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Influence of External Factors on the Production and Morphology of Biogenic Silver Nanocrystallites

Seyed J. Ashrafi[1,*], Mahrokh F. Rastegar[2], Motahare Ashrafi[3], Fatemeh Yazdian[1], Reza Pourrahim[4], and Anil K. Suresh[5,*]

[1] Department of Life Science Engineering, University of Teharn, Tehran, 14174, Iran
[2] Department of Plant Protection, Ferdowsi University of Mashhad, Mashhad, 9177948974, Iran
[3] College of Chemistry, Shahrood University of Technology, Shahrood, 36155-316, Iran
[4] Plant Virus Diseases Research Department, Plant Pests and Diseases Research Institute, Iran University of Tehran, Tehran, 14174, Iran
[5] Department of Molecular Medicine, Beckman Research Institute, City of Hope, Duarte, CA 91010, USA

Naturally existing biological materials have been garning considerable attention as environmentally benign green-nanofactories for the fabrication of diverse nanomaterials, and with desired size and shape distributions. In the present investigation, we report the size and shape controllable biofabrication of silver nanocrystallites using the growth extract of the fungus, *Rhizoctonia solani*. Influence of various factors such as growth medium; radiation, in the form of sun light; and seeding duration on the production of silver nanoparticles using aqueous 1 mm silver nitrate solution under ambient conditions is presented. Our results demonstrate that these factors can significantly influence the production, size and shape transformation, and the rate of nanoparticles formation. Multiple characterization techniques involving UV-visible and Fourier transform infrared spectroscopy, X-ray diffraction, energy dispersive X-ray spectroscopy and transmission electron microscopy measurements confirmed the production, surface and structural characteristics, purity and crystalline nature of the biosynthesized silver nanoparticles. Our biogenic synthesis process provides a simple, ecologically friendly, cost-effective synthesis route, and most importantly the ability to have control over the size and shape distributions that lends itself for various biomedical and opto-electronic applications.

Keywords: Biogenic, Cell-Free Environment, Radiation, Silver Nanoparticles, Seeding Duration, Size and Shape Control.

RESEARCH ARTICLE

1. INTRODUCTION

Nanoparticles are considered as building blocks of the next generation optoelectronics, electronics, therapeutics and diagnostics, chemical and biochemical sensors.[1–11] Advances in nanobiotechnology implications and the unintentional environmental release of nanomaterials demands for environmentally friendly and green fabrication routes for their manufacture. Unique optoelectronic, physicochemical and biological properties of nanoparticles are determined by their size and shape distributions, crystallinity and surface properties that lack in their respective bulk counterparts. Therefore, the synthesis of nanoparticles with controllable morphology and dimensions has become a challenge in nanobiotechnology. Although various physical and chemical methods are extensively used for the production of a wide variety of nanoparticles;[2,12,13] issues with regards to their stability and solubility in aqueous suspensions are the subject of paramount concern.[14] Additionally, the use of common toxic chemicals, substrates, precursors and surfactants, for example, cetyl trimethylammonium bromide, mercaptoundecanoic acid, mercaptopropionic acid, zinc precursor, and the use of non-polar solvents in the synthesis procedures limit their applications in various biomedicinal and clinical areas.[15] Therefore, development of facile, controllable, nanomaterials using ecologically friendly and green syntheses routes deserves merit. Recently, biological methods of synthesis that involve naturally existing microorganisms (uni- and or multi-cellular) and or biomaterials in the form of protein/peptides, plant extracts etc. are being utilized as nano-factories for the fabrication and assembly of various technologically important advanced functional nanomaterials for various applications.[2,16–19] These biomaterials based synthesis processes are regarded as safe, green, cost-effective, sustainable and environmentally friendly when

[*] Authors to whom correspondence should be addressed.

compared to that of chemical and or physical means of synthesis.[2] However, most of the microbial-based biosynthesis involves the use of whole microorganism for the synthesis, and our report is unique in the sense that we use microbial growth extract instead of a microorganism. The several benefits using this strategy are

(1) prevents additional steps that might be involved in the purification,

(2) avoids intracellular formation of nanoparticles (where purification itself becomes impossible), and

(3) as the employed method does not deal with a complex microorganism and just its extracellular secretions, size and shape control becomes an achievable task. In the present investigation we demonstrate for the first time the use of plant pathogenic fungus, *Rhizoctonia solani* extract for the biosynthesis of silver nanoparticles when incubated with aqueous $AgNO_3$ solution under ambient conditions. Additionally, the influence of various factors such as the growth medium; radiation; and seeding duration on the production, size and shape transformation and the rate of formation of silver nanoparticles is described. As size and shape control of nanoparticles has always been an issue of considerable interest. Multiple physical characterizations including the UV-visible and Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDX) and transmission electron microscopy (TEM) measurements revealed the production, surface and structural properties, purity and crystalline nature of the biogenic silver nanoparticles. Our results provide a simple, ecologically benign, cost-effective and controllable biogenic production of silver nanoparticles for various intended biomedical and opto-electronic applications.

RESEARCH ARTICLE

2. MATERIALS AND METHODS

The fungus *Rhizoctonia solani* used in our studies was isolated from the Rosemary plant (*Rosmarinus officinalis*)[20] from the campus of Ferdowsi University of Mashhad, Iran. All other chemicals and reagents were from standard commercial sources.

2.1. Biogenic Production of Silver Nanoparticles

The fungus *R. solani* was maintained on potato dextrose agar (PDA) slants (potato 20% w/v, dextrose 2% w/v, and agar 2% w/v) at 25 °C. The fermentation was performed by inoculating a 1 cm diameter mycelium from 7 day old PDA slant, into a 100 mL liquid MGYP medium (0.3% w/v malt extract, 1.0% w/v glucose, 0.3% w/v yeast extract, and 0.5% w/v peptone) and PGB medium (25% boiled potato extract, 2% glucose) separately (so as to evaluate the impact of growth medium) in 500 mL sterile bottles, followed by incubation at 26 ± 1 °C on a rotary shaker (200 rpm) for 72 h. After the fermentation periods, mycelia were separated from the culture broth by centrifugation (6000 rpm at 4 °C for 30 min) and to the culture supernatant $AgNO_3$ was added accordingly to obtain an effective concentration of 1 mM, in 500 mL sterile bottles and incubated at 26 ± 1 °C under standard light conditions (2 micro mol/m^2s^{-1}). Biogenic production and growth of the silver nanoparticles were monitored visually and by UV-vis spectroscopy measurements (250–650 nm). After completion of the reaction process, the reaction mixture was filtered using a sterile 0.22 μm syringe filter and the particles were collected by centrifugation (the process of filtration was skipped for the very big particles (300 ± 20 nm) so as to avoid membrane entrapment of particles). After washing twice with Milli Q water the biogenic silver nanoparticles were used for the below described physical characterizations. Control experiments involved similar reactions performed in the presence of growth mediums; PGB and MGYP and without the fungal extract separately.

2.2. Influence of Radiation and Seeding Duration

Separate experiments were performed to assess the influence of radiation (natural sun-light was used as light source) and seeding duration. To evaluate the impacts of seeding duration, separate reactions were performed as mentioned above, except carried out at various durations; $\sim$12 hours, 20 days and 30 days on a rotary shaker (200 rpm). Additionally, to assess the influence of radiation, solarization experiments were conducted involving similar reactions, however subjected to $\sim$140–1240 micro mol/m^2s^{-1} and variable temperatures 24–36 °C.

2.3. Physical Characterizations

Aliquots of the reaction mixture were removed at regular intervals and subjected to UV-vis spectrophotometric measurements performed on a S2100UV (Unico, USA) at a resolution of 1 nm over a spectrum range of 250 nm to 650 nm. Fourier transform infrared spectroscopy (FTIR) measurements of the freeze-dried (PD10 Freeze-dryer, Pishtaz Eng. Co., Iran) nanoparticle samples deposited on KBr pellets were performed on a Shimadzu FTIR spectrophotometer (Tokyo, Japan) at a resolution of 4 cm^{-1}. Dynamic light scattering measurements to assess the hydrodynamic size distributions of the silver particles were performed on a Brookhaven 90 Plus/BI-MAS Instrument (Brookhaven Instruments, New York). Samples of silver nanoparticles thin films formed on carbon coated copper TEM grids were analyzed by transmission electron microscopy using a Hitachi HD-2000 STEM operated at an accelerating voltage of 200 kV. EDX analysis of a layer of sample coated on carbon coated copper grid was performed on Hitachi HD-2000 STEM. X-ray diffraction analysis of the drop-coated film of the silver nanoparticles coated onto glass lam were performed using D8 Advance Bruker X-ray diffractometer, operated in transmission mode at 40 kV and at a current of 40 mA.

3. RESULTS AND DISCUSSION

Circumvent to the use of lethal or toxic solvent chemicals, surfactants and precursors that are commonly used in chemical and physical methods of synthesis of nanoparticles. Alternative route involving the utilization of naturally existing microbial-based technique for the synthesis of silver nanoparticles as green chemistry is reported. When aqueous solution of silver nitrate was incubated with fungal extract of *R. solani*, the reaction mixture turned from colorless to brown in 12 h, indicating the biosynthesis of silver nanoparticles (Fig. 1(A) and (A), inset). The biosynthesis process was monitored visually as well as following UV-visible spectroscopy measurements, by performing a spectral sweep from 250–650 nm. The appearance of surface plasmon resonance (SPR) peak at 410 nm indicated the formation of silver nanoparticles (Fig. 1(A)). In order to evaluate the impact of growth mediums on the nanoparticle synthesis, two different growth mediums, MGYP and PGB were used separately. Silver nanoparticles formation was observed only in the PGB medium (Fig. 1(A)), whereas in MGYP medium no nanoparticles synthesis was observed (Fig. 1(A)). Literature suggests that MGYP is the most commonly used growth medium for the biosynthesis of several metal and semiconductor nanoparticles.[21–24] Our observations that *R. solani* was unable to synthesize nanoparticles in MGYP medium and that can synthesize particles in PGB medium, clearly indicate that growth medium does influence on the production of nanoparticles. One possible explanation to justify such observation is that some or one of the unknown components, presumably from the yeast extract, in the MGYP medium might be inhibiting the catalytic site of the metal-reducing enzyme thereby preventing the bioreduction. Though details on such observed new phenomenon are yet to be understood, our results alerts researchers before drawing any conclusions. Control experiments included the reactions performed using MGYP and PGB medium only in the absence of cells or *R. solani* cell-free culture supernatant, and exhibited no change in color or absorbance, indicating the requirement of fungal cell extract or fungal secretions in the biosynthesis. Several investigations performed by various researchers demonstrated that microbial-based metal biotransformation might involve a complex of either capping proteins/peptides and reductases, quinones or cytochromes, phytochelatins or electron shuttles that are known to reduce various metals and metal oxides.[6, 7, 25, 26] After completion of the reaction process (12 h), the particles were purified by filtration using a sterile Millipore 0.22 μm syringe filter followed by dialysis and centrifugation, and were used for further characterizations. The purified nanoparticles were highly stable in aqueous solution with no observed particle aggregation or clumping even after several months of the reaction. FTIR spectroscopy measurements confirmed the presence of a capping protein or peptide bound to the surface of the biogenic silver nanoparticles. Spectral analysis carried out within the region of 800–3600 cm^{-1} revealed the presence of vibration bands centered at 1023, 1410, 1660, 2935 and 3302 cm^{-1} (Fig. 1(B)). The bands at 1023 and 1660 cm^{-1} correspond to the –N–H and carbonyl (–C–O–C– or –C–O–) stretch vibrations in amide linkages (amide I and amide II), the peak at 1410 cm^{-1} was also observed from the amide III linkage, clearly indicating the presence of protein or peptide on the nanoparticle surface that likely serves as a capping or stabilizing agent.[6, 7] The band at 2935 cm^{-1} is due to C–H stretch, and the band at 3302 cm^{-1} corresponds to carbonyl and hydroxyl functional groups in alcohols and phenol derivatives.[6, 7, 14] Our observations are in agreement with earlier such biosynthesized nanoparticles.[6, 7, 14]

Confirmation of the synthesis of silver nanocrystallites was based on X-ray diffraction analysis that showed the presence of intense peaks corresponding to (111), (200) and (220) (Fig. 2(A)) due to the broadening of Bragg reflections at 38.1, 41.3 and 58.6, respectively, in the 2θ range 20–70° based on the face-centered cubic structure

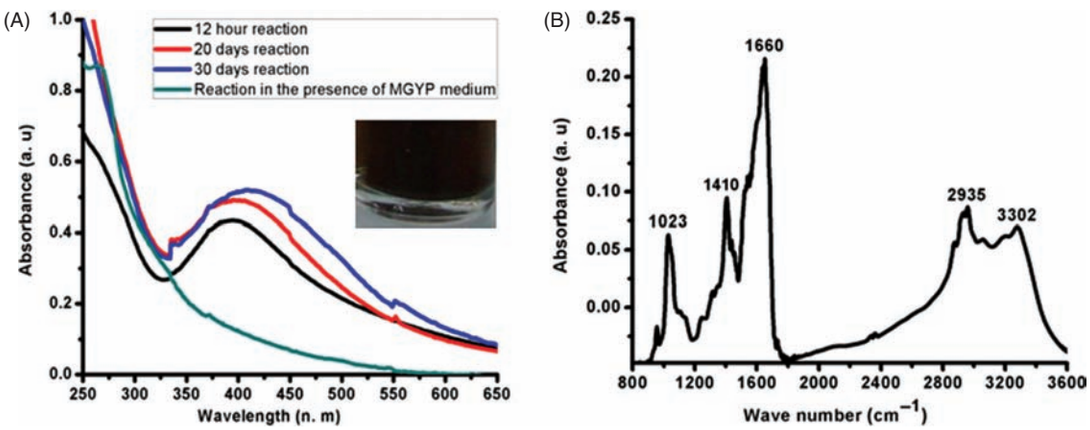

Fig. 1. UV-vis and FTIR spectroscopy measurements of the biogenic silver nanoparticles produced using the growth extract of fungus, *R. solani*. (A) UV-vis spectra recorded from the reaction mixture after the formation of silver nanocrystallites at different seeding durations. (B) FTIR spectra of the silver nanoparticles.

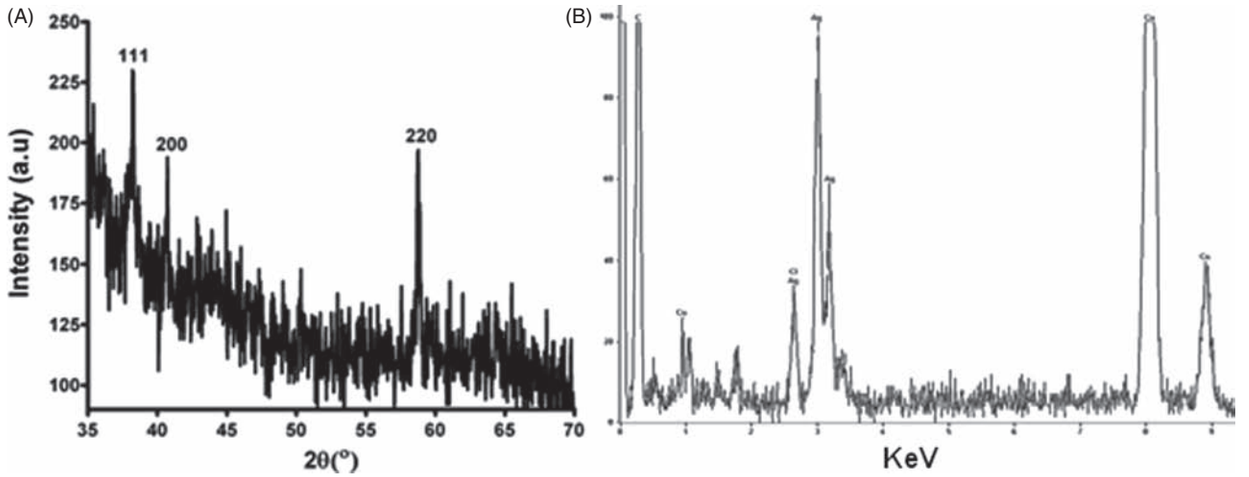

Fig. 2. X-ray diffraction and EDS analysis of the biogenic silver nanoparticles produced using the growth extract of fungus, *R. solani.* (A) XRD analysis of the drop-coated film of silver nanoparticles. (B) EDS of the biogenic silver nanoparticles. Strong signal from the silver can be observed.

of silver nanoparticles and are in agreement with similar reported silver peaks from the literature.[14]

EDS analysis of the particles revealed a strong signal for silver at 3 keV, characteristic of silver nanoparticles (Fig. 2(B)),[27] along with the C and O signatures that might be from the stabilizing protein encapping the surface of the particles. An additional peak for Cu was also observed due to from copper grids on which the nanoparticle samples were prepared.

Transmission electron microscopy analysis revealed homogeneous predominantly spherical and well-separated silver nanoparticles (Fig. 3(A)). Histogram size distributions obtained by counting ∼50 particles from the TEM micrograph revealed the particles to be in the size range

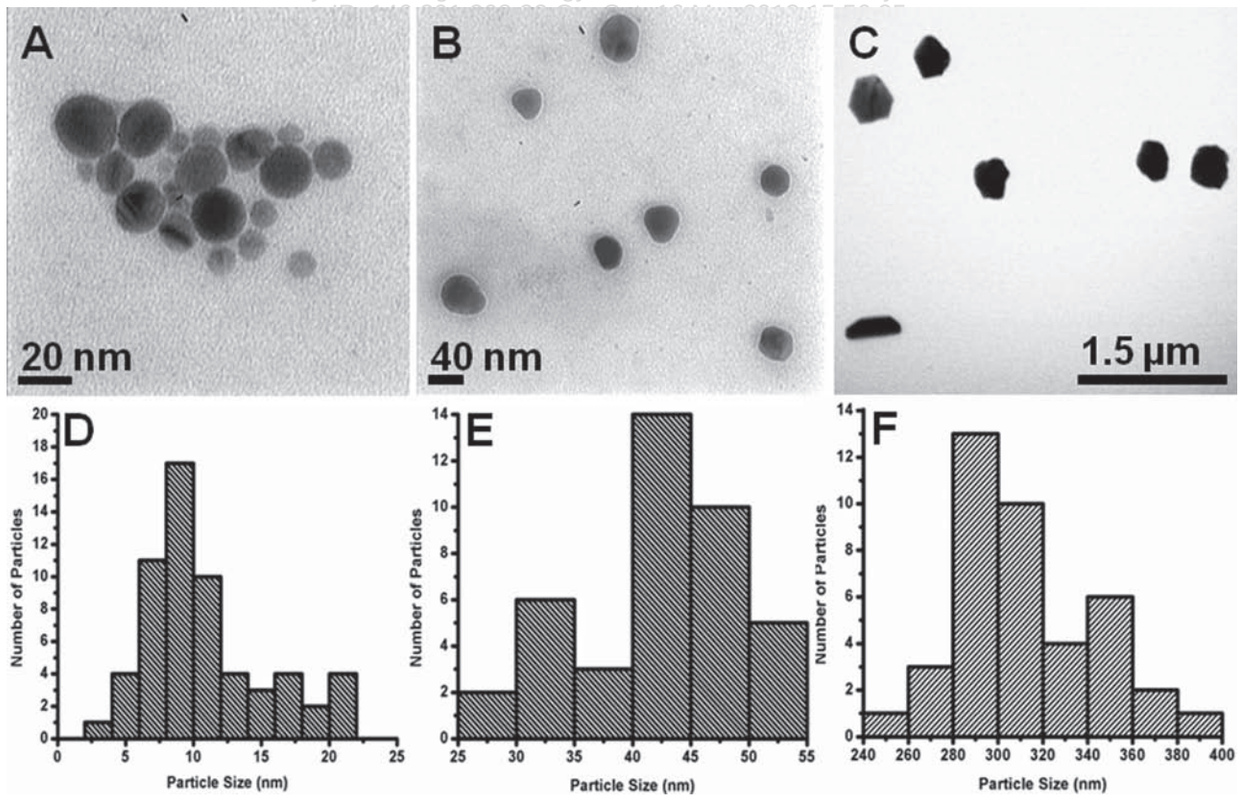

Fig. 3. TEM measurements of the biogenic silver nanoparticles formed using the growth extract of *R. solani.* TEM images and the respective histogram size distributions of the biogenic silver nanoparticles at reactions times 12 h (A) and (D), 20 days (B) and (E) and 30 days (C) and (F).

Table I. Size distribution and lambda max (λ_{max}) of the various silver nanoparticle samples under various reaction conditions.

Experimental condition	Reaction time (h)	λ_{max} (nm)	Particle mean diameter (TEM) (nm)	Hydrodynamic diameter (DLS) (nm)
Radiation (sun light)	~1–2	410	$\sim 12 \pm 4$	21 ± 3.1
Seeding Duration (12 h)	–	410	$\sim 9 \pm 2$	17 ± 5.3
Seeding duration (20 days)	–	430	$\sim 40 \pm 5$	73 ± 4.2
Seeding duration (30 days)	–	445	$\sim 300 \pm 20$	332 ± 7

of ~2–22 nm, and with a mean diameter of $\sim 9 \pm 2$ nm (Fig. 3(D)). An estimate of the size of the particles was also made from the line broadening of the (111) reflection pattern using Debye Scherrer's formula ($D4 = 0.94\lambda/\beta \cos\theta$), where D is the average crystalline domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half-maximum and θ is the diffraction angle,[28] and is in good agreement with the nanoparticles size assessed by TEM analysis. However, based on dynamic light scattering measurements the hydrodynamic diameter of the nanoparticles appeared to be larger when compared to that of TEM measurements (Table I). This may be attributed to overlapping particles and the electrical double layer phenomenon that occurs with charged particles which can affect DLS measurements, while TEM imaging allows latitude for eliminating aggregated particles from the analysis.[6]

As descried earlier in the materials and methods section, separate experiments were performed to evaluate the influence of radiation and seeding duration on the biogenic production of silver nanocrystallites. Based on our observations using radiation, sunlight being the light source, though there was no significant difference in the nanoparticle characteristics (Fig. 4) compared to that of non-radiated ones (Figs. 1, 2 and 3(A)), the overall rate of formation of the silver nanoparticles was drastically increased. In the presence of radiation the reaction was brought down to ~1–2 h from 12 h (Table I). Such combinatorial approach involving photogeneration methods have been successfully proven to increase the rate of nanoparticles production. For example, Mohamadian et al. in their report on the photo-induced biosynthesis of silver nanoparticles suggested that the reaction time can be brought down from 96 h to 1 h, however, their studies involved the use of fungal biomass and artificial light source using halogen-tungsten lamp (90000 lux).[29] Similarly, sun-light induced rapid biosynthesis of small (~3.4 nm) silver nanoparticles was reported using the plant extract of *Andrachnea chordifolia*.[30]

Additionally, our results suggested that seeding duration had a huge impact on the size and shape transformation of silver nanoparticles (Fig. 3). Over the course of seeding duration between 12 hours and 20 days, we observed that the initially formed small spherical shaped nanoparticles of $\sim 9 \pm 2$ nm (Figs. 3(A), (D)) somehow get transformed into bigger spheres and triangular shaped particles of average size distributions of $\sim 45 \pm 5$ nm (Figs. 3(B), (E)), and further prolonged seeding duration (30 days) resulted in the transformation of most of the nanoparticles into big platelets of size distributions between 240–400 nm with an average of $\sim 300 \pm 20$ nm (Figs. 3(C), (F)). TEM measurements correlate with the UV-vis spectroscopy measurements where a gradual shift in the surface plasmon resonance band was observed with increase in the size of the particles (Fig. 1(A)). Due to quantum size effects, where the electronic properties of metallic nanoparticles (less than 100 nm) are altered with reduction or increase in the particle size. The hydrodynamic diameter of the various seeding durations samples were also measured and are given in Table I. Though the exact mechanism on the formation of such big platelets is not known, we predict that the initially formed triangular shaped particles due to metallic attractions assemble into these big pentagonal/hexagonal

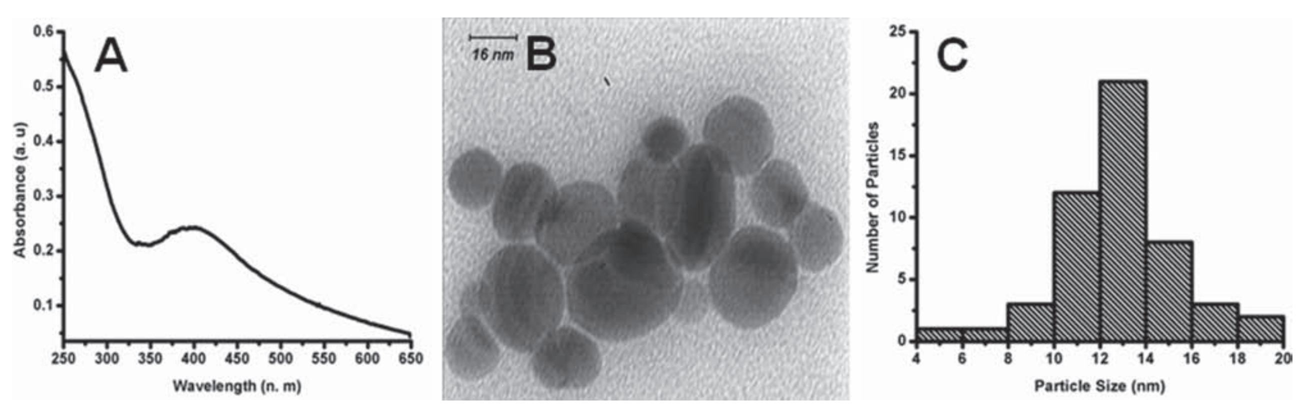

Fig. 4. UV-vis spectroscopy and TEM measurements of the biogenic silver nanoparticles produced using the growth extract of fungus, *R. solani* in the presence of radiation (sun-light) (A) UV-vis spectra recorded from the reaction mixture after the formation of silver nanocrystallites in the presence of light. TEM images (B) and the respective histogram size distributions (C) of the biogenic silver nanoparticles in the presence of light source.

platelets (Fig. 3(C)). However, the exact mechanism that might be involved is currently under investigation. Size and shape controlled synthesis of biogenic nanoparticles is an emerging novel area of research with potential benefits. Our observations are in correlation with the results from Cui et al. wherein using live yeast cells the authors showed the controllable biosynthesis of fluorescent quantum dots.[15] Per the authors different size distributions were obtained by simply controlling the incubation duration of the reaction process. Similarly, extracellular protein secreted by the bacterium, *E. coli* has been shown to produce triangular shaped of gold nanoparticles.[31] In another investigation, Sastry et al. reported the biosynthesis of gold nanoplates upon reacting auric chloride with the lemongrass leaf extract.[32] The authors opined that the oil extract acted as both reducing as well as stabilizing and or shape controlling agent. In a similar investigation Sneha et al. scrutinized the effects of several counter ions, temperature, pH, and reaction times on the morphology of gold nanoparticles.[33] Kumar et al. in an interesting investigation reported the biofabrication of heterogeneous shapes of gold nanocrystallites such as rods, triangles, hexagons, spheres, pyramids and stars using the plant pathogenic fungus *H. solani*.[34] The authors further showed that the gold nanoparticles could be easily size-fractionated following sucrose density gradient based table-top centrifugation and that the obtained smallest size fractions of < 5 nm can be used as in drug delivery application. In a recent investigation, Das et al. reported the microbial based biosynthesis of multiple shapes of gold nanoparticles using the cell extract of *Rhizopus oryzae*. The authors were able to generate anisotropic shapes of gold nanoparticles by varying minor factors such as protein concentration, reaction pH and the reaction duration.

4. CONCLUSIONS

The use of *R. solani* growth extract has been proven to be successful in the generation of silver nanocrystallites as a green nanofabrication technique. Based on our observations radiation, in the form of natural sun-light, had a significant influence on the rate of biogenic production of silver nanoparticles with the rate of reaction being brought down from ~ 12 h to ~ 1–2 h. Additionally, our results also demonstrate that cell-free environment can be exploited for the fabrication of nanoparticles and suggest that various factors such as the seeding duration and change in the growth medium can drastically influence the production and size and shape transformation of the silver nanoparticles. Overall these interesting observations will likely aid in diverse applications and permit the controllable fabrication of desired size and shape distributions, avoiding any purification.

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