Silver Nanoparticles Synthesized by Penicillium citreonigrum and Fusarium moniliforme Isolated from El-Sharkia, Egypt

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Abstract:
Background: Although nanoparticles can be made using various physicochemical methods that remains expensive and involves the use of hazardous chemicals. Biological synthesis of nanoparticles appears as a suitable process since it requires less energy, is environmentally safe, emerges as an eco-friendly, scalability, exciting approach, it has low manufacture costs of scalability, and better nanoparticle stabilization, compared to chemically synthesized nanoparticles.

This study illustrates simple, green synthesis of AgNPs in vitro using cell lysate supernatant (CLS) of fungal species and to investigate its potential antimicrobial, antiviral activities, and cytotoxic effects against some tumor cell lines. The production ability of silver nanoparticles was investigated by the means of UV-V spectroscopy, electron microscopy and X-ray microanalysis. Results: The production of silver nanoparticles by Penicillium citreonigrum and Fusarium moniliforme is reported in this study. The biosynthesized nanoparticles exhibited typical plasmon absorption maximum of silver nanoparticles (420nm). Spherical silver nanoparticles were found to have size between 10 and 50 nm by electron microscope analysis. X-ray pattern revealed the crystalline nature of the silver nanoparticles. Silver nanoparticles have broad spectrum antimicrobial activity against Gram positive and negative bacteria and fungi. In the meantime, AgNPs exhibited promising cytoprotective efficacy towards Herpes simplex type 2 virus and pronounced cytotoxic activity against some tumor cell lines. Conclusions: The studies showed that the microbial susceptibility to Ag-NPs is different for each microorganism. Penicillium citreonigrum and Fusarium moniliforme have capacity to biosynthesize silver nanoparticles, which are intracellularly accumulated. This property is present in whole cells and in free cell extracts indicating that this process is probably enzymatically mediated, due to the requirement of NADH as cofactor for this biological transformation. Moreover, Silver nanoparticles have different biological applications.

Key words: Silver nanoparticles, antimicrobial, antiviral activities, and tumor cell lines.

1- Introduction
The use of microorganisms in the synthesis of nanoparticles emerges as an eco-friendly and exciting approach, for production of nanoparticles due to its low energy requirement, environmental compatibility, reduced costs of manufacture, scalability, and nanoparticle stabilization compared with the chemical synthesis. The impact of nanotechnology in all areas of science and technology is evident[1].

The expanding availability of a variety of nanostructures with properties in the nanometer size range has sparked widespread interest in their use in biotechnological systems[2]. Nanoparticles are usually ≤ 100 nm in each spatial dimension and are commonly synthesized using top-down and bottom-up strategies[3]. They can be used as catalysts, adsorbers, membranes, water disinfactants and additives to increase catalytic activity and capability due to their high specific surface areas and nanosize effects[4]. Silver nanoparticles show very high chemical reactivity compared to bulk silver, well known for being inert. This kind of nanoparticles has multiple applications in drug delivery, gene transfer, disinfectants in medical institutions[5], as bioprobes in cells and for tissue analysis in visualization of micro-and nanoobjects, for observation of biological processes at nanoscale[6]. Because of their antimicrobial property, silver nanoparticles (Ag-NPs) have become the most frequently used nanoparticles in consumer products. By August 2009, there were 259 consumer products containing nano-silver, which ranked first in the “Woodrow Wilson International Centre for Scholars” study on emerging nanotechnologies[7].

Conventionally, nanomaterials are synthesized using either chemical or physical methods which include micelle, chemical precipitation, hydrothermal method, pyrolysis, or chemical vapour deposition[8]. Some of these methods are easy and provide control over crystallite size by restoring the reaction environment. But problem still exists with the general stability of the product and in achieving monodisperse nano size using these methods[9]. Biological methods "Nanobio-technology" have emerged as an alternative to the conventional methods for synthesis of nanoparticles. Synthesis of inorganic nanoparticles by biological systems makes nanoparticles more biocompatible and environmentally benign. Moreover, the process is cost effective too[10].

The synthesis of nanoparticles by fungi, and their subsequent application, particularly in medicine are
studied under Myconanotechnology. Myconanotechnology is the interface between ‘Mycology’ and ‘Nanotechnology’ and has considerable potential, partly due to the wide range and diversity of the fungi[11]. When focusing on the synthesis of nanoparticles using fungi, it was observed that nanoparticles of good mono-disperisty and well dimensions could be synthesized. As fungi are found to secrete high amount of protein they might result in the significant mass productivity of nanoparticles. The fungal proteins are capable of hydrolyzing metal ions. In addition to this, fungi are easy to isolate and culture. Moreover, the downstream processing and the handling of fungal biomass are less complex than the synthetic methods[13]. The fungal system has emerged as Bionanofactories synthesizing several nanoparticles.

The use of silver as a metal can be traced back to times even before Neolithic revolution. Moyer first recorded the medicinal use of silver during 8th century[13]. Nowadays, the tunable photophysical attributes of silver nanoparticles[14], their efficient addressability via optical and spectroscopic techniques, and rapid advances in nanoparticle synthesis and fabrication[9][15] have brought these nanoparticles to the forefront of nanotechnology research directed toward applications ranging from photonics[16] to biomedicine[13].

Researchers are continuously developing newer methods for synthesis of nanoparticles which are efficient in terms of synthesis rate as well as energy usage[10]. Here, we report the extracellular and intracellular synthesis of silver nanoparticles using fungi and to investigate its notable potential antimicrobial, antiviral activities, and cytotoxic effects against some tumor cell lines.

2- Materials and methods:
The preparation of microbial biomass that was used for biosynthetic reactions of nanoparticles was carried out according to method described by Fayaz[19]. The extracellular silver nanoparticles productions by the microbial species were carried out using whole living cells and cell-free lysates[10]. The change in the color from colorless into brown indicates a positive result for extracellular production. To verify reduction of silver ions, the solution was scanned in the range of 200-800 nm on Spectronic 1201 Milton Roy at 435 nm as described by Prakash[20]. The size and morphology of the nanoparticles were analyzed with the transmission electron microscope (TEM) JEOL® 1010 Japan. The sample was prepared by placing a drop of silver nanoparticles on carbon coated copper grid and subsequently drying in air before transferring it to the microscope. For Scanning Electron Microscopy (SEM) the drop of silver nanoparticles were mounted on specimen stubs with double-sided adhesive tape and coated with gold using sputter coater (SPI-Module). SEM micrograph confirmed the size and the morphology when the samples were examined under JEOL JSM-5500 LV scanning electron microscopy at 15 KV at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

The ability of microbial strain to produce intracellular AgNPs was estimated by Karbasian[21]. EDX microanalysis was carried to indicate the presence of silver nanoparticles inside the fungal species by using X-ray microanalyzer Oxford® 6587 INCA attached to JEOL® JSM-5500LV scanning electron microscopy at 20 KeV after gold coating using SPI-Module® sputter coater at the Regional Center of Mycology and Biotechnology, Cairo, Egypt. Then, transmission electron microscope (TEM) was used to confirm the presence of the silver nanoparticles inside the fungal species.

The antimicrobial ability of silver nanoparticles against pathogenic bacteria and fungi was carried by method described by Musarrat[22]. The minimum inhibitory concentration (MIC) was performed by testing different concentration from the synthesized nanoparticles using micro dilution method[23].

The silver nanoparticles were tested against three tumor cell lines i.e., Breast cancer cell line (MCF-7), Colon carcinoma cell line (HCT-116) and liver carcinoma cell line (HepG2). The cell cultures of the three tumor cell lines were prepared in complete growth medium (DMEM and RPMI-1640) depending on the type of cell line[24]. Antitumor viability assay was carried according to the method described by Saintigny[25].

Antiviral activity of silver nanoparticles was determined by studying the inhibition of herpes simplex type 2 virus activity by using silver nanoparticles where, the Vero cells were challenged with herpes simplex type 2 virus doses and simultaneously the cells treated with different dilutions of silver nanoparticles and incubated at 37º C for two days then compared to the control according to the method described by Harden[26].

3- Results and Discussion
The biological synthesis of nanoparticles is challenging concept which is very well known as green synthesis[27]. Green synthesis of nanoparticles is cost effective, easily available, ecofriendly, nontoxic, large scale production and act as reducing and capping agent in compared to chemical methods which is very costly as well as it emits hazardous byproduct which can have some deleterious effect on the environment[28]. Therefore, our work was devoted to exploit microbial isolates for achieving this task in the production of nanosilver.

After being subjected to Penicillium citreonigrum, the colour of AgNO3 was changed from pale yellow to intense brown colour (Figure 1) due to formation of extracellular nanoparticles after incubation for 72 h at 28 º
ions occurred extracellularly, resulting in the rapid reduction of the metal solution. This result was in accordance with that of plant pathogenic fungal strain, leading to the formation of metal nanoparticle in the fungus and reduced outside the fungal biomass, this secreted from microorganisms. The silver nanoparticles formed as a result of reduction of silver ions present in the aqueous solution. The exact reaction mechanism leading to the biosynthesis of silver nanoparticles is believed that NADH-dependent reductase involving in reduction of silver ions.[29 & 30].

Formation of nanosilver was also monitored using the UV-Visible spectroscopy which showed increments of optical density at 435 nm (Fig. 2) indicate silver nanoparticles formation, implying that the bioreduction of the silver nitrate has taken place following incubation of the AgNO₃ solution in the presence of the cell-free extract. Control experiments showed no change in colour of suspension when cells were not present. This result was in agreement with those results that reported the absorption spectrum of spherical silver nanoparticles present a maximum between 420 nm and 450 nm[31 & 32].

Identification, localization and nanoparticles size were verified by transmission electron microscopy (TEM). TEM micrographs of fabricated extracellular silver nanoparticles revealed variable shape with majority of them spherical in shape with size range of 10-50nm (Fig.3). Because of the metal ions are not exposed to the fungus and reduced outside the fungal biomass, this leading to the formation of metal nanoparticle in the solution. This result was in accordance with that of plant pathogenic fungal strain Fusarium oxysporum behaved considerably as the same way; the reduction of the metal ions occurred extracellularly, resulting in the rapid formation of highly stable silver[33] nanoparticles of 2- to 50-nm dimensions. The aqueous extract of the fungal biomass can reduce silver ions to the corresponding nanoparticles. Most probably, the reduction of silver ions occurs due to reductases released by the fungus into the solution, thus opening up a novel fungal/enzyme-based in vitro approach to nanomaterials. The long-term stability of the nanoparticles in the solution may be due to the stabilization of proteins[34].

SEM micrograph (Fig. 4) showed silver nanoparticles separated (Fig.4a and b) in low concentration of nanoparticles and aggregated (Fig.4 c and d) in high concentration. In the meantime, the nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent.

In chemical nanoparticle synthesis methods, a stabilizer is necessary to prevent the aggregation of fine particles to make them stable for a long period of time but with use of biological systems, it is clear, that even aggregated nanoparticles don’t have direct contact with one another. This is due to the fact that nanoparticles are stabilized in solution by capping proteins, which are secreted from microorganisms. The silver nanoparticles formed by this process are quite stable due to capping proteins for a period of 5 months at 25°C[35 & 36].

The ability of microbial strains to produce intracellular nanosilver was monitored by EDX that confirm the presence of silver signal after incubation of Fusarium moniliforme for 72hr in contact with silver nitrate (Figure 5). This result confirmed the formation of silver nanoparticles inside the Fusarium moniliforme cells.

It used to record the elemental composition of particles within the cell. It was found in addition to the elemental composition inside the cell which appeared in control (Fig. 5a) such as C, O, S, P, Na, Mg and K signals were likely to due to X-ray emission from proteins/enzymes present in the cell wall of the biomass[37], there was a signal for silver nanoparticles observed after incubation with silver nitrate (Fig. 5b).

Further evidence that showed, organization of the intracellular formation of silver nanoparticles is provided by a transmission electron micrograph (TEM). There were small particles 10-50 nm sized observed within the cells that are clearly seen populating both the cell wall and the cytoplasmic membrane of the single fungal cell (Fig. 6). Since the nanoparticles are formed on the surface of the mycelia and not in the solution, it is thought that the first step involves the trapping of the metal ions on the surface of the fungal cells possibly via electrostatic interaction between the ions and the negatively charged carboxylate groups in the enzymes present in the cell wall of the mycelia. In the next step, the metal ions which reduced by the enzymes within the cell wall leads to the aggregation of metal ions and formation of nanoparticles[33]. The ability of fungal cells to multiply after exposing to metal ions proves the capability of using microorganisms in the synthesis of nanomaterials[38 & 34].

The TEM analysis of the fungus also, depicts presence of nanoparticles on the cytoplasmic membrane as well as within cytoplasm also ensured. This shows the possibility that some nanoparticles diffuse through cell wall and are reduced by the enzymes present on the cytoplasmic membrane while, some of the smaller nanoparticles diffuse through the fungal cell wall and get trapped within the cytoplasm[39].

Silver has been studied and used since ancient times to fight infections and prevent spoilage. The antibacterial, antifungal and antiviral properties of silver nanoparticles have been extensively studied against different test organisms. Silver is also found to be non-toxic to humans and animals in low concentrations. The microorganisms are unlikely to develop resistance against silver as compared to antibiotics as silver attacks a broad range of targets in the microbes[40 & 41].

The AgNPs exposure to eleven different test microorganisms resulted in formation of the zones of inhibition. The efficient antimicrobial property of silver
nano-particles compared to the silver salts can be attributed to their extremely large surface area (Table 1 and 2).

Silver nanoparticles exhibit more activity than silver nitrate solution and the highest antibacterial activity was observed against Escherichia coli. The general idea about the mechanism of silver nanoparticles action against pathogenic bacteria was by attachment to the cell membrane and also penetrates inside the bacteria. The bacterial membrane contains sulfur-containing proteins and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity [42].

The size of the nanoparticle implies that it has a large surface area to come in contact with the bacterial cells and hence, it will have a higher percentage of interaction than bigger particles [43]. The nanoparticles smaller than 10 nm interact with bacteria and produce electronic effects, which enhance the reactivity of nanoparticles. Thus, the bactericidal effect of silver nanoparticles is size dependent [42].

Different types of antifungal agents that are available for treatment remains extremely limited. This is because fungi are eukaryotic organisms with a structure and metabolism that are similar to those of eukaryotic hosts [45]. The results of antifungal evaluation against various human pathogenic fungi indicating antifungal activity of silver nanoparticles more efficient than those of silver nitrate solution. Many studies have shown the antimicrobial effects of AgNPs, but the effects of AgNPs against fungal pathogens are mostly unknown. It was found that there is an ability of AgNPs to disrupt the fungal envelope structure and formation damage in their cell walls and pores in their plasma membrane [46].

Silver nanoparticles have been used to exhibit the antimicrobial efficacy against viral particles. Silver nanoparticles have been shown to exhibit promising cytoprotective activities towards Herpes virus (Table 3). The synthesized extracellular silver nanoparticles give the Strong antiviral activity at concentrations 50 µg/ml, moderate antiviral activity at concentrations 25 µg/ml and weak result at 12.5 µg/ml but the intracellular silver nanoparticles gives weak result at concentrations 50 and 25 µg/ml.

The surface plasmon resonance and large effective scattering cross section of individual silver nanoparticles make them ideal candidates for molecular labeling, silver nanoparticles binding to the disulfide bond regions of binding domain within the glycoprotein subunit in viral envelope. Binding of these nanoparticles to this subunit appeared to be size dependent as particles greater than 10 nm were not observed attached to the viral envelope [47 & 48].

The extracellular silver nanoparticles showed inhibitory activity against liver cancer cells (Hep G2) (figure 7) under experimental conditions with IC50 equal to 6.68 µg/ml but the intracellular nanoparticles showed inhibitory activity with IC50 equal to 9.5 µg/ml which provides better results compared with silver nitrate in water with IC50 equal to 20.8 µg/ml.

The extracellular silver nanoparticles showed inhibitory activity against breast cancer cells (MCF-7) (figure 8) under experimental conditions with IC50 equal to 5.69 µg/ml but the intracellular nanoparticles showed inhibitory activity with IC50 equal to 9.5 µg/ml which provides better results compared with silver nitrate in water with IC50 equal to 21.1 µg/ml.

The extracellular silver nanoparticles showed inhibitory activity against colon carcinoma cells (HCT-116) (figure 9) under experimental conditions with IC50 equal to 3.86 µg/ml but the intracellular nanoparticles showed inhibitory activity with IC50 equal to 5.69 µg/ml which provides better results compared with silver nitrate in water with IC50 equal to 11.9 µg/ml.

The cytotoxic effect of silver nanoparticles because of the nanosize, surface area and surface functionalization are chief factors that control biokinetics and thus toxicity leading to lesser cell viability, the possible mechanism of toxicity is projected which involves disruption of mitochondrial respiratory chain by nanosilver leading to creation of ROS and disturbance of ATP synthesis, which leads to DNA damage [49].

4- Conclusion

The studies showed that the green biosynthesis for silver nanoparticles was an eco-friendly process with low costs and better nanoparticle stabilization and it will be an important step in the field of application of nanotechnology.

Penicillium citreonigrum and Fusarium moniliforme are having the ability to produce extracellular and intracellular nanoparticles, respectively. The fungal system has shown its compatibility over other organisms as the handling of fungal biomass and its downstream processing is much simpler.

The fungal-derived nanoparticles have depicted a wide range of applications in different fields of science including medicines, pharmaceutical industry, agriculture and electronics because it showed a promising result as antimicrobial, antiviral and antitumor substance.

The effective and efficient focusing on the biosynthesized silver nanoparticles was required to know the more about the mechanisms of their action.
References


biosynthesis of silver nanoparticles using the plant pathogenic fungi, Pucciniagram inis.


Figure 1: Cell filtrate (72 h) of Penicillium citreonigrum with silver ion (1 mM). The right one at the beginning of the reaction and the left one was after 72 h of reaction.

Figure 2: The spectra (of the brown solution produced after 72 h of incubation) which produced from the screening by UV-visible spectroscopy.
Figure 3: TEM micrograph of negatively stained biosynthesized extracellular silver nanoparticles taken after 72 hrs of incubation of Penicillium citreonigrum cell free filtrate with silver nitrate.

Figure 4: Scanning electron micrograph showing silver nanoparticles produced after 72h of incubation. a) X15000 and b) X20000 which represented the separated silver nanoparticles. c) X22000 and d) X30000 which represented the aggregated silver nanoparticles.
Figure 5: X-ray microanalysis for silver nanoparticles produced intracellularly from Fusarium moniliform a) The control fungus before incubation with silver, b) The fungus after incubation for 72h.

Figure 6: Transmission electron micrographs for Fusarium moniliform, a) Control with magnification X10000; b) After incubation with silver nitrate with low magnification X15000 & with high magnification X25000

Table 1: Antibacterial inhibition zone measurements by mm of extracellular and intracellular silver nanoparticles and their MIC.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Extracellular AgNPs</th>
<th>Intracellular AgNPs</th>
<th>silver nitrate with water</th>
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Table 2: Antifungal activity of the extracellular and intracellular silver nanoparticles and their MIC

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Extracellular AgNPs</th>
<th>Intracellular AgNPs</th>
<th>silver nitrate with water</th>
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<tr>
<td></td>
<td>inhibition zone in mm</td>
<td>MIC by µg/ml</td>
<td>inhibition zone in mm</td>
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<tr>
<td>Aspergillus niger</td>
<td>26</td>
<td>12.5</td>
<td>18</td>
</tr>
<tr>
<td>Penicillium expansum</td>
<td>40</td>
<td>12.5</td>
<td>20</td>
</tr>
<tr>
<td>Penicillium notatum</td>
<td>42</td>
<td>6.2</td>
<td>22</td>
</tr>
<tr>
<td>Synthphalastrumracemosum</td>
<td>38</td>
<td>6.2</td>
<td>28</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>36</td>
<td>12.5</td>
<td>30</td>
</tr>
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Table 3: Antiviral activity of silver nanoparticles against Herpes Simplex type 2 virus

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample concentration (µg/ml)</th>
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<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Positive control</td>
<td>+</td>
</tr>
<tr>
<td>Extracellular silver nanoparticles</td>
<td>+++</td>
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<tr>
<td>Intracellular silver nanoparticles</td>
<td>+</td>
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(-) No antiviral activity; (+) Weak antiviral activity 5-30%; (+++) Moderate antiviral activity 30.1-60%; (++++) Strong antiviral activity 60.1-90%
Figure (7): Antitumor activity of silver nanoparticles on hepatocellular carcinoma (HepG2) cells.

Figure (8): Antitumor activity of silver nanoparticles on Breast carcinoma (MCF-7) cells.

Figure (9): Antitumor activity of silver nanoparticles on colon carcinoma (HCT-116) cells.

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