Biosynthesis of silver nanoparticles by *Aspergillus niger* and *Penicillium chrysogenum* and insights into the mechanism of production

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Abstract: Using microorganisms to reduce the particle size of metal particles and increase their surface area has opened up an exciting approach toward the development of natural "nano-factories". Thus, the main aim of the present study is selecting local resources for silver nanoparticles producers and studying the mechanism applied in the production process. Three different soil samples were treated with increasing concentrations of silver nitrate to isolate silver-tolerated fungi. Two fungal isolates out of six silver-tolerated fungi showed a high ability for silver nanoparticles synthesis, they are identified as *Aspergillus niger* and *Penicillium chrysogenum*. The resulted silver nanoparticles were characterized using transmission electron microscope (TEM), dynamic light scattering (DLS) and fourier transform infrared spectroscopy spectrophotometer (FTIR) in comparison with silver nanoparticles formed by chemical methods using trisodium citrate. All characterization studies showed the superiority of silver nanoparticles synthesized by biological methods (either *A. niger* or *P. chrysogenum*) over chemically formed ones. The present study proved the participation of electron shuttle as well as enzyme system in the synthesis of silver nanoparticles by selected fungi. The antimicrobial and cytotoxicity activities of produced silver nanoparticles against multi-drug resistant pathogenic microbes and cancer cell line (Hela); respectively were confirmed the superiority of silver nanoparticles synthesized by biological system over chemically formed ones. The most interesting finding is the neutral effect of silver nanoparticles synthesized by *A. niger* and *P. chrysogenum* on colon-9 normal cells. An experiment was designed to investigate the synergistic effect between different commercial antibiotics and silver nanoparticles. The promising results of this experiment opens up new horizons to the problem of antibiotics resistance.

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Key words: silver nanopaticles, *A. niger*, *P. chrysogenum*, TEM, SDL, FTIR, multi-drugs resistant microorganisms, Hela cells, Colon-9 normal cells, gel electrophoresis, reducing agents and enzymes

1.Introduction

Nanotechnology has attracted an interest in recent years due to its great impact on different fields including energy, medicine, electronics, and space industries. Nanoparticles have been synthesized by various physical and chemical processes; however, some chemical methods cannot avoid the possible hazards of toxic chemicals shared in the synthesis process **(Sau and Rogach, 2010)**. Therefore, there is an urgent need to develop a green process for nanoparticles synthesis; biological means using either microorganisms or plant extracts have offered reliable and eco-friendly alternative to chemical and physical methods.

In recent years, many fungi have been explored for nanoparticles synthesis such as *Aspergillus fumigates* **(Bhainsa and D'Souza, 2006)**, *A.niger* **(Gade *et al.*, 2008)**, *Cladosporium cladosporioides* **(Balaji *et al.*, 2009)**, *Coriolus versicolor* **(Sanghi *et al.*, 2009)**, *Fusarium oxysporum* **(Uddin *et al.*, 2008)**, *F. semitectum* **(Basavaraja *et al.*, 2008)**, *F. solani* USM3799 **(Ingle *et al.*, 2009)**, *Penicillium brevicompactum* **(Shaligram *et al.*, 2009)**, *Penicillium fellutanum* **(Kathiresan *et al.*, 2009)**, *Phaenerochaete chrysosporium* **(Vigneshwaran, 2006)**, *Phomaglomerata* **(Birla *et al.*, 2009)**, *Trichothecium sp., Trichoderma asperellum* and *T. viride* **(Ahmad *et al.*, 2005 and Fayaz *et al.*, 2010)**. Fungi are more advantageous compared to other microorganisms in many ways. They are easy to grow, handle and fabricate. The mesh of their mycelia can withstand flow pressure and agitation in bioreactors or other fermentation chambers compared to bacteria and plant materials.

Silver nanoparticles, due to their unique properties, find use in many day-to-day applications in human life.They are used in manufacturing of high performance delicate electronics, fabric cleaner and antireflection coating materials. They improve the transfer of heat from collectors of solar energy to their fuel tanks. The antimicrobial activity of silver nanoparticles is the most exploited application of silver nanoparticles in the medical field. They can also inhibit the activities of interferon gamma and tumor

necrosis factor alpha which are involved in inflammation **(Shin *et al.*, 2007)**. In addition, they are applied for many medical treatments including intravenous catheters, endotracheal tubes; wound dressings, bone cements, and dental fillings. Biosensors based on silver nanoparticles are excellent candidates for cancer diagnosis or treatment in favor to their plasmonic properties **(Loo *et al.*, 2005)**.

The objectives of this study are: (1) isolation and identification new candidates able to produce silver nanoparticles from local sources; (2) characterization of produced silver nanoparticles (biological origin) in comparison with chemical synthesized ones by standard techniques; (3) studying the mechanism involved in silver nanoparticles production by selected fungi; and (4) investigating the antimicrobial and the cytotoxicity of produced silver nanoparticles.

2.Material and Methods

Microorganisms and isolation process

Three samples of clay soils were collected from Al-Gharbia, Cairo and Giza Governorates. Each sample was distributed in pots; each one contains 100g soil. Increasing concentrations of silver nitrate (Ranging from 5mM to 30 mM) were added to the soil pots. Humidity of soil pots was maintained at 75% of complete saturation. The pots were incubated for 7days at room temperature. After that, isolation process was performed according to the standard methods and total fungal count for each pot was determined.

Biosynthesis of silver nanoparticles

The ability of isolated fungi to grow in the presence of high concentration of silver nitrate was individually studied in a preliminary experiment. Fungal isolates could grow at 20 mM silver nitrate were selected. Under aseptic condition, hundred milliliters of liquid media with following composition, in grams per liter: sucrose, 3g; $NaNO_3$, 2g; KH_2PO_4, 0.1g; $MgSO_4$. $7H_2O$, 0.5g; KCl, 0.5g were inoculated with fungal spore suspension of selected fungal isolates and incubated at 28±2°C. After 72h of incubation, the fungal pellets were filtered through Whatman No.1 filter paper and washed with double distilled water. For each tested fungal isolates, 10 g of well developed pellets were aseptically transferred into 250 ml Erlenmeyer flask containing 100 ml double distilled water and incubated for 72h in an orbital shaker (100 rpm) at 28±2°C. Then, pellets-free extract was obtained by filtration and silver nitrate (1mM) was added. The reaction mixture was kept as the same incubating conditions as previously described for 72 h, after which silver nanoparticles production was estimated. Conical flasks with either pellets-free extract or $AgNO_3$ were served as negative controls.

Characterization of silver nanoparticles

For comparison aim, silver nanoparticles were chemically synthesized by reduction of silver nitrate with trisodium citrate according to the procedure previously described **(Fang *et al.*, 2005)**. The production of silver nanoparticles by selected fungi was visually observed by changing the color of reaction mixture (containing silver nitrate and pellets-free extract) from colorless to yellowish-brown. The production of silver nanoparticles was confirmed by UV-visible spectra at different time intervals by periodic sampling using Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm. The fluorescence measurements were carried out on a Perkin-Elmer LS 50B luminescence spectrophotometer.

Characterization and stabilization of silver nanoparticles were studied using transmission electron microscope (Hitachi-H-7500) to determine the size and the shape of the produced nanoparticles. To confirm the size of synthesized silver nanoparticles and determine the actual size distribution, the produced silver nanoparticles were analyzed using dynamic light scattering in aqueous solution by PSS-NICOMP 380-ZLS particle sizing system St. Barbara, California, USA. In addition, the probable bio-molecules responsible for reduction, capping, and effective stabilization of the synthesized silver nanoparticles were recorded using fourier transform infrared spectroscopy spectrophotometer (Nicolet 6700, Thermo scientific) in the range of 500 to 4000 cm^{-1}.

Insights into the mechanism of silver nanoparticles production

To characterize enzymes that may participate in silver nanoparticles production by *A. niger* and *P. chrysogenum*, twenty grams of freshly developed pellets were suspended in 100 ml of sterile distilled water and incubated on an orbital shaker (100 rpm) at 28±2°C for 72h. Afterthat, pellets were removed by filtration, and the pellets-free extract of each fungus was concentrated by ultra-filtration against sucrose using a dialysis membrane (molecular weight cutoff 12 kDa). The concentrated aqueous extract containing proteins was analyzed by gel electrophoresis using sodium dodecyl sulphate: polyacrylamide (12% & 5% W/V) at pH 8 **(Laemmli, 1970)**.

In an individual experiment, three reaction mixtures containing silver nitrate and the pellets-free extract were prepared for each selected fungus. One of them was boiled at 100°C for 15 min to inactivate enzymes that may participate in silver nanoparticles production. Potassium permanganate at 0.1N concentration was added to the second treatment to inactivate probable reducing agents present in the

mixture in the second set whereas the rest set was served as positive control.

To identify the mechanism involved in silver nanoparticles, selected fungi were screened for carrying gene encodes the nitrate reductase enzyme (nai D). DNA extraction was made using the protocol of GeneJet Plant genomic DNA purification Kit (Thermo, # K0791). Polymerase chain reaction was performed by PCR (Techchni instrument). PCR reaction mixture consists of; 3 µg template DNA, 20 µl Maxima HotStart PCR Master Mix (2x), 5 µM of each primer and completed up to 50 µl of nuclease-free water. The amplification conditions were as the following; one cycle represents initial amplification at 95°C for 5 min. followed by 40 cycles of denaturation (95°C for 30 s), annealing (65°C for 1 min), extension (72°C for 1 min/kb) and finally the process ends by final extension at 72°C for 15 min. PCR mixture (5 µl) was loaded on 1% agarose gel to examine the PCR product against 3Kb plus ladder (Thermo).To avoid incorporation of mismatch of PCR products, at least three individual clones were analyzed. After completing electrophoresis, DNA bands were visualized using UV trans-illuminator and Gel Doc Biometra by the aid of the ethidium bromide fluorescence.

Applications studies

All Multi-drug resistant bacteria used in the present study (*Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus*) as well as *Candida albicans* were kindly provided by El-Mattaria Learning Hospital and Kept at Department of Radiation Microbiology, National center for radiation research and Technology as reference strains. The selected fungi were identified according to morphological characteristics at Mycological center, Assuit University, Egypt (AUMC).

For the biomedical applications of silver nanoparticles, the concentration of silver nanoparticles in nanograms was determined according to calculations previously reported **(Marquis *et al*., 2009)** and it is equivalent to 260 nM/ ml.

The antimicrobial activity of different concentrations of silver nanoparticles (20, 30 & 40 µl) was studied against 18h-old cultures of pathogenic strains mentioned earlier by well-plate technique on nutrient agar medium for bacteria and peptone dextrose agar for yeast. Disk diffusion assay was used to evaluate the combined effects of silver nanoparticles and standard antibiotics (ampicillin, cefotaxime, ceftazidime, ceftriaxone, doxycycline, gentamicine and nitrofurantoin). Standard antibiotics disks were purchased from Mast Co. (Liverpool, UK). For combining studies, standard antibiotic disks with known concentration were impregnated onto 10ml of freshly prepared silver nanoparticles suspension. All assays were performed in triplicate.

Hela cells and colon-9 normal cell lines were kindly provided by Department of virology and tissue culture, faculty of Medicine, Al-Azhar University, Cairo. These cells were used to evaluate the cytotoxicity of different concentrations of silver nanoparticles. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. The cell viability assay was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide dye reduction assay. Briefly, both cell lines were plated onto 96-well flat bottom culture plates with various concentrations of silver nanoparticles. All cultures were incubated for 24h at 37°C in a humidified incubator. After 24h, 10 µl of MTT (5mg/ml in PBS) was added to each well, and the plate was incubated for a further 4h. The resulting formazan was dissolved in 100 µl of DMSO with gentle shaking at 37°C, and absorbance was measured at 595 nm with an ELISA reader (Spectra MAX; Molecular Devices, USA). The results were given as the mean of three independent experiments. Concentration of silver nanoparticles that shows 50% reduction in the cell viability was calculated.

Statistical analysis

The significance of the data with respect to efficiency of different concentrations of silver nanoparticles either individually or coupled with different commercial antibiotics as antimicrobial agent; three replicates for each treatment; was evaluated using one-way analysis of variance ANOVA. All the analyses were performed with SAS software package version 6.12.

3.Results

Isolation of fungal strains that have the ability to produce silver nanoparticles

A preliminary experiment was carried out to isolate silver nitrate-tolerated fungi present in Egyptian soil samples. Results revealed that total fungal count in tested soil samples was decreased by increasing silver nitrate concentration. About 95% of total fungal count was declined by increasing silver nitrate concentration from 5 mM to 20 mM (Data not shown). At 30 mM concentration of silver nitrate, the fungal population was disappeared completely from all tested soil samples.

Six fungal isolates; belong to different fungal genera; able to grow at minimum inhibitory concentration of silver nitrate (20 mM) were selected and individually tested for silver nanoparticles production. Visual observation of the reaction mixture that changes from colorless to brownish-yellow color as well as recording the strength of surface plasmon resonance at 400-450 nm are the main criteria used as

indicators for transforming silver from elemental form into nanoparticles. Table (1) showed that there is no appreciable change in the UV-Vis spectra of the reaction product after 72 hours; thus the reaction reaches to the equilibrium state. It should be mentioned that the reaction extremely stable for almost a month without evidence of nanoparticles aggregation. The absorption spectrum also recorded the presence of other peaks at 220, 260 and 280 nm.Fungal isolates that achieved the maximum strength at 400-450 nm were selected as the most active silver nanoparticles producers and identified as *Aspergillus niger* and *Penicillium chrysogenum* at AUMC.

Characterization of silver nanoparticles produced by *Aspergillus niger* and *Penicillium chrysogenum*

TEM-images of silver nanoparticles synthesized by *Aspergillus niger* and *Penicillium chrysogenum* as well as chemically formed ones by trisodium citrate were represented in figure (1).These images show individual silver nanoparticles as well as number of aggregates; an individual one is spherical in shape for silver nanoparticles synthesized by biological system. The size range of silver nanoparticles produced by *A. niger* is 10-20 nm (Figure 1a) whereas a smaller size 5-10 nm was observed for nanoparticles synthesized by *P. chrysogenum* (Figure 1b). The chemically formed silver nanoparticles have a greater particle size (30-80 nm) and shaped in triangle or polygonal grains (Figure 1c). Results also showed the morphology and size of silver nanoparticles were stable even after 6 months (Data not shown). The size distribution of biologically produced by *A. niger* and *P. chrysogenum* or chemically formed silver nanoparticles was estimated by dynamic light scattering in aqueous solution. Figures (2a, 2b & 2c) cleared that the average size of the produced nanoparticles is 20, 10 and 69.4 nm; respectively.

In addition to silver nitrate and silver nanoparticles, the reaction mixture contains different chemical compounds having various functional groups that determined by FTIR (Figures 3a & 3b). FTIR spectrums of silver nanoparticles synthesized by *A. niger* and *P. chrysogenum* showed intense absorption bands between (1143.6 and 3899.8 cm^{-1}) and (1396 and 3530.1 cm^{-1}), respectively. After 6 months, FTIR spectrum analysis of silver nanoparticles synthesized by the both fungi revealed a little shift in the absorption spectrum (Data not shown). FTIR spectrum analysis of silver nanoparticles chemically formed by trisodium acetate showed intense absorption bands between 1144.7 and 3631.1 cm^{-1} (Figure 3c).

Probable mechanism involved in the silver nanoparticles production

To understand the mechanism involved in the reduction process of silver in metallic form by selected fungi (*Aspergillus niger* and *Penicillium chrysogenum*) into silver nanoparticles. FTIR results demonstrated the presence of chemically function groups as C=O stretch and N-H bend that may be attributed to the secondary structure of proteins. Identification and characterization of these proteins was studied using gel electrophoresis. Figure (4) clears the presence of more than seven different proteins (probably extracellular enzymes) produced by both selected fungi in the reaction mixture, their molecular masses are ranged between 11kDa and 180 kDa.

An experiment was conducted to predict whether proteins in the form of enzymes were the sole criteria involved in the reduction of silver nitrate to silver nanoparticles or there is another mechanism participates in the reduction process. For each selected fungus, three treatments are designed as previously described in material and methods section. Boiling pellets-free extracts represent silver nanoparticles synthesized by inorganic reducing agents present in the reaction mixture since enzymes are denaturized by boiling. In the second set, addition of potassium permanganate to pellets-free extract oxidize all inorganic reducing agents exist in the reaction mixture. Thus, reducing enzymes are responsible for silver nanoparticles production. It is worth to mention that the amount of potassium permanganate needed to oxidize all the inorganic reducing agents in the pellets-free extract of *A. niger* is higher than those exist in the pellets-free extract of *P. chrysogenum* (Nearly two folds). Results showed that the set of pellets-free extract of both selected fungi produce silver nanoparticles even after boiling at 100°C for 15 min. Thus, the reduction of silver nitrate may be occurred due to the presence of inorganic reducing agents (electron shuttle). Results revealed that electron shuttle is the main cause of silver nitrate reduction in *A. niger* but the electron shuttle participates with reducing enzymes in the biosynthesis of silver nanoparticles by *P. chrysogenum*.

There are many types of reducing enzymes that may participate in the silver nanoparticles synthesis by selected fungi but the most previous review mentioned that nitrate reductase is the greatened cause. Genetic tools were used to identify nitrate reductase gene in *A. niger* and *P. chrysogenum.* By aiding to gene bank database, two primers were designed for each studied fungus using Primer Plus® version 3 and oligonucleotides were purchased from Sigma Company.

For *A. niger*,
Left: 5'-AACAGACGGAAGGAGCAGAA-3'
Right: 5'-CGATATGGTAATCGGGCATC-3'
For *P. chrysogenum*,
Left: 5'-TGCAGGAAACAAACGACAAG-3'

Right: 5'-ACGGGTCAAGAAACCATCAG-3'

Figure (5) clears PCR amplified products of nitrate reductase gene (nia D), the amplified fragment was as expected 1500 bp. Results revealed the presence of nitrate reductase gene only in *P. chrysogenum* not in *A. niger*. Thus, the reduction of silver nitrate into nanoparticles by *A. niger* may be due to another reductase or dependent mainly on electron shuttle.

Comparing the activity of silver nanoparticles synthesized by *Aspergillus niger* and *Penicillium chrysogenum* with chemically synthesized analogue as antimicrobial and cytotoxic agents

Table (2) showed that all tested silver nanoparticles whatever their origin (chemically or biologically synthesized) have antimicrobial activity against pathogenic bacterial strains under investigation either Gram + ve or Gram –ve and *Candida albicans* that represents pathogenic yeast. The antimicrobial activity of biologically synthesized silver nanoparticles was more obvious than chemically synthesized one and in Gram - ve bacteria than Gram + ve bacterial pathogenic tested strains.

Coupling silver nanoparticles with different commercial antibiotics improves the antimicrobial activity of commercial ones as shown in table (3). The activity of some antibiotics such as cefotaxime, ceftazidime, ceftriaxone and ampicillin/sublactam was more obvious after coupling with silver nanoparticles against pathogenic microbes under investigation even they show no microbial activity before coupling process. In individual experiment, the Hela cancer cells or Colon-9 normal cells were treated with silver nanoparticles either chemically formed or biologically synthesized ones suggested that all tested silver nanoparticles reduced the cell viability of Hela cancer cells in a dose dependent manner however the biologically synthesized silver nanoparticles showed more obvious effect than chemically formed analogues (Figure 6a, 6b & 6c). It is worth to mention that the biologically produced nanoparticles have a neutral effect on colon-9 normal cells whereas chemically formed nanoparticles have a lethal effect but in lesser degree that observed for Hela cancer cells.

Table (1): Screening the silver*-tolerated fungal isolates as silver nanoparticles producers

Soil samples	Silver-tolerated fungal isolates	The intensity of λ maximum** at different incubation periods		
		24 h	72 h	96 h
Loamy soil collected from agricultural field in Al-Gharbia Governorate	F_1	1.077	1.990	2.100
	F_2	0.924	1.780	1.896
Loamy soil collected from special garden in Cairo Governorate	F_3	1.200	1.010	0.998
	F_4	0.900	1.313	1.00
Loamy soil collected from public park in Giza Governorate	F_5	0.590	0.210	0.200
	F_6	0.626	0.500	0.450

* Silver was supplemented in the form of silver nitrate.

** The intensity of λ maximum was measured at 400-450 nm as indication for silver nanoparticles production.

- Fungal isolates (F_1 and F_2) were identified as *Aspergillus niger* and *Penicillium chrysogenum*, respectively.

Table (2): The efficiency of different concentrations of silver nanoparticles as an antimicrobial agent

Pathogenicmicrobial strains	Efficiency of different concentrations of silver nanoparticles * (%)								
	Biologically- synthesized (*A. niger*)			Biologically- synthesized (*P. chrysogenum*)			Chemically- synthesized		
	10	20	30	10	20	30	10	20	30
B. cereus	137.5 ± 0.4^{C}	150.0 ± 0.1^{B}	175.0 ± 6.3^{A}	125.0 ± 3.8^{DE}	137.5 ± 0.1^{C}	162.5 ± 0.9^{B}	112.5 ± 3.6^{E}	125.0 ± 3.1^{D}	137.5 ± 7.3^{CD}
C. albicans	106.3 ± 0.1^{E}	131.3 ± 3.6^{C}	150.0 ± 0.1^{A}	137.5 ± 6.3^{C}	150.0 ± 0.1^{A}	156.3 ± 3.6^{A}	93.8 ± 0.1^{F}	118.8 ± 0.1^{D}	137.5 ± 1.8^{B}
E. coli	115.4 ± 2.2^{E}	123.1 ± 0.1^{D}	146.2 ± 0.1^{A}	107.7 ± 2.3^{F}	130.8 ± 2.2^{C}	146.2 ± 3.9^{B}	72.3 ± 2.2^{H}	100.0 ± 0.1^{G}	123.1 ± 0.1^{D}
P. aureoginosa	212.5 ± 0.1^{E}	225.0 ± 0.1^{D}	262.5 ± 7.3^{B}	200.0 ± 0.1^{F}	225.0 ± 0.1^{C}	275.0 ± 0.1^{A}	162.5 ± 7.3^{H}	187.5 ± 0.1^{G}	237.5 ± 0.1^{C}
S. typhi	104.1 ± 5.3^{E}	104.1 ± 5.3^{D}	172.7 ± 9.5^{A}	109.1 ± 5.3^{E}	136.4 ± 5.3^{C}	154.5 ± 9.4^{B}	90.0 ± 0.1^{F}	109.1 ± 5.3^{E}	127.3 ± 5.3^{D}
S. aureus	162.5 ± 7.3^{C}	187.5 ± 9.0^{C}	212.5 ± 1.3^{A}	162.5 ± 7.3^{C}	175.0 ± 7.3^{C}	200.0 ± 9.4^{B}	112.5 ± 0.1^{E}	137.5 ± 6.3^{D}	175.0 ± 7.3^{C}

* The efficiency of silver nanoparticles as antimicrobial agent compared to broad spectrum amoxicillin antibiotic.

- Statistical analysis was individually done for each tested pathogenic microbe, different symbols means there is a significant difference.

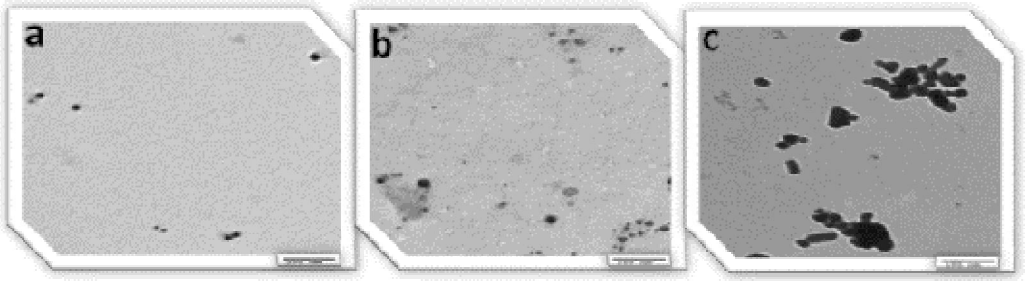

Figure (1): TEM micrograph of different silver nanoparticles synthesized by:

a- ***Aspergillus niger*** (The size is in range 10- 20 nm)

b- ***Penicillium chrysogenum*** (The size is in range 5- 10 nm)

c- **Trisodium citrate** (The size is in range 30- 50 nm)

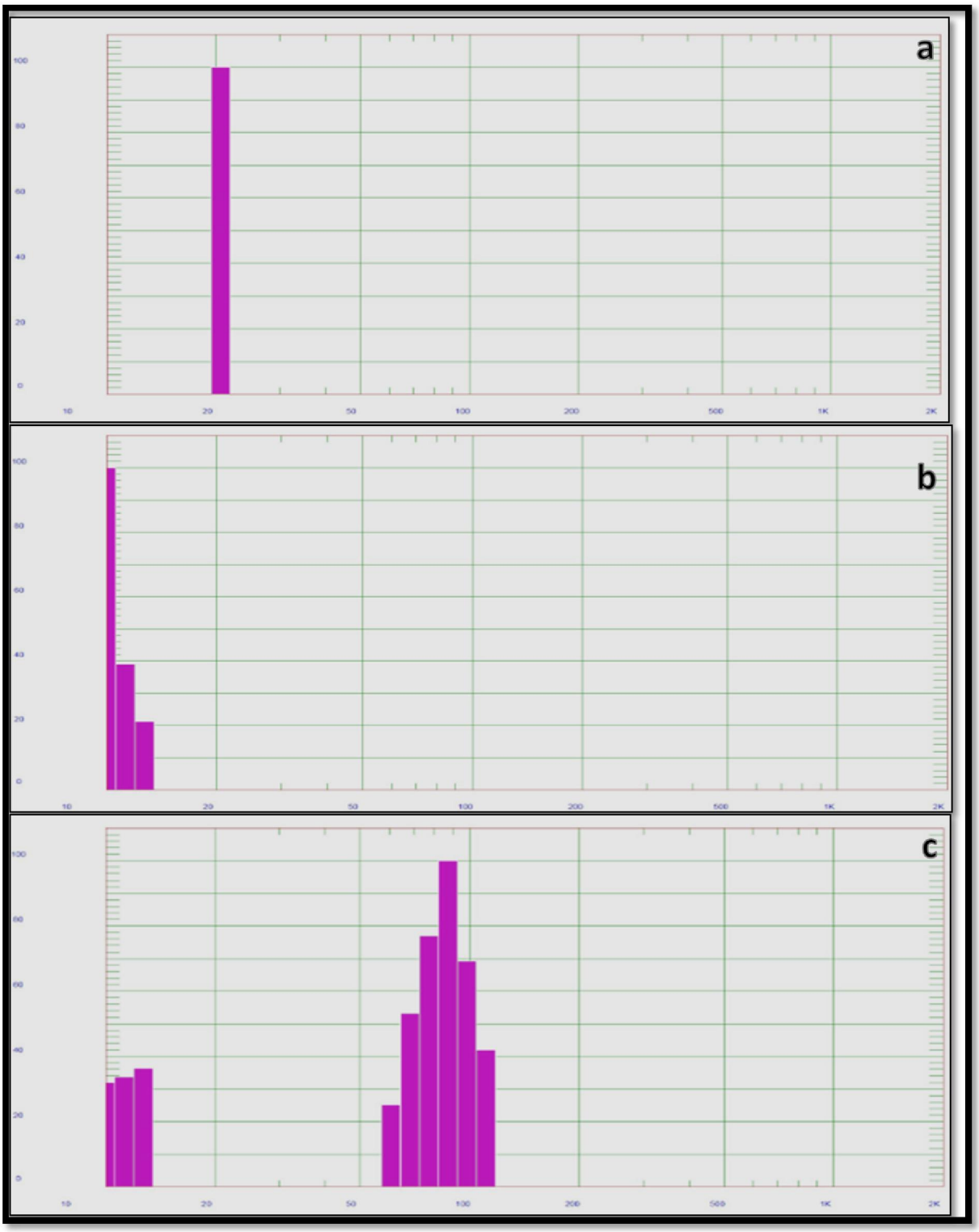

Figure (2): Particle size distribution of different silver nanoparticles measured by DLS

a- ***Aspergillus niger*** (Mean diameter = 20.6 nm & Fit error = 21.51)

b- ***Penicillium chrysogenum*** (Mean diameter = 10.7 nm & Fit error = 4.84)

c- **Trisodium citrate** (Mean diameter = 69.4 nm & Fit error = 28.66)

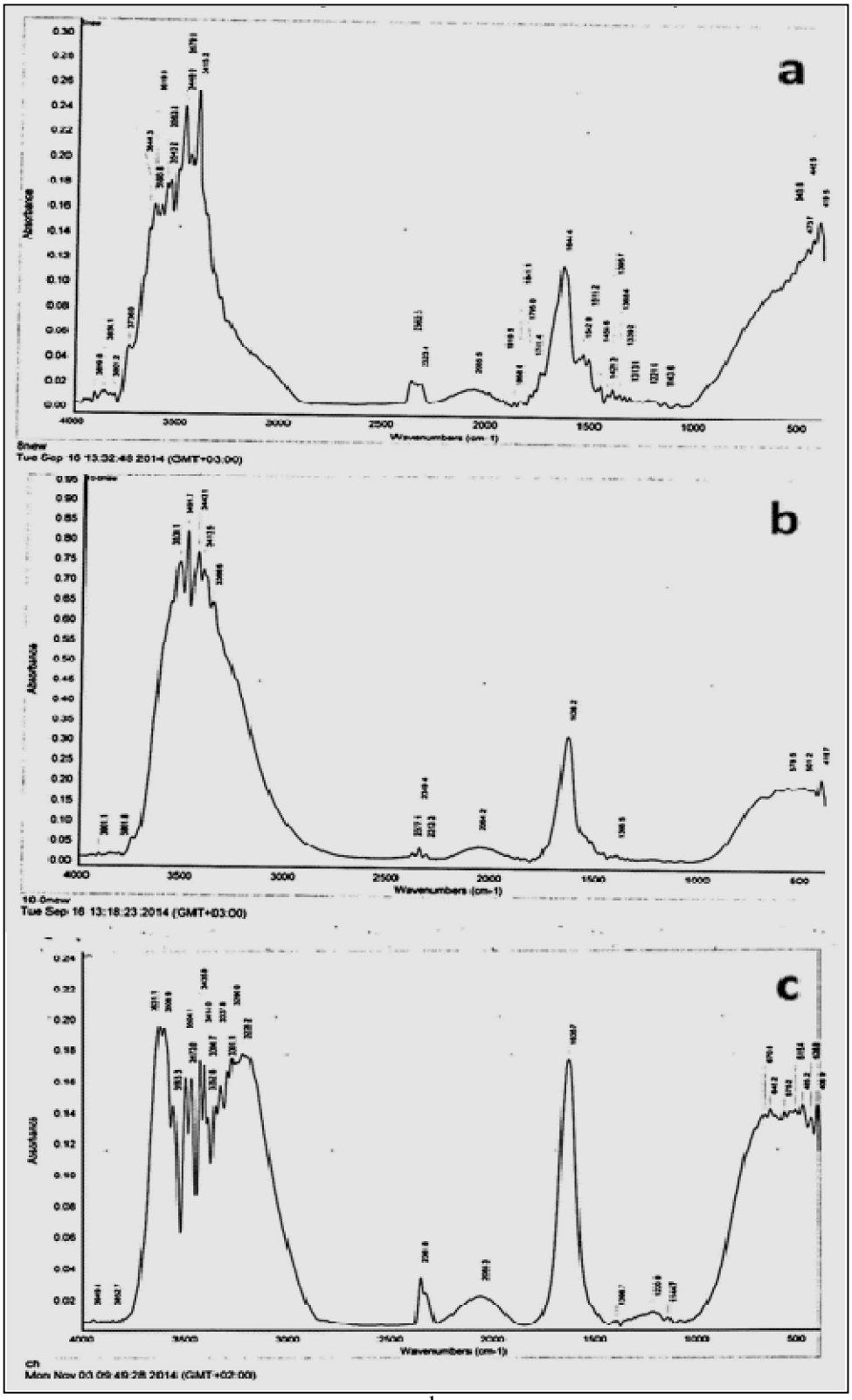

Figure (3): FTIR spectra in the rangeof 500 to 4000 cm^{-1} of different silver nanoparticles synthesized by: a- ***Aspergillus niger*****; b-*****Penicillium chrysogenum;*** **c- Trisodium citrate**

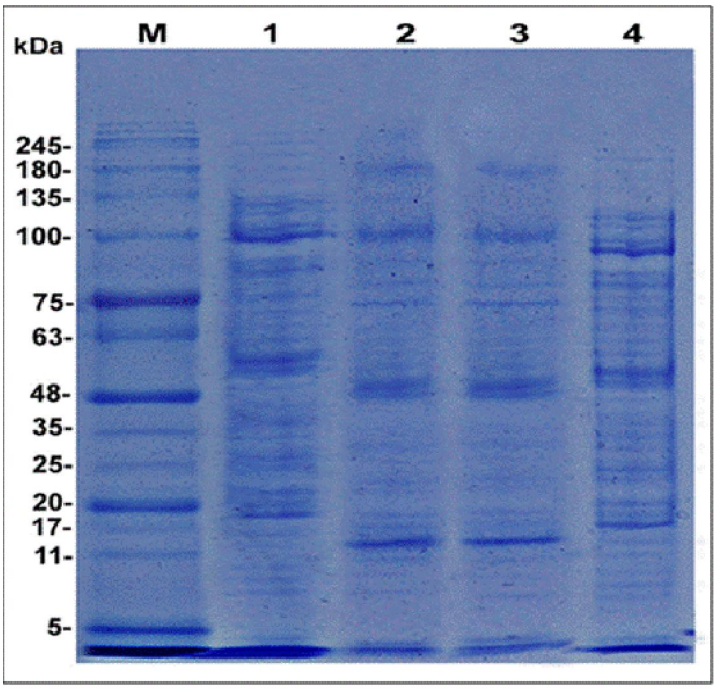

Figure (4): SDS-PAGE gel **ilver nanoparticles**
M is a marker protein (245 k
Lane 1: Crude proteins relea
Lane 2: Crude proteins relea °C.
Lane 3: Crude proteins relea
Lane 4: Crude proteins relea

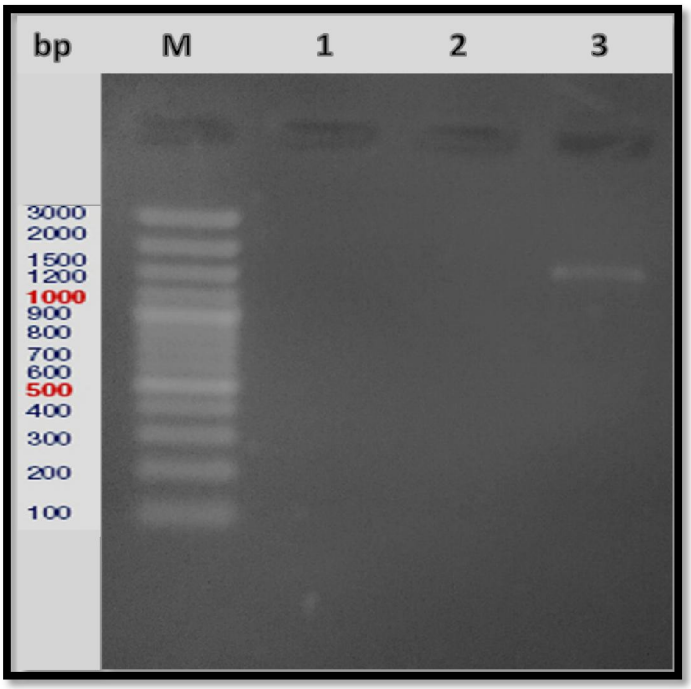

Figure (5): Agarose gel electrophoresis of PCR products amplified for detection nitrate reductase (nia D) gene
M: 3000 bp DNA ladder
Lane 1: Negative control
Lane 2: No detected band for nia D gene using genomic DNA extracted from *Aspergillus niger*
Lane 3: Amplified fragment with 1500 bp for nia D gene using genomic DNA extracted from *Penicillium chrysogenum*

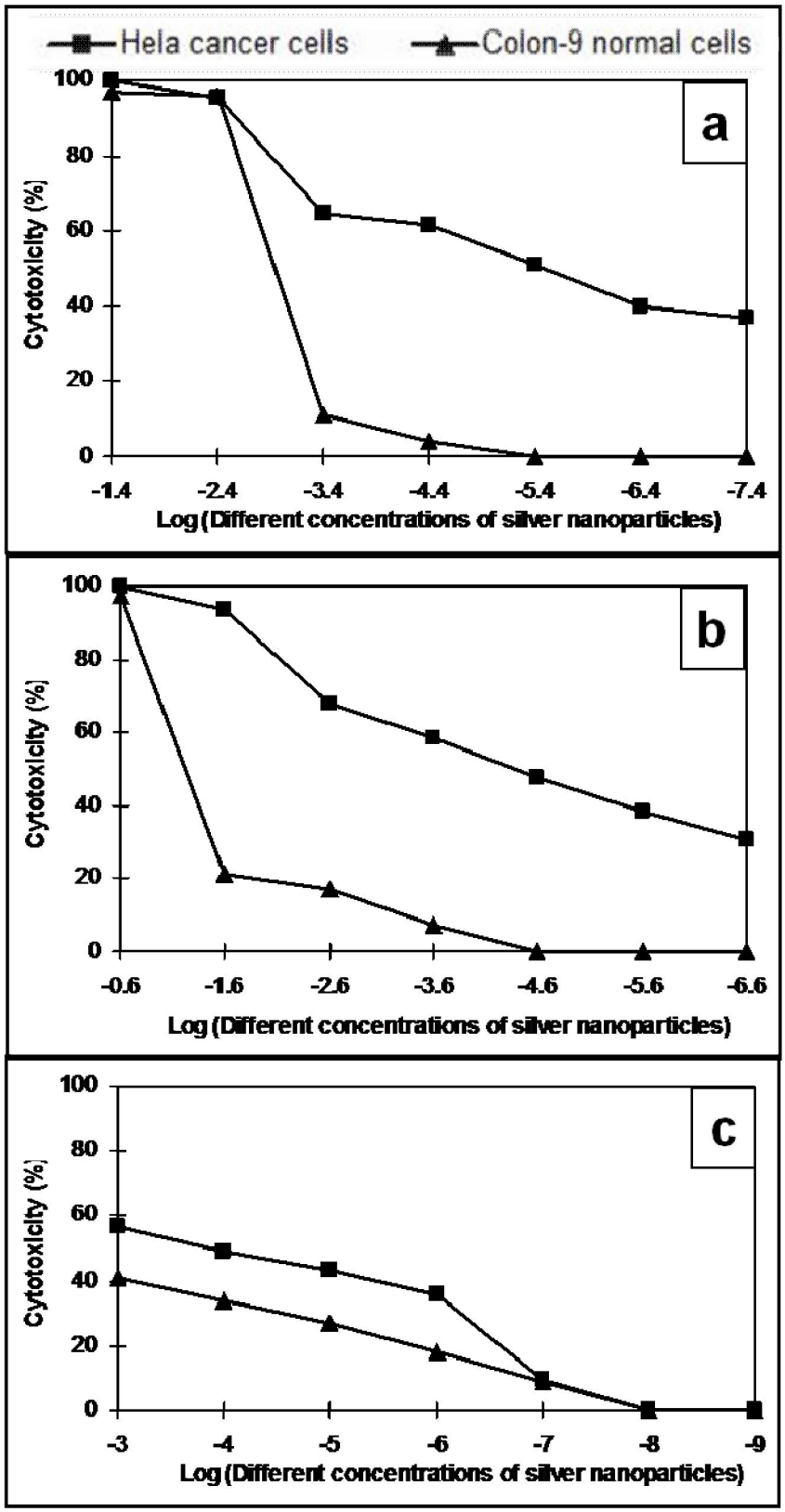

Figure (6): Cytotoxic effect of different concentrations of silver nanoparticles synthesized by: a- ***Aspergillus niger;*** **b-*Penicillium chrysogenum;* c- Trisodium citrate**

Table (3): The synergistic effect of silver nanoparticles synthesized by *Aspergillus niger* and *Penicillium chrysogenum* and different commercial antibiotics

Pathogenic microbial strains	Efficiency of silver nanoparticles coupled with different antibiotics compared to individual antibiotics (%)			
	Supplemented antibiotics	Biologically- synthesized (*A. niger*)	Biologically- synthesized (*P. chrysogenum*)	Chemically- synthesized
B. cereus	Ampicillin/subactam	275.0±3.63	237.5±3.63	212.5±6.25
	Cefotaxime	260.0±5.80	240.0±0.20	220.0±9.99
	Ceftazidime	260.0±6.00	240.0±0.20	200.0±5.80
	Ceftriaxone	260.0±5.80	240.0±5.80	220.0±5.80
	Doxycycline	141.9±0.32	138.7±0.94	129.0±0.94
	Gentamicin	181.3±3.63	175.0±1.81	156.3±3.63
	Nitrofurantoin	176.5±1.71	164.7±1.76	152.9±1.76
C. albicans	Ampicillin/subactam	237.5±1.81	243.8±1.88	225.0±1.88
	Cefotaxime	375.0±2.63	387.5±7.25	350.0±6.25
	Ceftazidime	260.0±6.00	240.0±6.00	220.0±5.80
	Ceftriaxone	250.0±2.14	264.3±2.07	194.1±1.71
	Doxycycline	150.0±0.73	152.5±0.75	118.0±0.58
	Gentamicin	257.1±2.14	271.4±2.14	250.0±2.14
	Nitrofurantoin	200.0±1.38	209.5±0.05	190.5±1.38
E. coli	Ampicillin/subactam	160.7±1.07	167.9±1.07	139.3±1.06
	Cefotaxime	360.0±5.80	380.0±6.00	300.0±6.09
	Ceftazidime	380.0±9.99	340.0±6.00	280.0±6.00
	Ceftriaxone	300.0±3.63	312.5±3.63	389.0±5.78
	Doxycycline	137.5±0.03	137.5±0.75	125.0±0.76
	Gentamicin	200.0±2.00	193.3±0.07	166.7±1.95
	Nitrofurantoin	200.0±2.00	193.3±0.07	173.3±3.30
P. aureoginosa	Ampicillin/subactam	260.0±4.98	240.0±0.20	220.0±6.09
	Cefotaxime	280.0±6.00	260.0±0.20	240.0±5.77
	Ceftazidime	260.0±5.99	240.0±6.00	220.0±5.81
	Ceftriaxone	280.0±6.37	260.0±5.84	240.0±5.99
	Doxycycline	166.7±1.11	174.1±0.04	140.7±1.05
	Gentamicin	220.0±2.00	680.0±5.82	173.3±1.99
	Nitrofurantoin	260.0±1.58	240.0±5.80	200.0±3.90
S. typhi	Ampicillin/subactam	245.5±2.64	218.2±0.09	200.0±2.64
	Cefotaxime	260.0±6.00	260.0±6.03	220.0±5.68
	Ceftazidime	280.0±6.04	260.0±6.00	200.0±5.45
	Ceftriaxone	280.0±0,20	260.0±5.81	220.0±5.82
	Doxycycline	154.8±0.71	154.8±0.02	142.9±0.02
	Gentamicin	185.0±1.53	175.0±1.45	160.0±1.50
	Nitrofurantoin	184.0±1.58	137.7±1.53	163.2±1.58
S. aureus	Ampicillin/subactam	170.0±0.05	165.0±1.50	150.0±1.52
	Cefotaxime	216.7±2.50	208.3±2.42	183.3±2.50
	Ceftazidime	260.0±6.07	240.0±6.07	200.0±0.21
	Ceftriaxone	225.0±2.50	225.5±0.08	191.7±4.17
	Doxycycline	150.0±0.89	150.0±0.89	128.6±0.86
	Gentamicin	177.0±3.21	172.2±3.17	155.6±0.06
	Nitrofurantoin	146.4±1.07	142.9±0.04	132.1±1.07

4.Discussion

The development of silver nanoparticles expected to open new avenues in many fields. The synthesis and assembly of nanoparticles would benefit for the development of clean, nontoxic and environmentally acceptable procedures, probably involving organisms ranging from bacteria to fungi and even plants **(Mohanpuria *et al*., 2008)**. The site of nanoparticles synthesis either intracellular or extracellular is a critical factor that effecting the development of the process in the industrial scale since the down-stream processing posses many difficulties and complications. In addition, unnecessary adjoining with cellular components of producer organism favors procedures based on the extracellular mechanism. Thus, fungi are regarded as ideal candidates for nanoparticles production because of their great enzyme system, which involves in the reduction and capping of nanoparticles.

The present study aims to select new candidates for industrial production of silver nanoparticles from local sources. In a preliminary experiment, it was found that the count of fungal populations was influenced by increasing silver nitrate concentrations. This is could be attributed to the toxic effect of silver nitrate on the microorganisms (**Morones *et al.*, 2005**). Two fungal isolates out of six silver-tolerated fungi showed high productivity to silver nanoparticles. On

the basis of colony characteristics and microscopic appearance, these fungal isolates are identified as *Aspergillus niger* and *Penicillium chrysogenum*. Few reports previously mentioned the synthesis of silver nanoparticles using *Penicillium* and *Aspergillus* genera **(Jaidev and Narasimha, 2010, Ganachari *et al*., 2012** and **Singh *et al*., 2014)**.

Biosynthesis of stable silver nanoparticles by *A. niger* and *P. chrysogenum* was preliminary indicated by visual observation since the color of reaction mixture turns from colorless or pale yellow into dark brown due to the excitation of surface plasmon vibrations specially groups of conduction electrons to external magnetic field. **(Balaji *et al*., 2009** and **Vahabi *et al.,* 2011)**. By studying the UV/Vis absorption spectrum of silver nanoparticles in the reaction mixture, the λ maximum of the absorption spectrum was at 430 nm for both tested fungi and its intensity proceeds with time. Previous studies mentioned the λ maximum for absorption spectrum of silver nanoparticles was between 420 and 450 nm **(Afreen *et al*., 2011 and Khalil, 2013)**.

Bio-molecules present in the reaction mixture such as polysaccharides, organic acids and proteins are believed to facilitate the synthesis of different crystal shapes of silver nanoparticles with favoring to spherical shape. **Jaidev and Narasimha (2010)** revealed the size and shape of the silver nanoparticles reflects excitation of surface plasmon vibrations since the absorption bands shift to longer wavelengths with increasing the size of nanoparticles. Results of the present study revealed that the produced silver nanoparticles haven't been in contact even within aggregation. This configuration could be due to certain proteins acts as capping agents. Most of earlier studies confirmed the well disparity of silver nanoparticles without agglomeration. The size of silver nanoparticles is varied according to producer organism, 7-35 nm for *Aspergillus fumigates* **(Navazi *et al.,* 2010)**, 5-50 nm for *Trichoderma reesei* **(Vahabi *et al*., 2011)**,10-40 nm for *Pestalotia sp*. **(Raheman *et al*., 2011)** and 5–30nm for *Penicillium diversum* **(Ganachari *et al*., 2012)**.

FTIR analysis was carried out to identify the bio-molecules responsible for the reduction of silver in metallic form and capping the reduced silver nanoparticles in the reaction mixture. The absorption band that appeared at 1450 cm^{-1} is assigned to methylene scissoring vibrations resulted from extracellular enzymes exist in the reaction mixture. **Gole *et al.* (2001)** revealed that the protein molecules not only act as reducing agent but also can act as stabilizing agent by binding to silver nanoparticles via free amino groups or cysteine residues or via electrostatic attraction of negatively charged carboxylate groups of extracellular enzyme of producer organism. **Ahmad *et al.* (2003)** observed the presence of two bands at 1650 cm^{-1} and 1540 cm^{-1} representamide I and II bands in the reaction mixture that arise due to C=O stretch and N-H bend, these bands are close to that reported for native proteins. **Kumar *et al.* (2007)** identified a capping peptide; phytochelatin during the synthesis of silver nanoparticles by nitrate reductase purified from *Fusarium oxysporum* in the presence of α-NADPH. TheFTIR spectral analysis of the reaction mixture of silver nitrate with pellets free extract of *A. clavatus* revealed the presence of –C–O–C– and C=C functional groups, which may be present between amino acid residues and protein synthesized during silver nanoparticles production **(Saravanan and Nanda, 2010)**. Similarly, **Singh *et al.* (2014)** demonstrated the presence of the bands at 1393, 1233, and 1074 cm^{-1} that are corresponding to C–N stretching vibrations of aromatic and aliphatic amines.

This study suggests a probable release of protein components into the pellets free extracts of *A. niger* and *P. chrysogenum*, these proteins play a crucial role in the reduction of silver ions into silver nanoparticles as well as bind to the nanoparticles thus imparting them with stability. One or more of these proteins may be belong to reductase family. Similarly, **Neal *et al.* (2004)** recorded the presence of NADH-dependent reductase in the outer membrane of *Geobacter sulfurreducens* during silver nanopartices production. **Kathiresan *et al.* (2009)** partially purified proteins that responsible for the reduction of silver ions by rhizospheric fungus, *P. fellutanum*. They revealed the involvement of fungal protein (70 kDa) in the reduction process. **Mukherjee *et al.* (2008**) studied the green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic fungus, *Trichodermaa sperellum* and demonstrated that the silver ions reduction process follows Michaelis–Menten type mechanisms with pseudo-zero-order kinetics.

There is another assumption discusses the participation of electron shuttles in the reduction of silver into silver nanoparticles. The present study concluded the great role that is played by reducing agents in the reduction process. Electrons or reducing agents involved in the reduction process are generated by different biochemical processes take place inside the producer organism. **Ahmad *et al*.(2003)** reported that a certain NADH-dependent reductase is involved in the reduction of silver ions by *Fusarium oxysporum* and the reduction of metal ions takes place due to the conjugation that occurs between the electron shuttles and a NADPH-dependent reductase. **Korbekandi *et al.* (2013)** investigated the addition of electron donor into the reaction mixture of silver nanoparticles production. They added glucose at 65 µM as electron

donor or cofactor for silver nitrate reduction by whole cells of *F. oxysporum*. By monitoring the UV-Vis absorption spectrum of the reaction mixture with time, they observed a decrease in the reaction rate up two hour followed by a gradual increase in the presence of glucose. Contrary, the absorption spectrum of silver nanoparticles reached the maximum intensity at 2 h then declined almost to zero after 48 h. They concluded the addition of electron donors accelerates the nanoparticles production process.

The antimicrobial activity of the biologically-synthesized silver nanoparticles produced by *A. niger* and *P. chrysogenum* was higher than chemically formed ones and is concentration dependent. This positive action was previously mentioned using different concentrations of silver nanoparticles on various pathogenic fungi **(Jaidev and Narasimha, 2010, Ninganagouda *et al.*, 2013** and **Singh *et al.*, 2014)**. The effect of biologically-synthesized silver nanoparticles against tested pathogenic microbes shows that silver nanoparticles have a less effect on the growth of Gram +ve bacteria than on Gram -ve bacteria and yeast. This is can be attributed to the structural difference in the cell wall composition between G +ve and G -ve bacteria, there is a lack of strength and rigidity in G -ve bacteria (**Madigan and Martinko, 2005**). In addition, the negative charges on lipopolysaccharides are attracted toward the weak positive charges available on silver nanoparticles **(Sui *et al.*, 2006)**. On the other hand, the cell wall in G +ve bacteria is principally composed of a thick layer of peptidoglycan consisting of linear polysaccharide chains cross-linked by short peptides to form a three-dimensional rigid structure **(Samuel, 1996)**. The rigidity and extended cross-linking not only endow the cell walls with fewer anchoring sites for the silver nanoparticles but also make them difficult to penetrate.

Due to the indiscriminate use of antibiotics, microorganisms have developed resistance against many antibiotics.Thus, increasing the efficiency of known antibiotics by combining them with silver nanoparticles is considered ecofriendly and cost effective solution. The synergistic effect of silver nanoparticles and commercial antibiotics against tested pathogenic microbes may be caused by the bonding reaction between antibiotics and silver nanoparticles in favor to hydroxyl and amide groups followed by dissipation of the proton motive force of the cell membrane **(Lok *et al.*, 2006)** or increasing the cell permeability by pitting the cell membrane by silver nanoparticles that causes an increase in the cell permeability and leads to cell death **(AshaRani *et al.*, 2008)**. Silver tend to have a higher affinity for phosphorous and sulphur compounds; the membrane of bacteria is well known to contain many sulphur containing proteins which might be the preferential sites for silver nanoparticles. As the nanoparticles enter the cell wall of the bacterium, they interfere with the bacterial growth signaling system by modulating the tyrosine phosphorylation putative peptide substances critical for cell viability and multiplication apart from forming ROS species **(Shivaraj*et al*, 2014)**.

In the present study, biologically-synthesized silver nanoparticles were relatively more toxic against Hela cancer cell line than chemically formed silver nanoparticles even at dilutions of seventh and ninth for silver nanoparticles synthesized by *A. niger* and *P. chrysogenum*, respectively. All silver nanoparticles treated cells showed great changes in the cellular structure especially losing of their spindle shape after treatment. **Park *et al.* (2011)** revealed that silver nanoparticles treated cells showed a decrease in the metabolic activity, which depends on the nature of cell line type and the size of nanoparticles. **Yen *et al.* (2009)** found that silver nanoparticles are cytotoxic to macrophages and fibroblasts at concentrations of 10 and 50 µg/ml; respectively. Silver nanoparticles induced apoptosis in malignant cells by heightening the ROS generation and activated c-Jun-N-terminal protein kinase signaling (JNK pathway) leading to mitochondria-dependent apoptosis **(Hsin *et al.*, 2008** and **Foldbjerk *et al.*, 2009)**.

Conclusion

Many microorganisms have been reported as silver nanoparticle producers but only few of them are capable of producing nanoparticles in quantity and quality encourage industrial application. The present study concluded that selected fungal strains; *Aspergillus niger* and *Penicilliumchrysogenum* synthesize silver nanoparticles by different mechanisms since the electron shuttle is mainly responsible for the former fungal strain, the silver nanoparticles production by the latter fungal strain is depended on enzyme system (nitrate reductase) encoded nai D gene. The harmless effect of silver nanoparticles synthesized by selected fungi on Colon-9 normal cells is very interesting finding and needs further studies on the reorganization and interaction between silver nanoparticles and living cells either healthy or abnormal ones.

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References

1. Afreen, R. V., and Ranganath, E.(2011). Synthesis of monodispersed silver nanoparticles by *Rhizopus stolonifer* and its antibacterial activity against MDR strains of *Pseudomonas aeruginosa* from burnt patients. *Int. J. Environ. Sci, 1*(7), 1582-1592.
2. Ahmad, A., Mukherjee, P., Senapati, S., Mandal, D., Khan, M. I., Kumar, R. and Sastry, M.(2003). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. *Colloids and Surfaces B: Biointerfaces, 28*(4), 313-318.
3. Ahmad, A., Senapati, S., Khan, M. I., Kumar, R., and Sastry, M.(2005). Extra-/intracellular biosynthesis of gold nanoparticles by an alkalotolerant fungus, *Trichothecium sp*. *Journal of Biomedical Nanotechnology, 1*(1), 47-53.
4. AshaRani, P., Low Kah Mun, G., Hande, M. P., and Valiyaveettil, S.(2008). Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS nano, 3*(2), 279-290.
5. Balaji, D. S., Basavaraja, S., Deshpande, R., Mahesh, D. B., Prabhakar, B. K., and Venkataraman, A.(2009). Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus. *Colloids and Surfaces B: Biointerfaces, 68*(1), 88-92.
6. Basavaraja, S., Balaji, S. D., Lagashetty, A., Rajasab, A. H., and Venkataraman, A.(2008). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*. *Materials Research Bulletin, 43*(5), 1164-1170.
7. Bhainsa, K. C., and D'Souza, S. F.(2006). Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. *Colloids and Surfaces B: Biointerfaces, 47*(2), 160-164.
8. Birla, S. S., Tiwari, V. V., Gade, A. K., Ingle, A. P., Yadav, A. P., and Rai, M. K.(2009). Fabrication of silver nanoparticles by Phoma glomerata and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Lett Appl Microbiol, 48*(2), 173-179.
9. Fang, J., Zhong, C., and Mu, R.(2005). The study of deposited silver particulate films by simple method for efficient SERS. *Chemical Physics Letters, 401*(1–3), 271-275.
10. Fayaz, A. M., Balaji, K., Girilal, M., Yadav, R., Kalaichelvan, P. T., and Venketesan, R.(2010). Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *Nanomedicine: Nanotechnology, Biology and Medicine, 6*(1), 103-109.
11. Foldbjerg, R., Olesen, P., Hougaard, M., Dang, D. A., Hoffmann, H. J., and Autrup, H.(2009). PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. *Toxicology letters, 190*(2), 156-162.
12. Gade, A., Bonde, P., Ingle, A., Marcato, P., Duran, N., and Rai, M.(2008). Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles. *Journal of Biobased Materials and Bioenergy, 2*(3), 243-247.
13. Ganachari, S. V., Bhat, R., Deshpande, R., and Venkataraman, A.(2012). Extracellular biosynthesis of silver nanoparticles using fungi *Penicillium diversum* and their antimicrobial activity studies. *BioNanoScience, 2*(4), 316-321.
14. Gole, A., Dash, C., Ramakrishnan, V., Sainkar, S., Mandale, A., Rao, M., Sastry, M. (2001). Pepsin-gold colloid conjugates: preparation, characterization, and enzymatic activity. *Langmuir, 17*(5), 1674-1679.
15. Hsin, Y.-H., Chen, C.-F., Huang, S., Shih, T.-S., Lai, P.-S., and Chueh, P. J.(2008). The apoptotic effect of nanosilver is mediated by a ROS-and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicology letters, 179*(3), 130-139.
16. Ingle, A., Rai, M., Gade, A., and Bawaskar, M.(2009). *Fusarium solani*: a novel biological agent for the extracellular synthesis of silver nanoparticles. *Journal of Nanoparticle Research, 11*(8), 2079-2085.
17. Jaidev, L., and Narasimha, G.(2010). Fungal mediated biosynthesis of silver nanoparticles, characterization and antimicrobial activity. *Colloids and Surfaces B: Biointerfaces, 81*(2), 430-433.
18. Kathiresan, K., Manivannan, S., Nabeel, M., and Dhivya, B.(2009). Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. *Colloids and Surfaces B: Biointerfaces, 71*(1), 133-137.
19. Khalil, N. M.(2013). Biogenic silver nanoparticles by Aspergillus terreus as a powerful nanoweapon against *Aspergillus fumigatus*. *African journal of microbiology research, 7*(50), 5645-5651.
20. Korbekandi, H., Ashari, Z., Iravani, S., and Abbasi, S.(2013). Optimization of biological synthesis of silver nanoparticles using *Fusarium oxysporum*. *Iranian journal of pharmaceutical research: IJPR, 12*(3), 289.
21. Kumar, S. A., Abyaneh, M. K., Gosavi, S., Kulkarni, S. K., Pasricha, R., Ahmad, A., et al.(2007). Nitrate reductase-mediated synthesis of silver nanoparticles from $AgNO_3$. *Biotechnology letters, 29*(3), 439-445.
22. Laemmli, U. K.(1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature, 227*(5259), 680-685.
23. Lok, C. N., Ho, C. M., Chen, R., He, Q. Y., Yu, W. Y., Sun, H., Tam, P. K., Chiu, J. F. ,Che, C. M. (2006). Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J Proteome Res, 5*(4), 916-924.
24. Loo, C., Lowery, A., Halas, N., West, J., and Drezek, R.(2005). Immunotargeted nanoshells for integrated cancer imaging and therapy. *Nano letters, 5*(4), 709-711.
25. Madigan, M. T.(2005). Brock Biology of Microorganisms, 11th edn: SciELO Espana.
26. Marquis, B. J., Love, S. A., Braun, K. L., and Haynes, C. L.(2009). Analytical methods to assess nanoparticle toxicity. *Analyst, 134*(3), 425-439.
27. Mohanpuria, P., Rana, N. K., and Yadav, S. K.(2008). Biosynthesis of nanoparticles: technological concepts and future applications. *Journal of Nanoparticle Research, 10*(3), 507-517.
28. Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramírez, J. T., and Yacaman, M.

(2005). The bactericidal effect of silver nanoparticles. *Nanotechnology, 16*(10), 2346.
29. Mukherjee, P., Roy, M., Mandal, B., Dey, G., Mukherjee, P., Ghatak, J.,Yacaman, M.J. (2008). Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. *Nanotechnology, 19*(7), 075103.
30. Navazi, Z. R., Pazouki, M., and Halek, F. S.(2010). Investigation of culture conditions for biosynthesis of silver nanoparticles using *Aspergillus fumigates*. *Iranian journal of biotechnology, 8*(1), 56-60.
31. Neal, A. L., Clough, L. K., Perkins, T. D., Little, B. J., and Magnuson, T. S.(2004). In situ measurement of Fe (III) reduction activity of Geobacter pelophilus by simultaneous in situ RT-PCR and XPS analysis. *FEMS microbiology ecology, 49*(1), 163-169.
32. Ninganagouda, S., Rathod, V., Jyoti, H., Singh, D., and Prema, K.(2013). Extracellular biosynthesis of silver nanoparticles using *Aspergillus flavus* and their antimicrobial activity against gram negative mdr strains. *International Journal of Pharma and Bio Sciences, 4*(2), 222-229.
33. Ninganagouda, S., Rathod, V., Singh, D., Hiremath, J., Singh, A. K., and Mathew, J.(2014). Growth Kinetics and Mechanistic Action of Reactive Oxygen Species Released by Silver Nanoparticles from *Aspergillus niger* on *Escherichia coli*. *BioMed research international, 2014*.
34. Park, M. V., Neigh, A. M., Vermeulen, J. P., de la Fonteyne, L. J., Verharen, H. W., Briedé, J. J., van Loveren, H. and de Jong, M.. (2011). The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles. *Biomaterials, 32*(36), 9810-9817.
35. Raheman, F., Deshmukh, S., Ingle, A., Gade, A., and Rai, M.(2011). Silver nanoparticles: novel antimicrobial agent synthesized from an endophytic fungus *Pestalotia sp*. isolated from leaves of Syzygium cumini (L). *Nano Biomed Eng, 3*(3), 174-178.
36. Samuel, B.(1996). Medical microbiology. *The University of Texas Medical Branch at Galveston, Texas*, 303-310.
37. Sanghi, R., and Verma, P.(2009). Biomimetic synthesis and characterisation of protein capped silver nanoparticles. *Bioresource technology, 100*(1), 501-504.
38. Saravanan, M., and Nanda, A.(2010). Extracellular synthesis of silver bionanoparticles from *Aspergillus clavatus* and its antimicrobial activity against MRSA and MRSE. *Colloids and Surfaces B: Biointerfaces, 77*(2), 214-218.
39. Sau, T. K., and Rogach, A. L.(2010). Nonspherical noble metal nanoparticles: colloid - chemical synthesis and morphology control. *Advanced Materials, 22*(16), 1781-1804.
40. Shahverdi, A. R., Fakhimi, A., Shahverdi, H. R., and Minaian, S.(2007). Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. *Nanomedicine: Nanotechnology, Biology and Medicine, 3*(2), 168-171.
41. Shaligram, N. S., Bule, M., Bhambure, R., Singhal, R. S., Singh, S. K., Szakacs, G. andPandey, A. (2009). Biosynthesis of silver nanoparticles using aqueous extract from the compactin producing fungal strain. *Process Biochemistry, 44*(8), 939-943.
42. Shin, S.-H., Ye, M.-K., Kim, H.-S., and Kang, H.-S.(2007). The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells. *International immunopharmacology, 7*(13), 1813-1818.
43. Singh, D., Rathod, V., Ninganagouda, S., Hiremath, J., Singh, A. K., and Mathew, J.(2014). Optimization and Characterization of Silver Nanoparticle by Endophytic Fungi *Penicillium sp*. Isolated from Curcuma longa (Turmeric) and Application Studies against MDR *E. coli* and *S. aureus*. *Bioinorganic Chemistry and Applications, 2014*, 8.
44. Sui, Z., Chen, X., Wang, L., Xu, L., Zhuang, W., Chai, Y., and Yang, C.(2006). Capping effect of CTAB on positively charged Ag nanoparticles. *Physica E: Low-dimensional Systems and Nanostructures, 33*(2), 308-314.
45. Uddin, I., Adyanthaya, S., Syed, A., Selvaraj, K., Ahmad, A., and Poddar, P.(2008). Structure and microbial synthesis of sub-10 nm Bi2O3 nanocrystals. *Journal of nanoscience and nanotechnology, 8*(8), 3909-3913.
46. Vahabi, K., Mansoori, G. A., and Karimi, S.(2011). Biosynthesis of silver nanoparticles by fungus *Trichoderma reesei* (a route for large-scale production of AgNPs). *Insciences J., 1*(1), 65-79.
47. Vigneshwaran, N., Kathe, A. A., Varadarajan, P., Nachane, R. P., and Balasubramanya, R.(2006). Biomimetics of silver nanoparticles by white rot fungus, *Phaenerochaete chrysosporium*. *Colloids and Surfaces B: Biointerfaces, 53*(1), 55-59.
48. Yen, H. J., Hsu, S. H., and Tsai, C. L.(2009). Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes. *Small, 5*(13), 1553-1561.

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