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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *ASPERGILLUS TERREUS* (KC462061)

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The biosynthesis of nanoparticles has found increasing attention due to environmentally friendly andcost-effective. In this report, silver nanoparticles (AgNPs) were synthesized using a reduction of aqueous Ag+ ion with the culture supernatants of *Aspergillusterreus*strain KC462061 were isolated from the roots of date palm and identified as *Aspergillusterreus* based. A fungal strain, SDP9, isolated from the roots of date palm (*Phoenix dactylifera*), was identified as *Aspergillusterreus* based on sequence analysis of the internal transcribed spacer regions (ITS1-5.8S-ITS2) and 28S rRNA. The bioreduction of AgNPs was monitored by ultraviolet-visible spectroscopy.The nanoparticles were characterized by UV–Vis spectrophotometry, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). These analyses revealed thatThe size of nanospheres was about 17.5 nm on average and in the range of 5–30 nm the and predominantly polydispersed and spherical.Moreover, the antimicrobial potential of AgNPs was evaluated. AgNPscould inhibit various bacteria and fungi

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1. Introduction

The biosynthesis of nanoparticles has received increasing attention due to thegrowing need to develop safe, cost-effective and environmentally friendlytechnologies fornano-materials synthesis[1]. The production of metal-based nanoparticles by thermal treatment [2] irradiation [3] and chemical reduction [4]Thus, it is essential to develop a green approach for AgNPs production without using hazardous substances such as organic solvents and toxic reducing agents to the human health and environment.The synthesis of silver particles using *Aspergillusniger*[5,6,7]and *Aspergillusterreus*[1] were isolated from soil.Fungi have been known to secrete much higher amounts than bacteria of bioactive substances, which made fungi more suitable for large-scale production[8],. In addition, the extracellular biosynthesis using fungi could also make downstream processing much easier than bacteria [9].

In this study, we have isolated *Aspergillusterreus*soil date palm from Riyadh, Saudi Arabia Riyadh, and used this filamentous fungusfor AgNPs synthesis. Molecular identification of the filamentous fungi was conducted based on sequence of mitochondrial cytochrome b gene [10]mycotoxin regulatory genes, alkaloids [11]. DNA topisomerase gene [12] , and ribosomal RNA identity [13]. However, ribosomal RNA regions as 18S rRNA and internal transcribed

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spacers (ITS 1 and ITS 2), between the small and large subunit, is a universal fungal probe as species-species identifiers for saprophytic filamentous fungi [14,15]

The silver nanoparticles can be characterized by different techniques such as Ultraviolet-Visible spectroscopy, fourier-transformed-infrared spectroscopy, the structure using powder X-ray diffraction (XRD), and microscopy techniques (TEM and SEM). These techniques can provide the information about the formation,size and morphology of the particles, capping, and stability. The antimicrobial activity of the nanoparticles was also examined

2. Materials and methods

Isolation and cultivation of fungus

The fungus was isolated from soil date palm from Riyadh, Saudi Arabia, Saudi Arabia on potato dextrose agar (PDA) and incubated at 28 C. The identities of the isolates were carried out by the Regional Center of Fungi and their Applications, Al-Azhar University, Cairo, Egypt .

Genomic DNA extraction

*Aspergillusterreus*isolates were cultured on double layer media in 50 mm Petri dishes, one solid and the other liquid. Base media solid, was potato dextrose agar as a film, and the top media, liquid, was peptone yeast glucose (PYG, 1200 µl). Fungi were incubated at 25°C for two days. Fungi mycelia (50 mg) were scraped using slides covers and transferred to sterile Eppendorf tubes (1.5 ml) for DNA isolation. Fungal genomic DNA was extracted according to the protocol of [16].

PCR amplification and sequencing of internal transcribed spacer regions (ITS1-5.8S-ITS2), and 28S rRNA

Fungal isolates were molecularly identified on the basis of their 18S rRNA gene, internal transcribed spacer regions (ITS1-5.8S-ITS2), and 28S rRNA sequences in a similar manner to the studies performed by [17].PCR primer ITS-1 (5′-TCC GTA GGT GAA CCT GCGT G -3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR reaction contains 4 µL DNA, 5 µL of 10x PCR buffer, 1 U of Taq DNA polymerase (BioLabs), 0.4 µLdNTP mix, 200 mM of each dNTP (BioLabs), and 1 µL of each ITS-1 and ITS-4 and 37.6 µL dH_2O for total volume 50 µL. The PCR protocols was adjusted as follow, denaturation at 95°C for 1 min, annealing at 50°C for 1 min, elongation at 72°C for 20 s, for 34 cycles with final elongation at 72°C for 10 min. The amplified products were loaded on a using 1% agarose gel containing ethidium bromide in 1x TAE, then visualized by gel documentation system (Biorad, USA). The amplified PCR product was sequenced using ITS-1 ITS-4 primers and using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA) at the DNA Sequencing Facility, King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia.

Multiple sequence alignments and phylogenetic analysis

Nucleotide sequences were compared with those maintained in the GenBank Database through NCBI Blast (http://www.ncbi.nlm.nih.gov). Alignment of nucleotide sequences was done using a cluster method W of the DNASTAR software program (DNASTAR Inc., Madison, WI, USA). Data analysis was performed using bootstrapped data set characterized with 1,000 replicates. In order to determine the genetic relationship between *Aspergillusterreus* strain SDP9 and other strains, a phylogenetic tree was generated based on the percentage difference between the sequences using the neighbor-joining method in the MEGA 7.01 software [18].

Biomass Preparation:

To prepare biomass for biosynthesis, *Aspergillusterreus*was grown in the liquid medium containing (g/L): KH2PO4 7.0; K2HPO4 2.0; MgSO4 7H2O 0.1; $(NH4)_2SO4$ 1.0; yeast extract 0.6 and glucose 10.0. After the incubation, the biomass was separated and extensively washed with distilled water. Fresh and clean 20 g. of biomass was collected with 100 ml of deionized water and further incubated for 72 h in an Erlenmeyer flask and agitated in similar conditions as described

earlier. After the incubation, the supernatant was obtained by passing suspension through Whatman filter paper No. 1.

Synthesis of silver nanoparticles by the *Aspergillusterreus*:

silver nanoparticles were synthesized using 50 ml of AgNO3 1mM solution was mixed with 50ml of cell filtrate in an Erlenmeyer flask and filtrate (without silver nitrate) as positive and pure silver nitrate solution (without cell-free filtrate) as negative controls were also run simultaneously along with the experimental flask in threereplicates and incubated at 28 ºC on a rotary shaker (150 rpm) in dark.

Characterization of AgNPs:

Ultraviolet-Visible Spectrophotometer

The formation of the reduced silver nanoparticles in colloidal solution was monitored byusing UV-Vis Spectrophotometer. Color changes in the supernatant were monitored both by visualinspection and absorbance measurements using double beam UV–Vis spectrophotometer, Cintra10e GBC (Victoria, Australia). The spectra of the surface plasmon resonance of AgNPs in the supernatants were recorded usingUV–Vis spectrophotometer at wavelengths between 200 to 800 nm.

X-ray diffraction analysis

XRD is an important technique to evaluate the formation of silver nanoparticles and to determine the particle size. The fungal supernatant containing AgNPs was freeze-dried using a HetoLyophilizer (Heto-Holten, Denmark) and stored in lyophilized powdered form until used for further characterization. The finely powdered sample was analyzed by an X'pert PRO PAN alytical diffract meter using CuK_{α} radiation (k = 1.54056 Å) in the range of $20 \leq 2\theta \leq 80 \leq$ at 40 keV.

Fourier transforms infrared.

For Fourier transform infrared (FTIR) spectroscopy measurements, the bio-transformed products present in cell-free filtrate after 72 h of incubation were freeze-dried and diluted with potassium bromide in the ratio of 1: 100. FTIR spectrum of samples was recorded on FTIR instrument mode Nicolet 6700 spectrometer of resolution 4 cm-1) attachment. All measurements were carried out in the range of 400– 4000 cm-1 at a resolution of 4 cm-1.

Transmission electron microscopy

Transmission electron microscopy was performed on JEOL (JEM-1010) instrument, withan accelerating voltage of 80 kV after drying of a drop of aqueous AgNPs on the carbon-coatedcopper TEM grids Samples were dried and kept under vacuum in desiccators before loading themonto a specimen holder. The particle size distribution of silver nanoparticles was evaluated usingImageJ 1.45s software1493

Scanning electron microscopy

Scanning electron micrographs were taken using JEOL (JSM-6380 LA) instrument.Samples were filtered and dried before measurements.

Antibacterial activity of AgNPs

The silver nanoparticles synthesized from *Aspergillusterreus* KC462061were tested as antibacterial activities against *Aspergillusflavus, Aspergillus fumigates, Aspergillusniger, Candida albicans*and *Staphylococcus aureus*using the agar well diffusion method. Using sterile micropipette 20 µl of the sample of nanoparticles solution was poured onto each of the wells in all three concentration (1.0, 2.0, 4.0 mM) onto the plates. After incubation at 35ºC for 24hrs for bacteria and 28ºC for 7 days for fungi the different levels of zone of inhibition were measured using a meter ruler and expressed in millimeter.

3. Result and discussion

3.1 ITS and 28S rRNA-based characterization and phylogenetic analysis of *A. terreus* strain SDP9

Amplification and sequencing of the ITS1-5.8S-ITS2 and 28S rRNA regions of *A. terreus* strain SDP9 resulted in 780 bp long nucleotide sequence, which has been deposited in NCBI GenBank (Accession Number: KC462061). The Phylogenetic tree and genetic distances revealed two sequence alignment analyses have 99.9% identity between the sequence of *A. terreus* strain SDP9 and sequence of *A. terreus* strain JGS11 fig (1). The sequence of ITS1-5.8S-ITS2 region appears the reliable molecular approach for fungal identification [19].Practically, ITS regions of rRNA are the most reliable molecular approach for fungal discrimination [14,15]. Unlike the broader classification of *Aspergillus* spp. depending on the sequence analysis of more conserved rDNA region (18S rRNA), ITS1-ITS2 regions shown a plausible accuracy on molecular identification as ensured by [14,15].

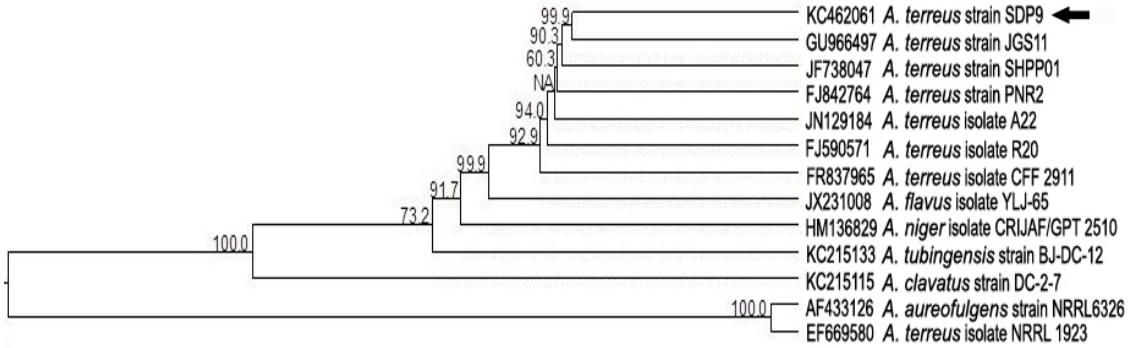

Fig 1. Phylogenetic relationship between the Aspergillusterreus (KC462061) and ITS sequences of other fungal strains retrieved from NCBI GenBank, Arrow represents the status of Aspergillusterreus (KC462061) strain from this study in phylogenetic tree.

3.2Synthesis and Characterization of AgNPs Using *Aspergillusterreus*(KC462061)

Thesynthesis of silver particles using *Aspergillusterreus*was investigated [1]**.**After addition of AgNO3 to filtered cell-free culture, the color of the mixture changed from light yellow to brown with intensity increasing during the period of incubation which indicated the formation of nanoparticles [20].

(Fig.2). Show that picture of conical flasks containing the filtrate of the*Aspergillusterreus*(KC462061)biomass in aqueous solution of 10-3M AgNO3 at the beginning of thereaction (A) and after 3 days of reaction (B). Control (without silver ions) showed no change in colour of the cell filtrates when incubated in the same conditions.

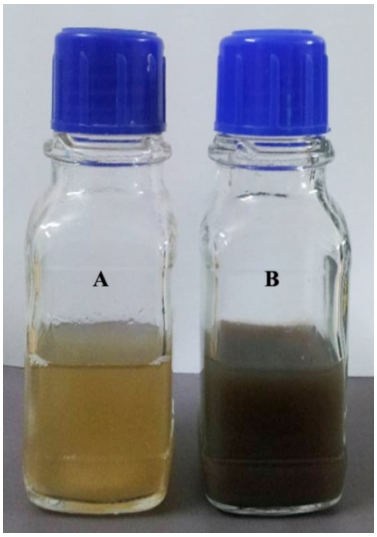

Fig. 2. Picture of two bottles containing the filtrate of theAspergillusterreus (KC462061)biomass in aqueous solution of 10^{-3} M AgNO3 at thebeginning of the reaction (A) and after 3 days of reaction (B).

3.3 Characterization and stability of fungal AgNPs

The formation nanoparticles in colloidal solution were monitored by using UV-vis spectral analysis.It was observed from spectra that the silversurface Plasmon resonance band showed an absorbance peak at 440 nm at different time intervals of reaction(Fig.3),which was specific for the silver nanoparticles and no further increase in intensity was recorded indicating complete reduction of silver ions [9]. Hypothesized that the silver ions required the NADH-dependent nitrate reductase enzyme for their reductionwhich was secreted by the fungus in its extracellular environment. The presences of NADH-dependent nitrate reductase enzyme are involved in reduction of silver ions[21, 22]. The reduction of metal ions occurs on the surface by the enzymespresented in the cell wall [23] this reduction through a nitrate-dependentreductase and a shuttle quinine extracellular process and the extracellular enzymes such asnaphthoquinons and anthraquinones they can act as electronshuttle in silver ions reduction [24].

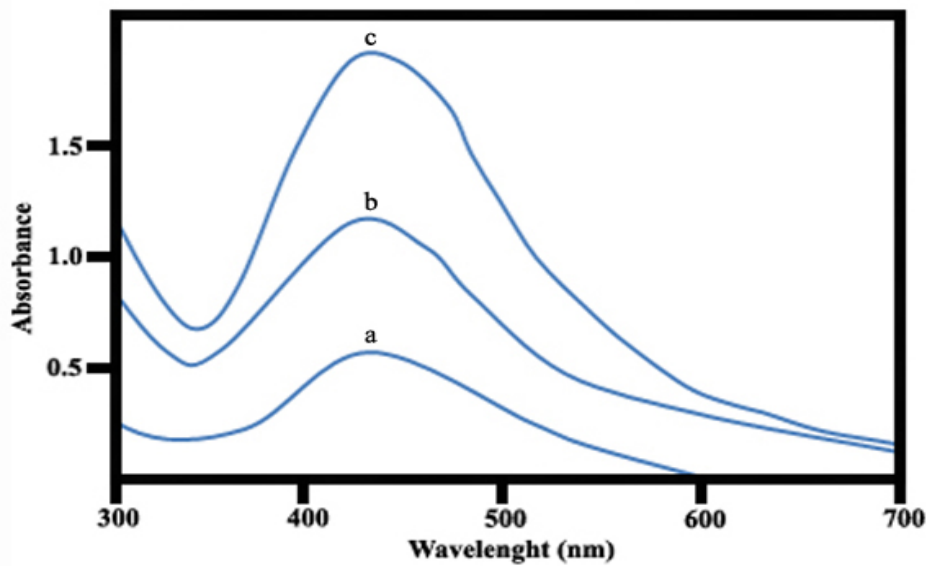

Fig. 3. The UV–Visible absorption spectra of extracellularly synthesized AgNPs byAspergillusterreus (KC462061)at 420 nm exhibiting time-dependent increase in typical SPR bands upon (a) 24 h, (b) 48 h, (c) 72 h of incubation. The inset shows the change in SPR as a function of time.

For the crystalline nature of the AgNPs, intense XRD peakswere observed corresponding to the (111), (200), (220), (311) planes at 2θ angles of 38.25°, 44.48°,65°, and 77.68°, respectively (Fig.4). These agreement with the unit cell of the face centered cubic (fcc) structure (JCPDS File No. 04-0783).

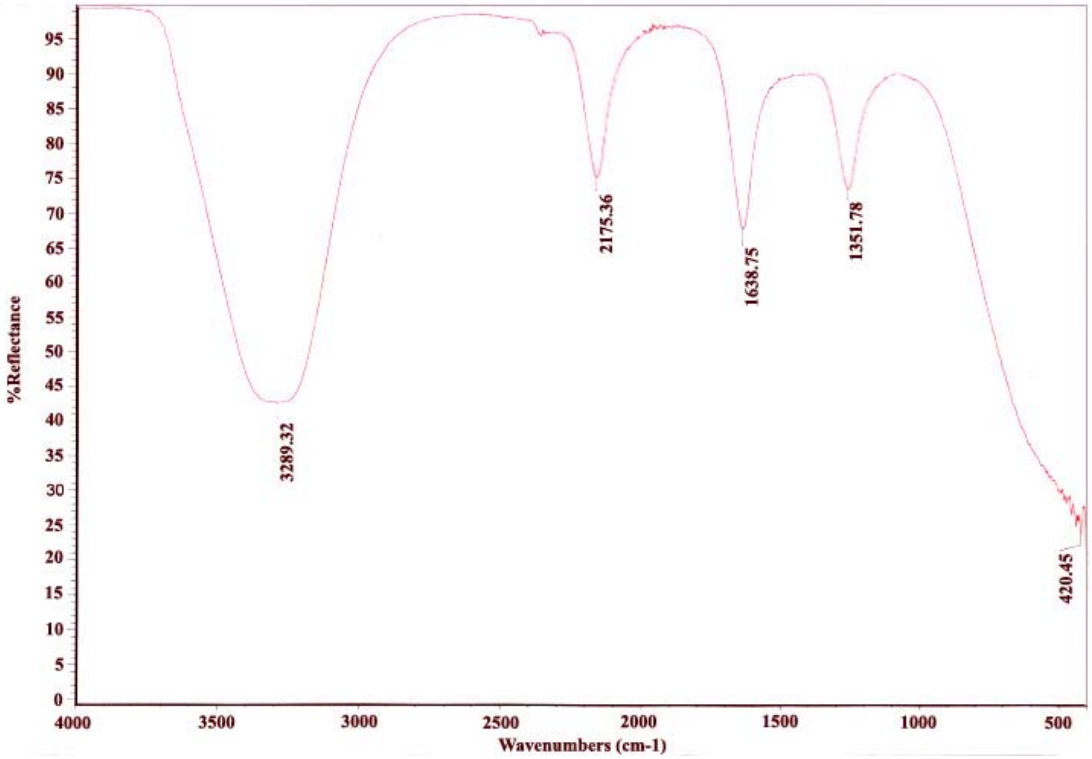

Fig. 4. Fourier Transform Infrared Spectroscopy (FTIR) spectrum of Ag nanoparticles synthesized by reduction of Ag+ ions byAspergillusterreus (KC462061)

The FTIR spectroscopy is very important to characterize the protein binding with thesilver nanoparticles and it is possible to quantify secondary structure in metal nanoparticle-proteininteraction and to confirm that amino acid and peptides have formed a coat covering the silver nanoparticles to prevent agglomeration. FTIR measurements of the dried and powdered samples of Ag-NPs showed the presence of four bands at 3289.32, 2175.36, 1638.75 and 1351.78 cm–1 (Fig.5). The bandsat 1638.75 developed for C–C and was commonly found in the proteins[25,26) and that corresponds to the bendingvibrations of the amide I and amide II bands of the proteins [27]while their correspondingstretching vibrations of primary amines were seen at 3289.32cm–1.Also revealed peak at 1351.78 for C–N stretching vibrations of aromatic amines [26].These observations indicate the presence and binding of proteins with silver nanoparticles which can lead to their possible stabilization. FT-IR study has confirmed that amino acid and peptides have formed a coat covering the silver nanoparticles to prevent agglomeration. The presence of the signature peaks of amino acids supports the presence of proteins in cell-free filtrate as observed in UV-Vis. spectra [28]. TEM measurements were used to determine the morphology and shape of nanoparticles.TEM micrographs (Fig. 6) observed that the particles of AgNPsare spherical in shape spherical or nearly sphericaland without significant agglomeration. The particle size histogram (Fig. 7)of silver nanoparticles shows that the particle size ranges from 5 to 30 nm. These results arecompatible with [1,7,29].The size of nanospheres was about17.5 nm on average. The size and shape of the biosynthesized nanoparticles appears to depend on the type of the microorganisms and the factors like temperature and pH of the medium, and thus a considerable size variability in AgNPs produced by different Aspergillusspp. [10, 30].

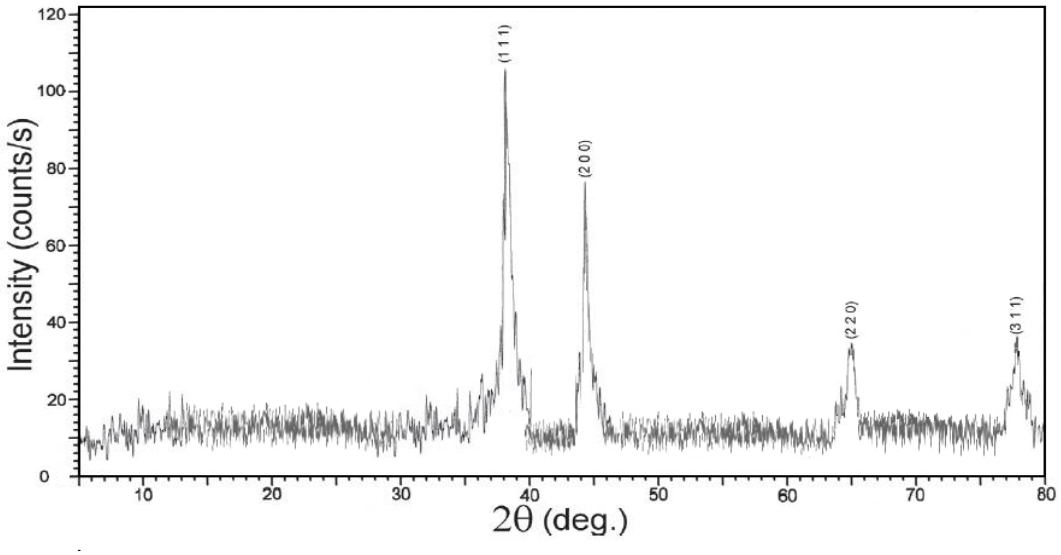

Fig. 5.Representative X-ray diffraction patterns of AgNPs synthesized by Aspergillusterreus (KC462061).

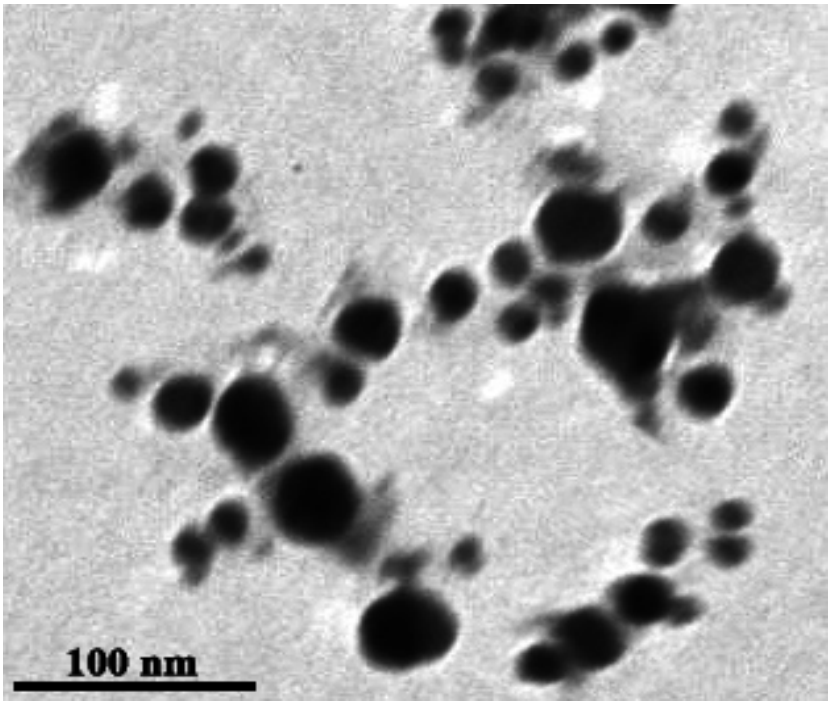

Fig. 6. Transmission Electron Microscopy (TEM) images of synthesized silver nanoparticles by Aspergillusterreus (KC462061)

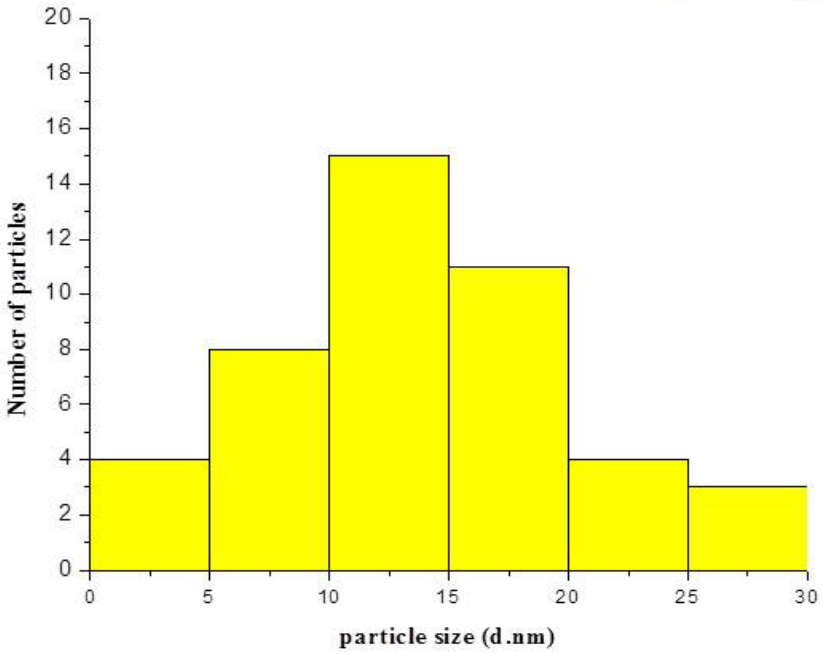

Fig. 7. A particle size distribution histogram of as synthesized silver nanaoparticles determined from Transmission Electron Microscopy (TEM) images.

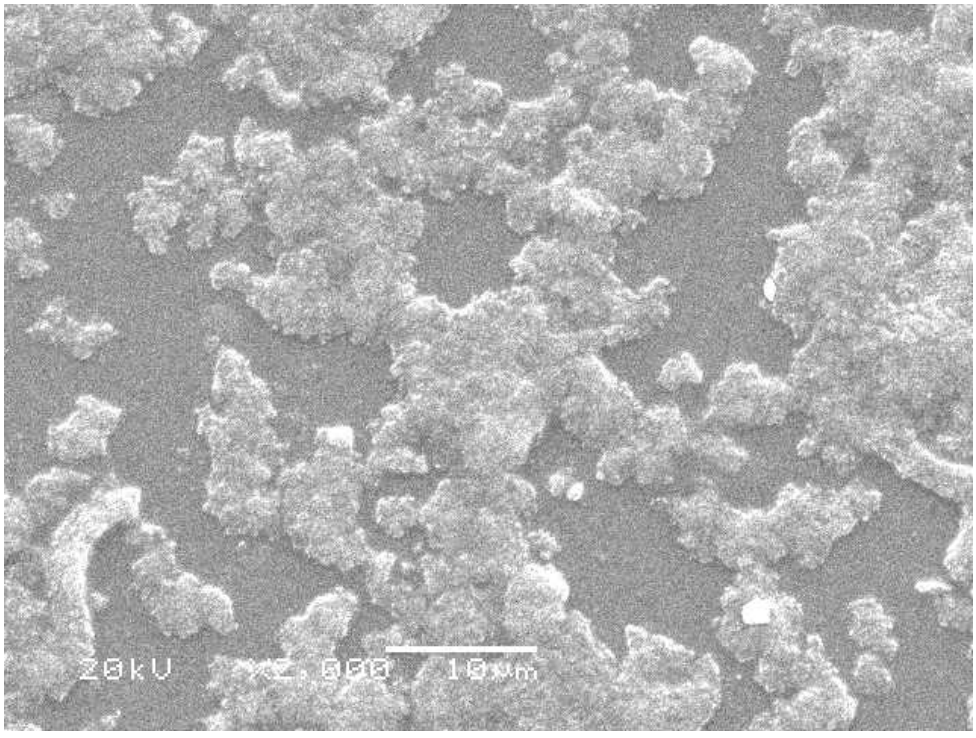

Fig. 8Transmission Electron Microscopy (TEM) images of synthesized silver nanoparticles byAspergillusterreus (KC462061)

Scanning electron micrograph (Fig.8) it was observed that the nanoparticles are partially aggregated due to the drying process can affect theirsize and shape [5]. SEM micrograph of silver nanoparticles shows, in many cases, aggregated particles due to the capping agent. Therefore, the particles size measured by SEM can be larger than the size measured byTEM [24]. The

antimicrobial activity of AgNPs against various pathogenic organisms including bacteria and fungi was investigated. Compared with the control, the diameters of inhibition zones based upon the concentration of silver used (Table 1). The AgNPs produced could inhibit including *Staphylococcus aureu* as previously described [31]**.**The AgNPs produced by *A. terreus*exhibited potent antifungal activity against *Candida albicans* which were the most important pathogenic fungi [32]. Additionally, the AgNPs showed good inhibition activity towards three kinds of filamentous fungus, which were naturally resistant to the common antifungal agent Fluconazole [33]**.**

Table 1. The inhibition zone for AgNPs synthesized by Aspergillusterreus KC462061

Tested organisms	Concentration (mM)	Inhibition Zone (mm)
Aspergillusflavus	1	12
	2	17
	4	22
Aspergillus fumigates	1	11
	2	14
	4	20
Aspergillusniger	1	13
	2	16
	4	19
Candida albicans	1	16
	2	18
	4	24
Staphylococcus aureus	1	15
	2	17
	4	20
Control	-	-

The antibacterial potency of silver is directly proportional to the concentration of silver ions in solution [34]**.** Silver ions exhibit an oligodynamic effect by denaturing the cellular proteins, inhibition of DNA replication and alteration in cell membrane permeability [35] salts is due to their extremely large surface area, which provides better contact with the microorganisms. The AgNPs release silver ions into the bacterial cells, which enhance their bactericidal activity [35]**.**

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