيوزاريوم اوكسيسبورم

أشـــرف الســـيد خلـــود حشيـــش
عبد الدايـــم شريـــف

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جزيئات الفضة النانومترية هي عبارة عن جسيمات صغيرة يتراوح حجمها من ١ الي ١٠٠نانوميتر ,يتم تصنيع هذه الجزيئات بالطرق الكيميائية والفيزيائية والحيوية حيث تتضمن الطريقة الحيوية استعمال البكتيريا والفطريات والمستخلاصات النباتية ,يفضل استعمال الفطريات في عملية التخليق الحيوي لسهولة التعامل معها معمليا وانتاجها بكميات كبيرة مما يسهل من استعمالها علي نطاق اوسع كما تتميز الفطريات بانتاج الأنزيمات المخلقة لجزيئات الفضة النانومترية خارج الخليه مما يسهل من عملية التنقية بعد التصنيع لذا تم في هذا البحث استعمال فطر ال *Fusarium oxysporum* f. sp *lycopersici* في عملية التخليق الحيوي .

عند اختبار ثلاثة عازالات من فطر الفيوزاريوم من حيث القدره علي انتاج جزيئات الفضة النانومترية وجد ان فطرال *Fusarium oxysporum* f. sp *lycopersici* هو الافضل من حيث الانتاج . وعند اختبار قدرة هذا الفطر علي انتاج جزيئات الفضة النانومترية وجد ان لون مستخلص الفطرقد تغير بعد معاملته بنترات الفضة من اللون الوردي للون البني الغامق وهذا اللون هو تأكيد علي عملية التخليق الحيوي , وايضا أمكن التأكد من انتاج جزيئات الفضة النانومتريه عن طريق قياس ال Surface Plasmonic Resonance (SPR) وقد وجد ان هذه القيمه تقع في المدي الخاص بوجود جزيئات الفضة النانومترية والذي يتروح من ٤٠٠ الي ٤٥٠ نانوميتر. وعند دراسة الانتشار الحجمي والشكل الجزيئي لجزيئات الفضة النانومترية باستخدام الميكرسكوب الالكتروني النافذ وجد انها تتواجد بشكل كروي ذات حجم يتراوح من 0.5الي ٥٠ نانوميتر وعند فحص جزيئات الفضة النانومترية عن طريق ال Selected Area Electron Diffraction (SAED) وجد ان هذه الجزيئات بالفعل تأخذ الشكل البللوي المميز لجزيئات الفضة النانومترية وتعتبر هذه الجزيئات عالية الثبات لان قيمة ال Zeta potential تعدت ٦١ مما يؤكد علي ثبات هذه الجزيئات بمرور الوقت . نستخلص من كل التحليلات السابقة ان فطر ال *Fusarium oxysporum* f. sp *lycopersici* يعتبر مصنع حيوي جيد لأنتاج جزيئات الفضة النانومترية بديلا عن الطرق الفيزيائية والكيميائية القديمة.

JOESE 5

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