Eco. Env. & Cons. 29 (2) : 2023; pp. (946-952)
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ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2023.v29i02.067

Biosynthesis of Silver Nanoparticles from *Penicillium sps* and their potential antioxidant activity

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(Received 10 January, 2023; Accepted 25 March, 2023)

ABSTRACT

Nanotechnology is a field of science that involves the production of particles with at least one dimension less than 100 nm. These nanoparticles have found various applications in the field of biomedicine, diagnostic, antimicrobial and anticancer agents etc. The present investigation focuses on the development of economical and eco-friendly methods for the biosynthesis of Silver Nanoparticles using cell free extract of *Penicillium sps.* The production of nanoparticles was monitored by scanning through UV Visible Spectrophotometer in the range of 300-70 nm. Size and shape of nanoparticles were determined by Scanning Electron Microscope (SEM) while the elemental composition was studied by Energy- dispersive X-ray spectroscopy (EDX). The presence of various functional groups that might have contributed for stability of silver nanoparticles was determined by Fourier transform infrared spectroscopy (FTIR). Antioxidant activities of silver nanoparticles were studied by 2,2- diphenylpicrylhydrazyl (DPPH) scavenging, Hydrogen peroxide (H_2O_2) radical scavenging and Reducing Power Assay (RPA). The nanoparticles showed dose dependent increase in antioxidant activities with IC_{50} value of 314.47 + 4.96 µg/ml and 317.34 + 4.40 µg/ml respectively for DPPH scavenging and H_2O_2 radical scavenging activity.

Key words: *Fungal isolates, Silver Nanoparticle, SEM, EDX, FTIR, Antioxidant activity.*

Introduction

Nanotechnology has evolved as a multidisciplinary field and has found multiple applications in all the branches of science such as chemistry, physics, biology etc. (Rahman *et al.,* 2019.*)* Nanoparticles have size ranging from 1-100nms in diameter. They possess distinct properties such as optical effect, surface effect, quantum size effect etc. (Xue *et al.,* 2016). The uniformity and unique properties of nanoparticles enabled their usage in nanomedicine, drug delivery, catalysis, water treatment and optical sensors (Konappa *et al.,* 2021). Antimicrobial nature of Silver nanoparticles (AgNPs) has made them valuable in treatment of diseases, food preservation and water purification (Khan *et al.,* 2017). AgNPs have broad spectrum potential (Taha *et al.,* 2019). AgNPs possess antimicrobial, anti-inflammatory, anti- angiogenesis properties (Elsharawya *et al.,* 2020). Studies showed that nanoparticles synthesized biologically using plants, bacteria, algae, and fungi have various applications as antioxidant, antimicrobial and anticancer agents (Rajoka *et al.,* 2020). Biosynthesis of AgNPs are found to be economical and eco-friendly relative to the traditional physical and chemical methods used for the synthesis (Durán *et al.,* 2016). Biological methods are environmentally friendly as metabolites of living organisms are used for the synthesis which do not involve the usage of toxic reagents and can also produce stable nanoparticles.

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Nanoparticles produced by these methods have uniform size and shape when compared to other methods (Ramos *et al.,* 2020). Biological methods produce stable nanoparticles, and are economical. They can be synthesized in a variety of hosts using mild reaction conditions (Xue *et al.,* 2016). Due to harmful chemicals secreted by chemical methods it is essential to find reliable, eco- friendly, inexpensive methods for the production of nanoparticles (Rajoka *et al.,* 2020). Studies also suggest that biologically synthesized AgNPs are found to be less toxic to organs when compared to those produced by conventional methods. The metabolites produced during biosynthesis provide nontoxic coating to the nanoparticles (Elsharawya *et al.,* 2020). In biological synthesis, capping of nanoparticles takes place by biomolecules derived from the organisms which not only increases the stability of the nanoparticles but also may add biological activity. Thus produced NPs are biocompatible (Guilger- Casagrande *et al.,* 2019). Microbiological methods are economical, reliable, eco-friendly and are biocompatible. Mycosynthesis of AgNPs have greater advantages such as low expenses and high production, and easy handling of biomass (Taha *et al.,* 2019). Compared to other microorganisms, fungi can secrete large amounts of secondary metabolites, which makes them suitable for the synthesis of nanoparticles (Wang *et al.,* 2021).

In the biosynthesis of nanoparticles by fungus, the fungal mycelium is exposed to the metal salt solution. This exposure to the metal salt solution will prompt the fungi to produce extracellular enzymes and metabolites for their survival. In this process the metal salt is reduced to metallic solid nanoparticles through the catalytic effect of extracellular enzymes and metabolites of fungi. The nitrate reductase was apparently essential for ferric ion reduction. Fungi that exhibit these characteristic properties, in general, are capable of reducing silver and gold (Muraleedharan *et al.,* 1994). The present study is focused on the mycosynthesis of AgNPs using cell-free extracts of *Penicillium sps* as reducing and capping agent. The study is also focused on optimization of parameters for production of AgNPs with better size and yield.

Materials and Methods

Isolation and Identification of Fungi

Soil samples were collected from the different regions of Baba Budangiri. Samples from 6-10 inches depth were collected aseptically from 10 different zones of deep forests of Western Ghats (Rohilla *et al.,* 2012). The samples were directly transferred into an appropriate, labeled zip lock cover aseptically under ambient temperature (Sanghi *et al.,* 2009). The soil samples were processed with an isolation procedure using the soil dilution plate method and the inoculated plates were incubated for 4-7 days at 28^0C. Single separate colonies on the agar plates were selected at random and streaked onto the MRBA media and were incubated for 4-7 days at 28°C±2. Codes were given to each of the isolated samples and stored at 15°C for identification by cotton blue staining methods (Panneerselvam *et al.,* 2012).

Biosynthesis of Silver nanoparticles using soil fungi

The pure cultures of soil fungi were inoculated in 500ml of sterile broth medium with following composition grams/ml: $(NH_4)_2SO_4$ – 1.0, $KH_2PO_4^-$ 7.0, $MgSO4.7H_2O$ – 0.1, K_2HPO_4- 2.0, yeast extract -0.6, and glucose – 10 and were incubated in a fungal incubator at growth temperature 28°C to produce fungal biomass for 5 days (Xue *et al.,* 2016). After optimum growth of the fungi, they were used for the biosynthesis of AgNPs. Followed by the five days of fungal biomass production, the fungal mat was washed with milliQwater three times and finally 20g of fungal mats were inoculated in 200ml of MilliQwater. The biomass was incubated at 28°C for 48 to 72 hours. After three days the fungal mat was filtered and the filtrate collected was used for the biosynthesis of AgNPs (El-Kahky *et al.,* 2021).

For biosynthesis of AgNPs, 100 ml of filtrate of all fungal isolates were taken in sterile 250 ml conical flasks. To these flasks, 0.025g silver nitrate (1.5 mM $AgNO_3$) was added in the dark room. The flasks were incubated at 30°C and immediately after adding the $AgNO_3$ and reading at 0 hour was recorded by scanning the filtrate at 435 nm for all fungal flasks using filtrate containing no $AgNO_3$ as blank. Incubation was continued for 2 hours after which again the filter was scanned in the same nanometer range. The scanning was continued for every two hours like 4h, 6h, 8h, 24h, 48h and 72h (El-Kahky *et al.,* 2021).

Characterization of Silver nanoparticles

The AgNPs obtained were concentrated by centrifugation at 10000 rpm for 10 minutes. The precipitate

collected was washed with deionised water by centrifugation at 10000 rpm. The above washing procedure was repeated thrice to remove any traces of metabolites present. AgNPs were further dried in an oven to ensure the removal of moisture content. Thus obtained AgNPs suspension was dried in an oven to get dry particles (Singh *et al.,* 2011). The pure form of AgNPs was further characterized by SEM, EDX and FTIR.

Size and morphology of AgNPs was characterized by SEM (Vanaja *et al.,* 2013). A small amount of dried AgNPs was placed on a carbon coated copper grid as thin films. Extra amount was removed from the grid using blotting paper. The film was allowed to dry by placing it under a mercury lamp for 5 minutes. The elemental composition of AgNPs s was analyzed by EDX (Gowramma *et al.,* 2015).

FTIR analysis of AgNPs was carried out to detect the presence of functional groups in the fungal extract (Jyoti *et al.,* 2015) that are responsible for reducing silver ions to AgNPs (Nagar *et al.,* 2016). The FTIR analysis was performed using Potassium bromide pellet technique and the results were recorded from 4,00 to 4000 cm^{-1} wavenumber (Khalir *et al.,* 2016).

Antioxidant activities of Silver nanoparticles

Sample (AgNPs) Preparation: Dissolve 1000 µg of above AgNPs in distilled water separately to obtain a solution of 1000 mg/ml concentrations. Serially dilute each of these solutions separately to obtain lower concentrations consisting of 62.5, 125, 250, 500 and 1000 mg/ml.

Antioxidant activity for DPPH radical

The assay was carried out according to Bhakya S *et al.,* 2016, with some modification. The assay was carried out on a 96 well microtiter plate. 200 µl of methanolic DPPH solution was added as test and control and 200 µl of methanol as test blank and control blank. Added 10 µl of each of the AgNPs or the standard from the lowest concentration and 10 µl DMSO served as control and control blank. The plates were incubated at 37 ºC for 30 minutes and absorbance of each solution was taken at 490 nm, using ELISA reader and the values were recorded (Bhakya *et al.,* 2016).

Scavenging of hydrogen peroxide (H_2O_2) radical

A solution of hydrogen peroxide (40 mM) was prepared in a phosphate buffer (pH 7.4). The 1 ml of AgNPs of various concentrations in distilled water was added to 0.6 ml hydrogen peroxide solution. After 10 min incubation at room temperature, the concentration of hydrogen peroxide was determined at absorbance 230 nm using a spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide (Keshari *et al.,* 2020).

Determination of antioxidant activity by reducing power assay

Spiked the AgNPs and Ascorbic acid solutions with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferric cyanide while distilled water was taken as test blank and control blank. The mixture was kept in a 50 °C water-bath for 30 minutes. The resulting solution was then cooled rapidly to room temperature, spiked with 2.5 ml of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 minutes. 5ml supernatant was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride.

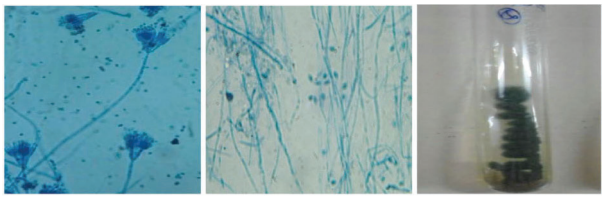
Incubated for 10 minutes. Pipetted out 0.1 ml to microtiter plate, test and control in triplets and test blank and control blank in singlet. Measured the absorbance at 700 nm using ELISA reader and the values were recorded. The reducing power assay was expressed in terms of Ascorbic acid equivalent per gram of dry weight basis (Keshari *et al.,* 2020).

Reducing power = Absorbance of test – absorbance of blank

Results and Discussion Results

Isolation and Identification of Fungi

A total of 24 fungal isolates were obtained from the soil sample. All the isolates were tested for biosynthesis of AgNPs. Out of all, 8 isolates showed promising results in the synthesis of AgNPs. In the present study *Penicillium sps* was selected as it showed good synthesis of AgNPs.



(A) (B) (C)
(A) (B) (C)

Fig. 1. (A and B) Microscopic observation of *Penicillium* sp (C). *Penicillium* sub-cultured on slants

The test filtrates incubated revealed color change from colorless to pink signifying the production of AgNPs whereas no color change was noticed in the control sample containing only filtrate and no $AgNO_3$. The results indicated that test filtrate color change to brown color is due to synthesis of AgNPs (Figure 2).

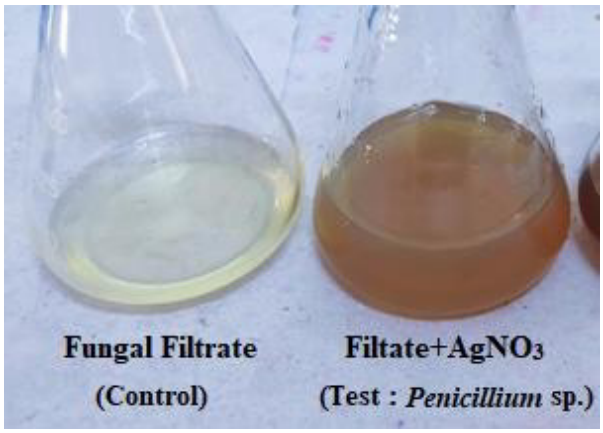


Fig. 2. Biosynthesis of Ag nanoparticles by *Penicillium sps* revealed color change

Change in color of the cell free extract from colorless to brown indicated the production of nanoparticles represented in Fig. 2 (Wang *et al.*, 2021). The reduction of silver was further subjected to spectral analysis by UV-Visible Spectrophotometer. This showed the absorbance peak at around 430 nm (Table 1) which was specific for AgNPs (Anandalakshmi *et al.*, 2016). UV-Visible analysis showed absorption peaks at 435 nm indicated the formation of AgNPs (Yassin *et al.*, 2021).

Characterization SEM and EDX

The SEM images showed the AgNPs were mostly aggregated. Figure 3 shows AgNPs are predominantly in circular shape but on aggregation they have formed particles without defined morphology (Gowramma *et al.*, 2015).

Different vibrations were identified in FTIR results to determine the various functional groups present (Balashanmugam *et al.*, 2015). FTIR studies revealed the presence of functional groups of capping agents. It is very important to find the capping agents as they provide stability and carry out reduction of silver ions to AgNPs. The FTIR spectrum showed broadband at 3435.71 cm^{-1} which indicated O-H stretch (Periasamy *et al.*, 2022). The bands at 821.99 cm^{-1} and 665.96 cm^{-1} may be assigned to eth-

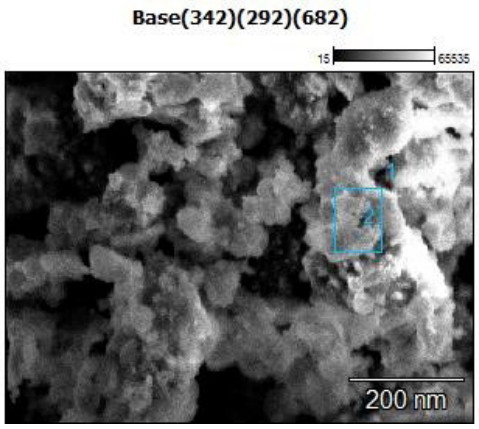


Fig. 3. SEM image of AgNPs

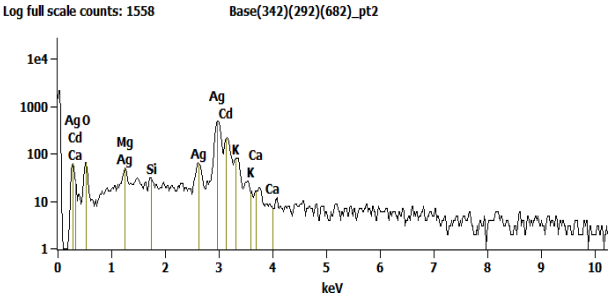


Fig. 4. EDX pattern of AgNPs

ylene systems -CH=CH (cis) (Devaraj *et al.*, 2013).

Antioxidant activities of AgNPs were represented in Table 1. DPPH is a stable compound which can be reduced by accepting hydrogen or electrons from the donors (Bhakya *et al.*, 2016). The AgNPs showed an IC50 value of 314.47 + 4.96 µg/ml. With increase in the concentrations, there was an increase in the antioxidant activity of DPPH radical by AgNPs (Salari *et al.*, 2019). Scavenging of H2O2

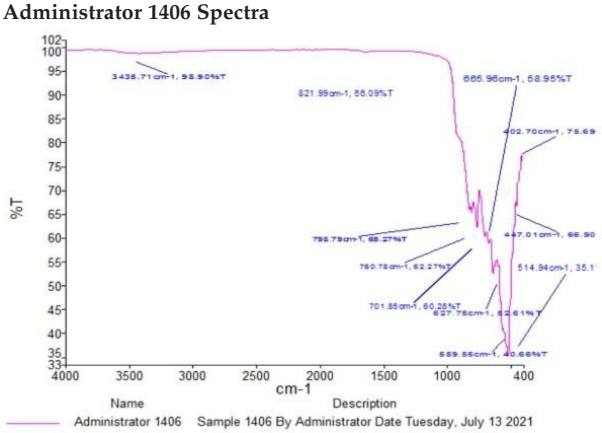


Fig. 5. FTIR results of AgNPs

radical is shown in Table 1. There was a dose -dependent increase in the inhibition of AgNPs for H_2O_2 radical (Patil *et al.,* 2015). The AgNPs showed an IC_{50} value of 317.34 + 4.40 µg/ml. The Antioxidant property of AgNPs have resulted in the reduction of Fe_3^+/ferricyanide. The increase in the absorbance value at 700 nm indicated an increase in the reductive ability (Subramanian *et al.,* 2013).

The present study explains the synthesis of AgNPs by *Penicillium sps*. The production of large quantities of proteins and enzymes, low toxicity, easy handling and high yield has made fungi a potential bio factory for the synthesis of AgNPs (Guilger-Casagrande *et al.,* 2019). *Penicillium sps* are considered good biological agent for AgNPs synthesis. Yassin *et al.,* (2021) have reported synthesis of AgNPs by *Penicillium verrucosum*. When AgNO3 was added to the cell free fungal extract, the colour of the solution changed from colourless to pink colour. The distinct pink colour formation in the test sample can be attributed to the surface plasmon resonance in metals which indicated the formation of AgNPs.

Colour change was observed only in the test sample while the control remained the same with no change in color. With increase in the incubation time, intensity of the colour change was also increased. The color change might have occurred due to the secretion of extracellular reductase enzymes by fungi, which might have reduced the silver ions in $AgNO_3$ solution, leading to the formation of AgNPs. As reductase enzyme was not present in the control, no color change was observed (Nida *et al.,* 2016). Control set showed no change in colour when the same experimental conditions were provided (Anandalakshmi *et al.,* 2016). Nanoparticles obtained were predominantly in circular shape which on agglomeration might have lost the defined morphology leading to irregular shapes. The SEM results obtained were in agreement with the results of Vanaja *et al.,* 2012. The aggregation might have happened due to the presence of secondary metabolites (Vanaja *et al.,* 2013). EDX spectrum of AgNPs shows the presence of AgNPs in large amounts compared to other elements (Gowramma *et al.,* 2015). Studies shows that the bioactive compounds present in the fungal extract might have been responsible for the formation of AgNPs. FTIR results confirmed the presence of various such functional group surface of bioactive compounds which might also be responsible for the capping of AgNPs and thereby stabilizing their nanosize (Keshari *et al.,* 2020). The antioxidant activity of AgNPs was found to be dose dependant which is in agreement with the results of study conducted by (Renganathan *et al.,* 2021). This strong antioxidant potential could make them a plausible drug delivery candidate. Various authors have suggested that the higher antioxidant properties of AgNPs might be result of their size and their crystalline nature. (Patra and Baek, 2017). The results have suggested that AgNPs can be considered as a potential antioxidant agent. These AgNPs have potential to balance the antioxidant and ROS level, thus have potency to prevent cell damage and also to prevent the degeneration of the cellular contents (Sharma *et al.,* 2022).

Conclusion

The results of this study highlight the application of *Penicillium sps* as a potential biofactory for the biosynthesis of AgNPs. The main aim of this study was to develop an economical and environmentally friendly method for the effective synthesis of AgNPs. The above-mentioned method does not involve the use of toxic materials thus making the process cheap and eco-friendly. The study also investigates the antioxidant activities of AgNPs which may help in developing new treatment regimens for treatment of various diseases including cancer.

Acknowledgements

The authors sincerely thank Sahyadri Science College, Kuvempu University, Shivamogga, for their extensive help in the completion of the work successfully.

Conflict of Interests

Authors declare no conflicts of interests to disclose.

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