Controlled Biosynthesis of Silver Nanoparticles Using Culture Supernatant of Filamentous Fungus

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ABSTRACT: *The focus of this study was to evaluate the effects of some parameters influencing the size and size distribution of the silver nanoparticles (AgNPs) produced by culture supernatant of Fusarium oxysporum. Results revealed that in the reaction solution containing equal volume of silver nitrate and culture supernatant; pH, temperature, and light source can control the AgNP's characteristics. The particle size decreased with an increase in pH. The average size of AgNPs, formed in reaction solutions, decreased as temperature increased from 40 °C to 121 °C. The smallest AgNPs with the highest polydispersity (average size of 14nm and PDI of 0.37) were obtained in reaction solution incubated at 121 °C. Also, the use of UV radiation in reaction solution resulted in the production of the very small AgNPs with the narrowest size distribution (average size of 9.7nm and PDI of 0.2). X-ray diffraction analysis verified the crystalline nature of synthesized AgNPs. Also, transmission electron microscopy analysis confirmed the production of spherical shape nanoparticles.*

KEYWORDS: *Silver nanoparticles (AgNPs); Controlled Biosynthesis; Fusarium oxysporum; culture supernatant.*

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INTRODUCTION

Nanoparticles have attracted increasing attention due to their unique chemical, physical, optical, and electronic properties compared to their bulk counterpart [1-2]. Among nanoparticles, silver nanoparticles (AgNPs) are employed in wide ranges of applications such as antimicrobial agent, biomolecular detection, biological labeling, photonics, catalysis, etc. [3-4] There are various physical and chemical methods for the synthesis of nanoparticles. Using organic solvents and toxic reducing agents is common in conventional methods. Due to the environmental issues that can be caused by using toxic reagents, eco-friendly alternative synthesis methods have been come into focus [5-6]. Therefore, green methods based on utilization of non-toxic, environmentally benign solvents, and renewable materials for nanoparticle synthesis were explored [7-8]. Using biological entities such as bacteria, fungi, actinomycetes, algae, and viruses have been investigated as promising green methods for nanoparticle synthesis [9-11]. Filamentous fungi have some advantages over other microorganisms including higher secretion of enzymes and proteins and easy scale up for large scale production of nanoparticles [12]. There are reports on the use of some fungi such as *Aspergillus flavus* [13], *Fusarium oxysporum* (*F. oxysporum)* [14-16], *Aspergillus fumigatus* [17], *Cladosporium cladosporioides* [18], and *Neurospora intermedia* [19] for nanoparticle synthesis.

Because the AgNP's properties are highly dependent on their size and size distribution, the ability to control over these parameters is important and a major challenge of the AgNP biosynthesis [11]. The colloidal AgNPs produced using *F. oxysporum* culture supernatant has been characterized previously in our laboratory [15-16]. In this research, we focus on controlled biosynthesis of AgNPs and finding the optimum conditions for size and size distribution of nanoparticles using culture supernatant of *F. oxysporum* fungus. The effects of parameters such as temperature, pH, and light source on the size and size distribution of the AgNPs are discussed.

EXPERIMENTAL SECTION

Fusarium oxysporum strain and chemicals

Fusarium oxysporum (PTCC 5291) was obtained from National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. The pure culture was maintained on a Potato Dextrose Agar (PDA) and the resulting medium was stored at 4 °C. This strain was sub-cultured periodically to sustain its viability during the period of this study. All chemicals, including silver nitrate, hydrochloric acid, sodium hydroxide, and culture media were of analytical grade and purchased from Sigma-Aldrich Company.

Fungal growth conditions

F. oxysporum inoculum (10^7 spores/ml) was grown aerobically in MGYP media containing 0.3% (w/v) malt extract, 1.0% (w/v) glucose, 0.3% (w/v) yeast extract, and 0.5%(w/v) peptone. The pH of the media was set to 6.5±0.2, which is the optimal value for *F.oxysporum* growth; using 1 N HCl. Flask containing the inoculated medium was incubated on an orbital shaker operating at 28 °C and 200 rpm for 96 h. After the cultivation period, the fungal culture was centrifuged at 6,000 rpm for 20 min and the resultant supernatant was used for subsequent experiments.

Biosynthesis of AgNPs using culture supernatant

The separated culture supernatant was added to 1 mM and 2 mM silver nitrate solutions with volume ratios of 1:100 and 1:1, respectively. These ratios were selected based on the preliminary experiments. The mixtures were incubated at 28 °C and 200 rpm and their absorbance intensities were measured continuously until the maximum absorbance intensity at λ_{max} was met which corresponds to the completion of the biosynthesis of the nanoparticles.

Controlled biosynthesis of AgNPs

The effects of pH, temperature and light source on both of the size and the size distribution of AgNPs in reaction solutions with equal volume of culture supernatant and silver nitrate was evaluated. Also, the effect of pH in reaction mixtures with culture supernatant to silver nitrate volume ratio of 1:100 was investigated. In all reaction mixtures, AgNP formation progress was investigated using a spectrophotometer. No noticeable change in the absorbance intensities of a reaction mixture indicates reaction completion.

Effect of temperature

In order to assess the effect of temperature on AgNP formation, mixtures of silver nitrate solution and culture supernatant were subjected to different temperatures of 28, 35, 40, 50 and 121 °C.

Effect of pH

The effect of various pH values from 8 to 12 in AgNP formation, in the case of same mixing volume ratio, was also investigated. Furthermore, the pH of the reaction mixture with supernatant to silver nitrate volume ratio of 1:100 was set to 9. Finally, all reaction mixtures were incubated in an orbital shaker operating at 28°C and 200 rpm.

Effect of light source

To evaluate the effect of light source on AgNP characteristics, the reaction mixtures containing silver nitrate and culture supernatant were exposed to different light sources including a halogen lamp, a UV lamp, and sunlight.

Characterization of AgNPs

UV-Visible analysis

The appearance of color changes of the reaction mixtures was used as the initial evidence of AgNP formation. 1ml sample was taken from each flask containing colloidal AgNPs at regular time intervals and its absorbance spectrum was obtained using a double beam UV–visible spectrophotometer (Cary 100, Varian) with a resolution of 1nm in the range of 200–800 nm.

Dynamic light scattering (DLS) analysis

The size distribution and the average size of the synthesized AgNPs were determined by DLS (Malvern Instruments LTD., UK). Polydispersity indexes (PDI) determined by DLS experiments were used as an indication of particle aggregation. Values of the PDI change from 0.01 for monodispersed particles up to values of 0.5–0.7 for aggregated particles [20].

X-ray Diffraction (XRD) analysis

XRD was performed using a Philips PW-1730 system (Philips, Netherlands) operating at the Co Kα radiation wavelength of 1.7889 A°, 30 mA, and 40 kV. Sample was prepared by drop-casting of colloidal AgNPs on a silicon substrate.

Transmission Electron Microscopy (TEM) analysis

The morphological investigation of the produced nanoparticles was carried out using Zeiss EM-900 TEM (Carl Zeiss AG, Germany) operating at 50kV. TEM sample was prepared by drop-casting of the colloidal AgNPs on a carbon-coated copper grid and was allowed to dry at room temperature.

RESULTS AND DISCUSSION

The AgNP formation in all reaction mixtures was preliminarily detected by visual observation of the color changes of the reaction solutions. These color changes were attributed to the excitation of Surface Plasmon Resonance (SPR) in the metal nanoparticles [21]. The absorbance intensities of the UV-Visible spectra provide insight into the bioreduction of silver ions [22]. The SPR band appeared in UV-visible spectra is affected by the size and the shape of the synthesized AgNPs. Regarding the UV-visible spectra, the lower-wavelength region implies the formation of smaller nanoparticles [23].

AgNP biosynthesis using culture supernatant and silver nitrate with volume ratio of 1:100

The color of the reaction solutions containing culture supernatant and silver nitrate with volume ratio of 1:100 was changed more rapidly than the color of the solutions containing equal volume of silver nitrate and culture supernatant. In fact, no color changes were observed for the latter solution. The UV–visible spectras of the AgNPs synthesized at different time intervals, for both volume ratios of 1:100 and 1:1, are presented in Fig. 1. As shown in Fig. 1, for supernatant to silver nitrate mixing ratio of 1:100 , the spectral intensities steadily increased as a function of time (from 24h to 72 h), indicating the increasing number of AgNPs in the solutions. There is no noticeable change in the intensities of the spectra in this case after 72 h, which is the indication of the reaction completion. The influence of pH in this volume ratio on the synthesis of AgNPs was investigated by comparing the UV-Visible spectra of the reaction solutions with and without adjusting pH. As shown in Fig. 2, the peak area and height of the UV–visible spectrum obtained for the reaction solutions with pH adjusted to 9 were considerably.69 higher than that of the solution with the pH 6.5, which is a reaction mixture without pH setting. This finding shows that the higher pH leads to the higher AgNP productivity. The SPR bands are centered at 436 and 422 nm for spectra acquired from colloidal AgNPs formed in reaction solutions with initial pH values of 6.5 and 9, respectively. This finding reveals that increasing the pH of the reaction mixtures from 6.5 to 9 leads

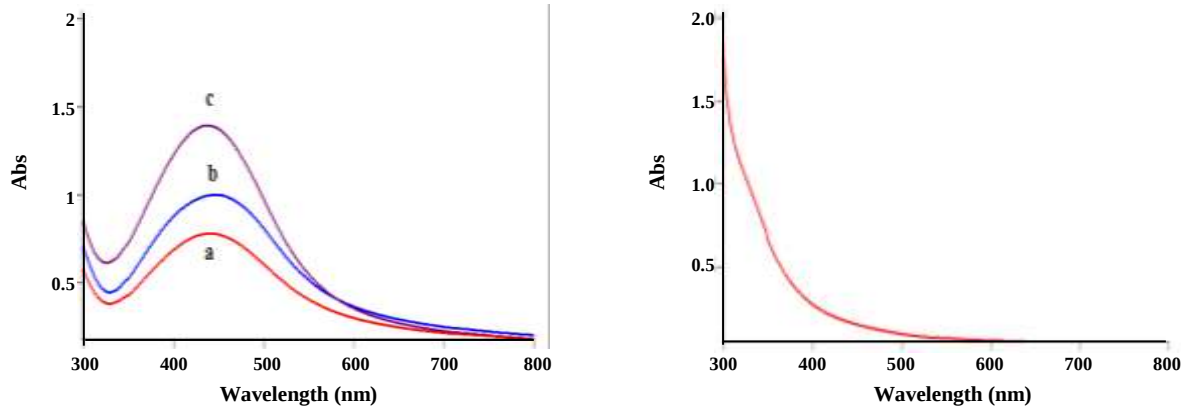

Fig. 1: UV-Visible spectra of colloidal AgNPs in reaction solutions containing A) 1:100 volume ratio of culture supernatant of and $AgNO_3$ at 24 h (a), 48 h (b) and 72 h (c); B) equal volumes of culture supernatant and $AgNO_3$.

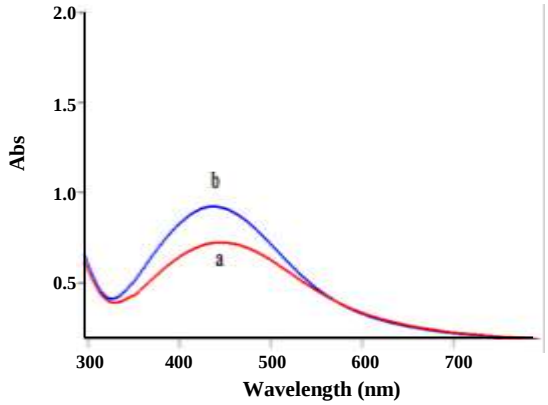

Fig. 2: UV-Visible spectra of the colloidal AgNPs produced in reaction solutions containing culture supernatant and $AgNO_3$ with volume ratio of 1:100 a) at initial pH of 6.5; b) at adjusted pH of 9; after reaction completion.

to the formation of smaller nanoparticles. Overall, it can be concluded that there is a strong relation between pH of the reaction solution and AgNP formation. The average particle size, size distribution, and Polydispersity Index (PDI) of the synthesized AgNPs were determined by DLS and the results are shown in Fig. 3. As illustrated in Fig. 3, the average sizes of AgNPs produced in reaction mixtures with initial pH values of 9 and 6.5 were 32 and 46 nm, and their PDI were 0.16 and 0.14, respectively. DLS analysis confirmed the results obtained from UV-Vis spectroscopy.

AgNP biosynthesis using equal volumes of culture supernatant and silver nitrate

As mentioned earlier, mixing of the same volumes of culture supernatant and silver nitrate solution led to no production of AgNPs. Regarding the effects of volume ratio of supernatant to silver nitrate on the synthesis of AgNPs, one can conclude that the performance of secreted proteins, as reducing agents, depends on the operating conditions. Therefore, the effects of factors, such as pH, temperature, and UV irradiation on AgNP formation were investigated only in the supernatant to silver nitrate volume ratio of 1:1.

Effect of pH on AgNP formation

The pH value of reaction solutions containing equal volumes of culture supernatant and silver nitrate solution was measured as 5.5. This acidic condition might be the cause of the secreted proteins efficiency loss. To prove this, the pH values of the reaction solutions were adjusted at various values of 8, 9, 10, 11 and 12. UV-Visible spectra of the colloidal AgNP in the reaction mixtures of supernatant and silver nitrate at different pH are presented in Fig. 4. The appearance of SPR bands in UV-Visible spectra at pH values higher than 8 verifies the pH-dependent performance of proteins in the mixtures as reducing agents which is in agreement with previous studies [24]. As shown in Fig. 4, with increasing pH values, the absorbance intensity increased and the λ_{max} of the spectra shifted to lower-wavelengths. Therefore, the mixture of the culture supernatant and the silver nitrate with the same volume ratio at pH 12 produced AgNPs with the highest productivity and the smallest size. These results are corroborated with the results of other researchers. The gold nanoparticle formation using both biomass and culture supernatant of *Coriolus versicolor*

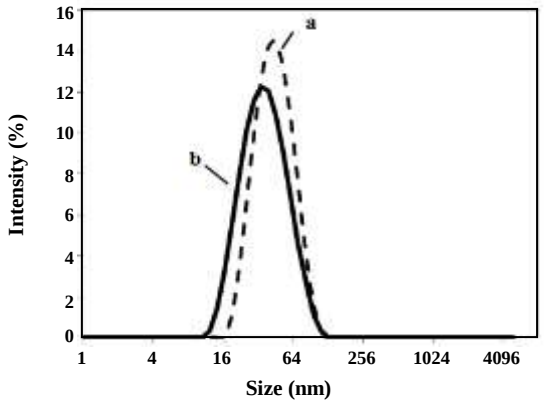

Fig. 3: DLS analysis of colloidal AgNPs generated by the mixtures of culture supernatant and $AgNO_3$ with volume ratio of 1: 100 at a) initial pH of 6.5; b) initial pH of 9.

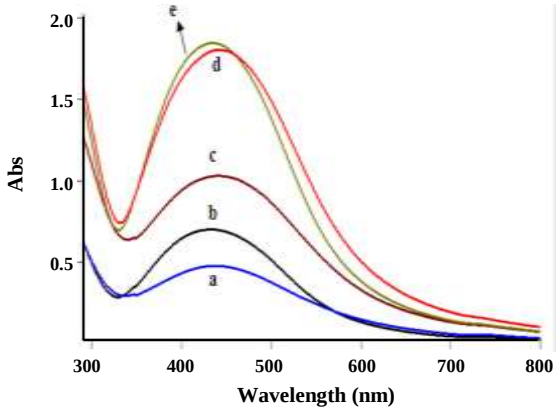

Fig. 4: UV-Visible spectra of colloidal AgNPs in reaction solutions containing 1:1volume ratio of culture supernatant and silver nitrate at a) pH 8; b) pH 9; c) pH 10; d) pH 11 and e) 12.

fungus at pH range of 2-11 has been reported. Researchers have revealed that the extracellular production of nanoparticles at low pH values (acidic solutions) does not occur. Whereas, the alkali condition (pH 11) leads to the formation of gold nanoparticles in both cases [24].

It seems that due to the high concentration of proton at low pH, all functional groups which are important in reduction process become positively charged. This leads to decrease in the interactions between these groups and metal ions and consequently decrease in the efficiency of these groups as reducing agents [25]. On the other hand, the proteins which are responsible for Ag^+ reduction could bound to silver ions in thiol (-SH) groups and form the –S-Ag bond at higher pH. This bond can act as an appropriate intermediate which accelerates conversion of Ag^+ into Ag^0 [24].

Effect of temperature on AgNP formation

Five flasks each containing the same volumes of culture supernatant and silver nitrate solution were prepared and their pH were adjusted to 8 which is the minimal pH value for AgNP formation. Then, flasks were incubated at various temperatures of 28, 30, 35, 40 and 50 °C. Also, one flask containing same volume ratios of culture supernatant and silver nitrate was kept at temperature of 121 °C and pressure of 15 psi for 15 min in an autoclave. The production of AgNPs was monitored in these reaction solutions using UV-visible analysis. The formation of nanoparticles occurred at temperatures higher than 40 °C was occurred. Comparing the absorbance intensities of the colloidal AgNPs in reaction solutions at different incubating temperatures after reaction completion in Fig. 5, revealed that the AgNP productivity depends on the reaction temperature and it increases at higher temperatures. Strong SPR observed near 418, 410, and 403 nm for AgNPs synthesized using culture supernatant at different temperatures of 40, 50 and 121 °C, respectively. Furthermore, Fig. 5C indicates that incubating reaction solutions (containing the same volumes of culture supernatant and silver nitrate) at 121 °C results in the formation of the smallest AgNPs with the highest productivity. As shown in Fig. 5, bioreduction of silver ions completed after 72 h and 48 h for mixtures subjecting to 40 and 50 °C, respectively. This indicates the more rapid formation of AgNPs at higher temperature.

The average particle size, size distribution, and polydispersity index (PDI) of the synthesized AgNPs were determined by DLS, and the results are shown in Fig. 6, The average sizes of the AgNPs in reaction solutions incubated at 40, 50 and 121 °C were 36, 22 and 14 nm, and their PDIs were 0.29, 0.24 and 0.37, respectively. DLS results shows that incubating the reaction mixture at 50 °C produces AgNPs with smaller size and higher monodispersity than incubating at 40°C. A comparison of the average particle size and PDI under various temperatures reveals that incubating the reaction mixtures at 121 °C produces AgNPs with the smallest size and the highest polydispersity. The high polydispersity of AgNPs produced at 121 °C which verifies the formation of aggregates, may be due to vaporization of the reaction mixture at this

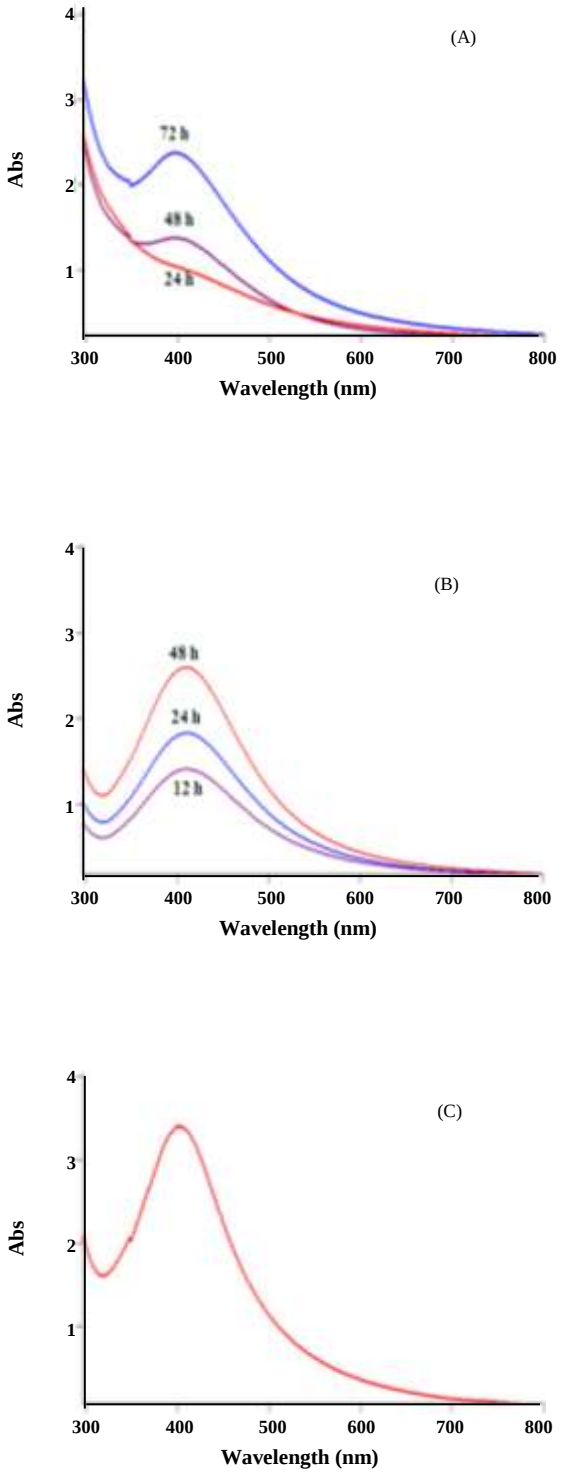

Fig. 5: UV-Visible spectra of colloidal AgNPs at different temperatures of A) 40, B) 50 and C) 121 °C in reaction solutions with equal volumes of culture supernatant and silver nitrate.

high temperature [26]. DLS analyses showed that increasing temperature results in decreasing the nanoparticle average size which is in agreement with the results that obtained from UV-Visible analysis. Temperature dependence of AgNP characteristics; such as average size, size distribution, and productivity; revealed in this study is in agreement with results reported by other researchers [27].

As mentioned earlier, the rate of the nanoparticle formation depends on the incubation temperature of the reaction mixture. Also, increased temperature levels leads to accelerated rate of particle nucleation and growth. With increasing temperature, the electron transport rate increases via reducing agent, which is a rate limiting step [27-28].

Effect of light source on AgNP formation

Nanoparticle formation did not occur in reaction mixture in the presence of sunlight and halogen lamp. UV-visible analysis of colloidal AgNPs produced using the same volumes of culture supernatant and silver nitrate after exposure to UV-lamp is presented in Fig. 7. As shown in Fig. 7, the absorbance intensities steadily increased as a function of exposure time. The occurrence of absorption band at 280 nm (Fig. 7) is attributed to the aromatic amino acids of proteins that arises due to electronic excitations in tryptophan and tyrosine residues of the proteins [29]. This may be due to the secretion of proteins into the solution by *F. oxysporum* fungus and suggests a possible mechanism for the reduction of silver ions in the reaction mixture. As shown in Fig. 7, during the AgNP formation the absorption band at 280 nm disappeared. This observation reveals the importance of the aromatic amino acids in Ag^+ reduction. The absorption of UV irradiation leads to split or oxidation of peptide bonds; aromatic rings, such as tryptophan and tyrosine; and disulphide groups present in proteins [30].

Particle size distribution of the synthesized nanoparticles is presented in Fig. 8. The average size of colloidal AgNPs in the presence of UV-irradiation was 9.7 nm and their PDI equals to 0.20. As mentioned earlier, in comparison to using variation of temperature to decrease the particle size of AgNPs, in reaction mixtures containing the same volume ratios of culture supernatant and silver nitrate; using UV-visible light source led to the formation of smaller AgNPs. UV-radiation leads to

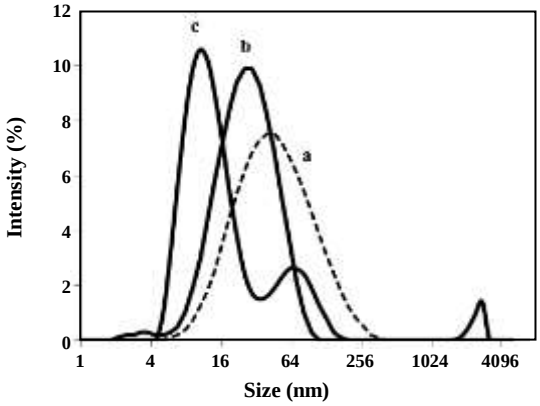

Fig. 6: DLS analysis of colloidal AgNPs formed by the mixtures of culture supernatant and $AgNO_3$ with same volume ratio at a) 40 °C; b) 50 °C and c) 121 °C.

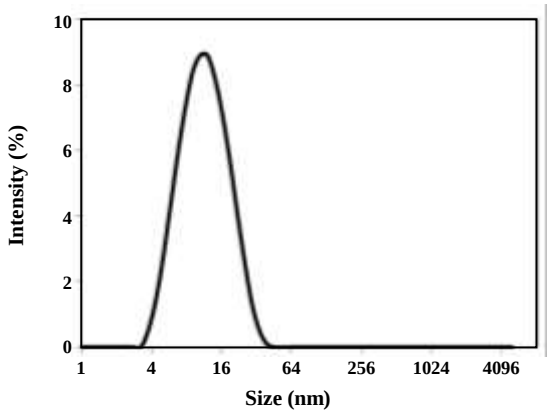

Fig. 8: DLS analysis of the AgNPs synthesized using same volumes of F. oxysporum culture supernatant and silver nitrate solution at the presence of UV-Lamp irradiation for 10 h.

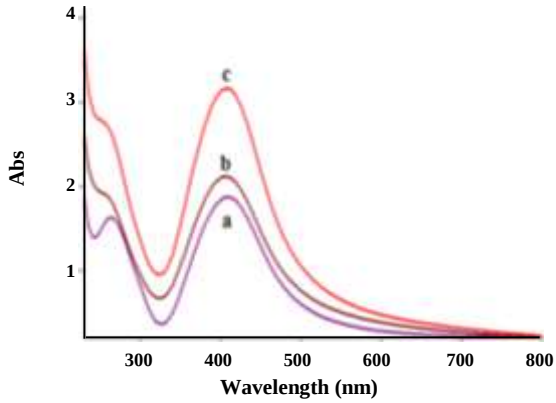

Fig. 7: UV-Visible spectra of colloidal AgNPs generated in the reaction mixture with same volume ratio of culture supernatant and $AgNO_3$ solution after a) 1 h; b) 2 h and c) 10 h exposing to UV-lamp irradiation.

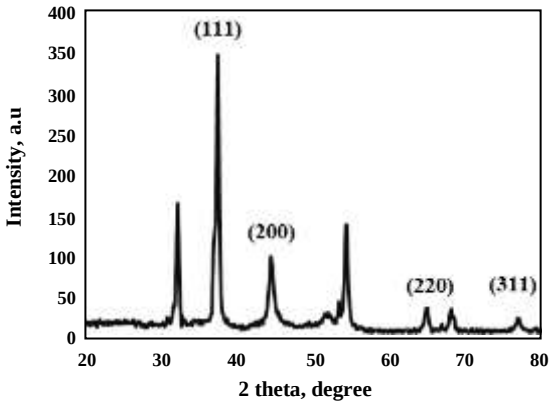

Fig. 9: XRD diffractogram of colloidal AgNPs originated from UV-irradiation of the reaction solution containing same volumes of culture supernatant of F. oxysporum and $AgNO_3$.

the formation of hydrated electrons in the reaction mixture that can reduce Ag^+ ions without excessive reducing agents and this consequently leads to the formation of higher amounts of initial nucleation centers. This may be the reason of producing nanoparticles with the smallest size. Moreover, the uniform distribution of these electrons in the reaction solution resulted in the production of stable nanoparticles with high monodispersity [31-32].

Structural and morphological study of AgNPs

The XRD pattern for AgNPs produced using the same volumes of the culture supernatant and silver nitrate at the presence of UV-lamp irradiation showed peaks with 2θ values of 37.52°, 44.49°, 64.77° and 73.41° which are assigned to (111), (200), (220), and (311) reflection planes of face-centered-cubic (fcc) silver, respectively. The observed peaks are in agreement with the pure crystalline silver structure database of the Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. This verifies the crystalline nature of the AgNPs synthesized using culture supernatant of the fungus.

Fig. 10, shows the TEM image of AgNPs formed by UV-irradiation of the reaction solution containing the same volumes of culture supernatant and silver nitrate. As shown in this figure, the shape of the nanoparticles is predominantly spherical.

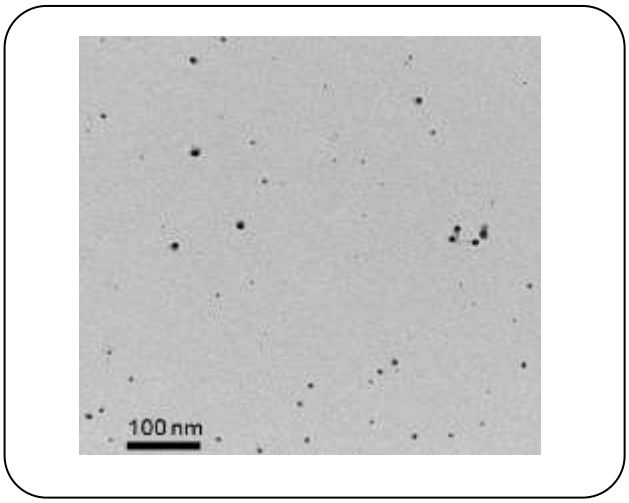

Fig. 10: TEM image of colloidal AgNPs synthesized using same volumes of F. oxysporum culture supernatant and $AgNO_3$ at the presence of UV-Lamp radiation.

CONCLUSIONS

The rate of silver nanoparticle formation and the size of the nanoparticles in reaction solution containing the same volumes of fungal culture supernatant and silver nitrate were found to be depend on pH, temperature and light source. No extracellular production of AgNPs was observed at pH lower than 8. Whereas, under pH range of 8-12, the color changes with time, indicates the formation of silver nanoparticles. Result showed that AgNPs with the highest productivity and the smallest size was formed at pH 12. Investigating the effect of temperature for the reaction solutions consisting of the same volume ratio of culture supernatant and silver nitrate proved that the nanoparticle formation was occurred at temperatures higher than 40 °C. Also, increasing the temperature decreases the size of nanoparticles. The smallest nanoparticles, with average size of 14 nm, were obtained at 121 °C. Exposing UV-light to the reaction mixture led to the formation of the highest amount of AgNPs with the smallest size of 9.7 nm.

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