Saudi Journal of Biological Sciences 28 (2021) 2123–2127

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Biosynthesis of silver nanoparticles using *Penicillium verrucosum* and analysis of their antifungal activity

Mohamed A. Yassin [a,b,*], Abdallah M. Elgorban [a,b,c], Abd El-Rahim M.A. El-Samawaty [a,b], Bandar M.A. Almunqedhi [a]

[a] Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia
[b] Agricultural Research Centre, Plant Pathology Research Institute, Giza, Egypt
[c] Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 24 December 2020
Revised 29 January 2021
Accepted 31 January 2021
Available online 13 February 2021

Keywords:
Nanoscience
Antifungal activity
Nanomaterials
Fungus
Characterisations

ABSTRACT

The present study describes the biosynthesis of silver nanoparticles, using the fungus *Penicillium verrucosum*. The silver nanoparticles were synthesised by reacting silver nitrate ($AgNO_3$) with the cell free filtrates of the fungal culture, and were then characterized by UV–visible spectroscopy, transmission electron microscopy, scanning electron microscopy, energy-dispersive, and X-ray diffraction analysis to further evaluate their successful biosynthesis, optical and morphological features (size and shape), and crystallinity. The bioactivity of the synthesized nanoparticles against two phytopathogenic fungi i.e: *Fusarium chlamydosporum* and *Aspergillus flavus* was evaluated using nanomaterial seeding media. These biogenic silver nanoparticles were polydisperse in nature, with a size of 10–12 nm. With regard to the antifungal activity, 150 ppm of the nanoparticles suppressed the growth of *F. chlamydosporum* and *A. flavus* by about 50%. To the best of our knowledge, this is the first report on the use of *P. verrucosum* to synthesise silver nanoparticles. The present study demonstrates a novel, simple, and eco-friendly process for the generation of biofunctionally useful biogenic nanoparticles.

1. Introduction

The field of nanoscience is a prospering one that holds a great future. It deals with materials in the 1–100-nm size range, the properties of which are different from those of their bulkier counterparts (Saxena et al., 2014). Nanoscale-sized materials exhibit novel chemical, physical, electronic, and magnetic properties, granting them tremendous potential for use in a wide range of applications in agriculture (Thul et al., 2013), medicine (Nosrati et al., 2021), and various areas of technological importance (Thiruvengadam et al., 2018). Over the years, researchers have put much effort into understanding the different characteristics of nanomaterials, such as their size, shape, and chemical compositions (Murray et al., 1993; Manna et al., 2002). Noble metals have served as the materials of choice ever since such research began, given that these nanoproducts can come in direct contact with humans in the form of jewellery and ornaments (Pienpinijtham and Thongnopkun, 2015).

Although chemical and physical methodologies are readily available for the synthesis of nanomaterials, the techniques usually require high temperatures, employ toxic chemicals, and release hazardous by-products (Elgorban et al., 2016a, 2016b). To address these concerns, researchers have shifted their focus towards biological methods that occur at ambient conditions, as happens in nature. For example, microbes that have been used to synthesise materials at the nanoscale include bacteria (Elblbesy et al., 2014), cyanobacteria (Chakraborty et al., 2009) algae (Mata et al., 2009), and diatoms (Schröfel et al., 2011).

In the laboratory, bacteria (Dahikar and Bhutada, 2013), fungi (Syed et al., 2013), and even viruses (Dujardin et al., 2003) have been successfully used to generate nanomaterials; for example, the production of silver nanoparticles using bacteria from a silver mine (*Pseudomonas stutzeri* AG 256) (Klaus et al., 1999) and from buttermilk (*Lactobacillus*) (Nair and Pradeep, 2002). Fungal-based

* Corresponding author at: Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia.
E-mail addresses: myassin@ksu.edu.sa (M.A. Yassin), aelgorban@ksu.edu.sa (A.M. Elgorban), balmunqedhi@ksu.edu.s (B.M.A. Almunqedhi).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.01.063
1319-562X/

approaches have been preferred over those employing plants for the generation of nanomaterials, as fungi secrete more enzymes and are easier to cultivate and grow in the laboratory.

Microbial-based nanomaterials, in particular fungal-based ones, are being extensively investigated for novel properties. Fungal species is chosen widely as it could produce particles with high stability and thus preventing aggregation and enhance longevity (Castro-Longoria et al., 2011). Fungi are relatively more resourceful then bacteria in the biosynthesis of nanoparticles due to the presence of a number of bioactive metabolites, high accumulation and enhanced production (Alghuthaymi et al., 2015). In that context, it is interesting that *Verticillium* sp. (Mukherjee et al., 2001), *Fusarium oxysporum* (Ahmad et al., 2003) and *Phoma leveillei* (Yassin et al., 2017a, 2017b) produce intracellular and extracellular nanomaterials respectively. Various fungal strains are adding to the menu of nanomaterials which are being extensively researched for their potential applications (Siddiqi and Husen, 2016). These microbial processes are being used for the generation of nanomaterials with different chemical compositions, morphologies (size and shape), and biological activities. In the current study, we screened a number of fungi in the laboratory and identified *Penicillium verrucosum* as a good candidate for the synthesis of silver nanoparticles. Upon their biosynthesis, we evaluated the physical characteristics of the myco-synthesised silver nanoparticles as well as their antifungal activities against *Fusarium chlamydosporum* and *Aspergillus flavus in vitro*.

2. Materials and methods

2.1. Biosynthesis of the silver nanoparticles

All the materials (chemicals and medium) used in the experiment were procured from Sigma-Aldrich, Darmstadt, Germany, and used as received (i.e. without further purification).

The processes for the fungus cultivation and biomass production were adopted from our previous report (Elgorban et al., 2016a, 2016b). *P. verrucosum*, isolated from vegetable-cultivated greenhouse soil (Alharj, Riyadh, Saudi Arabia), was maintained on potato dextrose agar (PDA) medium. For the biosynthesis of silver nanoparticles, the fungus was grown in liquid medium containing malt extract (0.3%), yeast extract (0.3%), glucose (1.5%), and peptone (0.5%). Erlenmeyer flasks were inoculated with a spore suspension of *P. verrucosum* and incubated at 25 °C on a rotary shaker (150 rpm) for 7 days. Thereafter, the mycelial mat was collected by paper filtration (Whatman No. 1) and washed with sterile water. A 20 g sample of this mycelial mat was added to a flask containing 200 mL of sterilised distilled water and incubated at 25 °C for 24 h. The fungal biomass was refined, and the resultant cell-free crude filtrate was used for the synthesis of silver nanoparticles. In brief, 100 mL of the cell-free filtrate was mixed with 20 mL (1:5 ratio) of an aqueous 10 mmol/L silver nitrate solution in a 250-mL Erlenmeyer flask and incubated at 25 °C in the dark. A flask containing fungal filtrate without silver nitrate solution was used as the control. The silver nanoparticles obtained by this green synthesis approach were collected by centrifugation at 11,000 rpm (twice for 20 min each time) and then stored for further study (Yassin et al., 2017a, 2017b).

2.2. Characterisation of the biogenic silver nanoparticles

The biogenic silver nanoparticles were characterised using standard techniques; namely, UV–Vis spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray diffraction (XRD) analysis (Carvajal, 1993; Prema, 2010). To obtain the UV–Vis absorption spectrum, a stock solution of the silver nanoparticles was diluted to 5 mg/5 mL and the spectrum of this diluted solution was recorded in the range of 300–800 nm using a LAMBDA 35 UV–Vis spectrophotometer (PerkinElmer, Rodgau, Germany). The size of the synthesised nanoparticles were measured by TEM with a JEM-1011 microscope (JEOL, Peabody, MA, USA), operated at 120 kV accelerated voltage. The TEM samples were prepared by drop casting of the silver nanoparticles onto a copper grid. After about 6 h of drying in an 80 °C oven, the grid was observed at high magnification for the size determination of the nanoparticles. The morphological analysis was conducted by SEM with a JSM-6380 LA microscope (Japan) operating at 2 kV. EDS was carried out on an Altima IV system (Rigaku, Tokyo, Japan) to confirm the purity of the nanoparticles. The spectrum was recorded using a copper substract in the spot profile mode over the nanoparticle-coated surface. The XRD analysis was carried out by powder XRD on a Rigaku Miniflex X-ray diffractometer (Kα; λ = 1.5406 A). The patterns were recorded in the 2θ range from 10° to 90°, with a scanning rate of 0.05 mV/s.

2.3. Inhibitory activity of the biogenic silver nanoparticles

The fungal growth inhibition assay method was adopted from our previous report (Yassin et al., 2013). In brief, the antifungal activity of the silver nanoparticles against *Fusarium chlamydosporum* and *Aspergillus flavus* was evaluated using nanomaterial seeding media. PDA medium was first autoclaved and cooled to about 45 °C. The silver nanoparticles were then added to the medium to obtain final concentrations of 0, 50, 100, 150, and 200 ppm. Mycelial plugs of about 3-mm diameter, cut out from the periphery of 7-day-old cultures of the tested fungi, were inoculated (aseptically) upside down on the nanoparticle-containing PDA. The plates (in triplicate per treatment) were incubated at 27 ± 2 °C. The growth of the tested fungi was recorded for 7 days and the percentage inhibition of mycelial growth was compared with that of the control (0 ppm silver nanoparticles). The median effective dose (ED_{50}) and the dose required for a desired effect in 95% of the fungal culture (ED_{95}) were also determined.

Statistical analysis of the antifungal activity of the silver nanoparticles was carried out using Statistics for the Social Sciences (SPSS) software, and data are presented as the mean ± standard error of the mean (SE). The ED_{50}, ED_{95}, and slope of the activity of the nanoparticles against the tested fungi were obtained using probit analysis (Bahkali et al., 2015).

3. Results

3.1. Physical characterisation of the biogenic silver nanoparticles

The fungus *P. verrucosum* bioreduced the silver nitrate, resulting in silver nanoparticles that could be seen by visual inspection of the reaction flasks. As shown in Fig. 1(A), the aqueous silver nitrate solution was colourless before the reaction. Upon incubation with the fungal cell-free extract, the silver ions were bioreduced and a distinct brown colour. The synthesized Ag NPs were subjected to UV–visible spectrophotometric analysis and the particles showed a plasmonic peak at 420 nm (Fig. 1b).

The representative TEM image (Fig. 2(A)) shows the size and shape of the resultant nanoparticles, clearly revealing them to have a polydisperse nature. The size distribution histogram showed the nanoparticles to be between 2 and 20 nm, with an average size of 10 nm (Fig. 2(B)).

SEM analysis showed the synthesised silver nanoparticles to have an irregular morphology (Fig. 3(inset)). The EDS spectrum revealed the presence of C, O, and Ag elements in the reacted solution (Fig. 3).

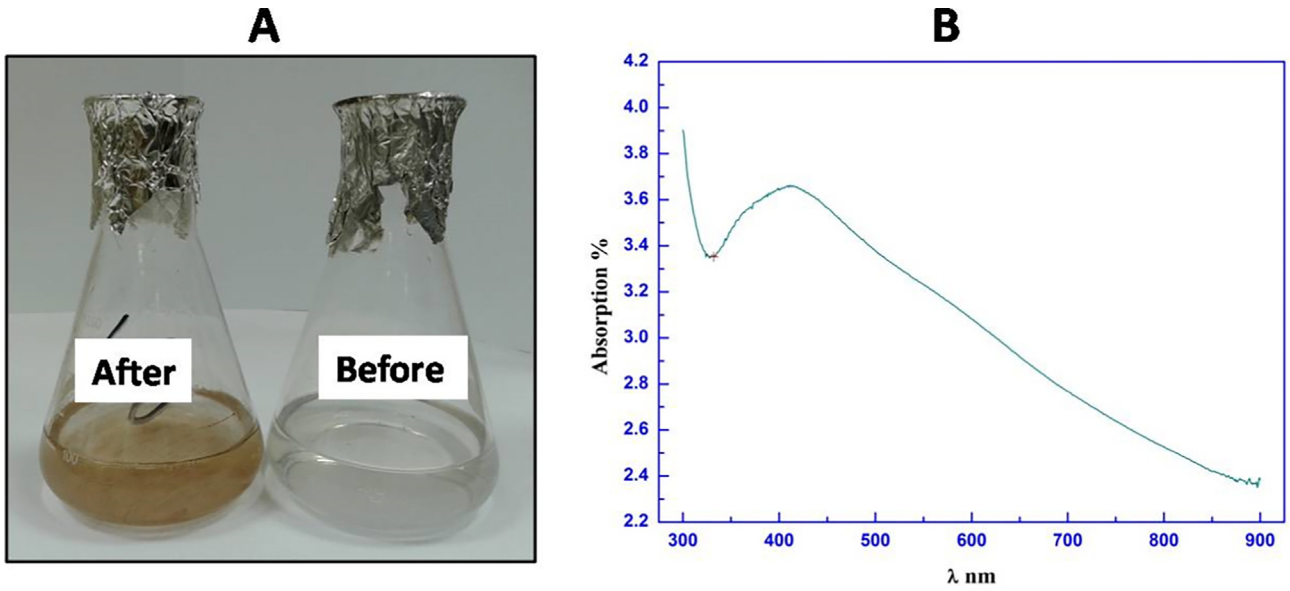

Fig. 1. (A) Flasks containing aqueous silver nitrate solutions before and after biosynthesis of the silver nanoparticles. (B) UV–Vis spectrum of the biogenic silver nanoparticles.

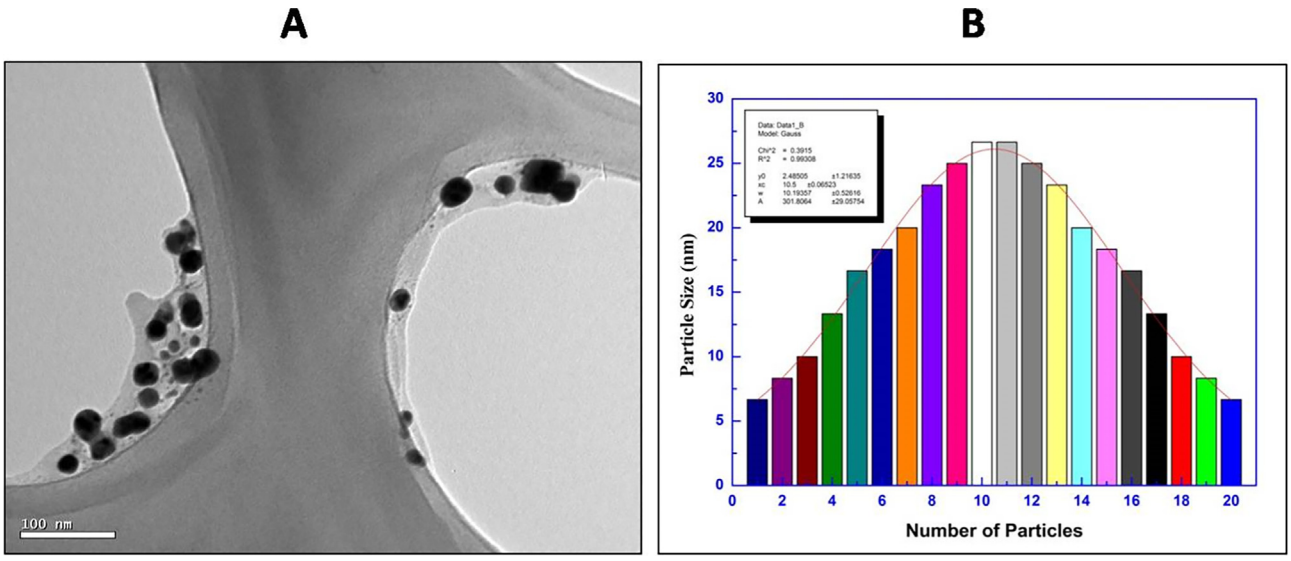

Fig. 2. (A) Transmission electron micrograph and (B) particle size distribution histogram of the biogenic silver nanoparticles.

Fig. 4 shows the results of the XRD analysis to further confirm the crystalline nature of the synthesised silver nanoparticles. The Bragg reflections that corresponded to the (111), (200), (220), (311), and (222) planes agreed with those reported for silver nanoparticles.

3.2. Inhibitory effect of the biogenic silver nanoparticles

The data in Table 1, indicate that the silver nanoparticles inhibited the linear growth of *F. chlamydosporum* and *A. flavus* by about 50% at the concentration of 150 ppm (ED_{50} = 174.68, ED_{95} = 838.3, slope = 2.33 ± 0.11) and by more than 50% at 200 ppm (ED_{50} = 156.08, ED_{95} = 1936.6, slope = 1.76 ± 7.1).

4. Discussion

Distinct brown colour following the bio-reaction of fungal cell-free extract and silver ions were attributed to the surface plasmon resonance in metals (Skottrup et al., 2007), indicating the formation of silver nanoparticles. The excitation at 420 nm was due to the strong SPR property of particles (Li et al., 2008; Tilaki et al., 2006). Even months past reaction, the solution showed high stability with no flocculation evidence of nanoparticles. It is established that capping and stabilization of the nanoparticles is brought about by different protein and other biomolecules secreted by microorganisms (Mazumdar et al., 2015). The extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes (Li et al., 2011). Recently, different filamentous fungi were reported to be proficient in the biosynthesis of noble metal NPs. The authors proposed that the fungal compounds, reductase enzymes and fungal media components potentially played a role in stabilizing the nanoparticles (Molnár et al., 2018). The researchers reported that the biosynthesis occurred by fungi and reducing sugars were involved to tailor spherical metal NPs. They studied the biosynthesis of Au NPs and established the role of specific fungal proteins in

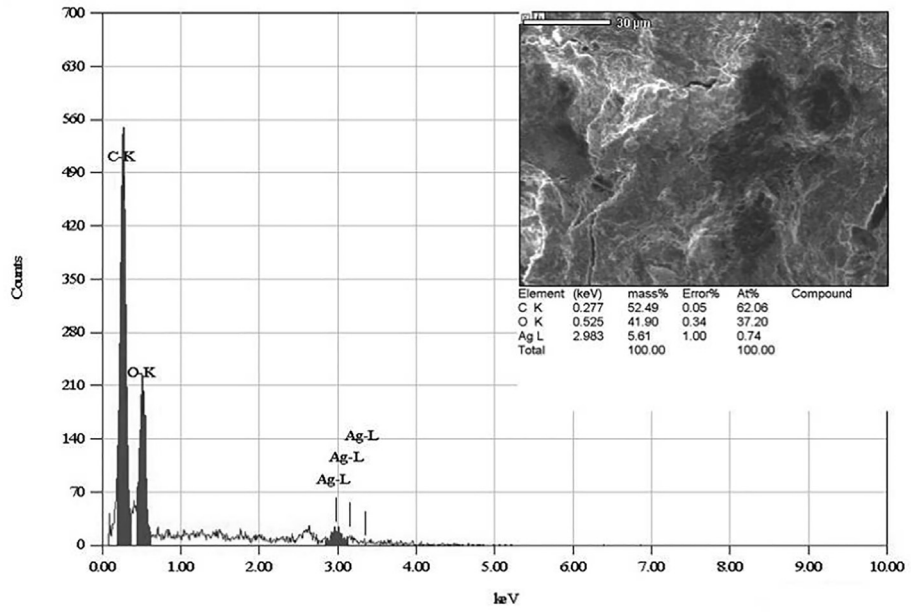

Fig. 3. The EDS micrograph of biosynthesized silver nanoparticles..

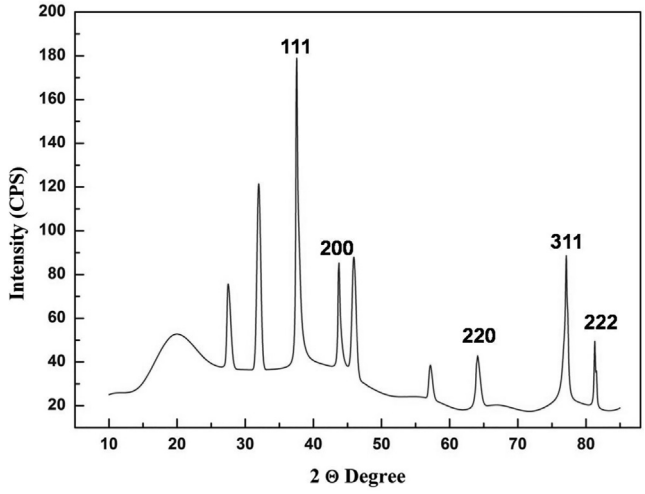

Fig. 4. X-ray diffraction spectrum of the biogenic silver nanoparticles.

the capping of the metal NPs (Zhang et al., 2011). Chowdhury et al. (2014) synthesized AgNPs by using a fungus *Macrophomina phaseolina* and reported that the NPs are stabilized by proteins. Hence, the stability of green ZV-AgNPs due to the capping proteins could have an additional advantage as antimicrobial agents. The results on particle size and nanocrystal structure revealed by XRD analysis (Fig. 4) were in line with Kalishwaralal et al. (2008), who reported the synthesis of AgNPs from *B. licheniformis* with similar diffraction peaks.

At the higher concentrations, the inhibitory effect of the silver nanoparticles was higher against *A. flavus* than against *F. chlamydosporum*. Antifungal activity could be attributed to the size, shape, and capping proteins attached to the AgNPs. These finding are in agreement with those obtained by Elgorban et al. (2016a, 2016b) and Yassin et al. (2017a, 2017b). Elgorban et al. (2017) also found that Ag^{+} and silver nanoparticles were highly effective against *Cladosporium fulvum*, which causes tomato leaf mould. Lamsal et al. (2011) reported that silver nanoparticles inhibited the growth of *Colletotrichum* species (the causal agent of pepper anthracnose) both *in vitro* and *in vivo*. This high antifungal activity of silver nanoparticles may be related to their high concentration in the solution at which they can saturate and adhere to hyphae. On the other hand, Feng et al. (2000) confirmed that both *Escherichia coli* and *Staphylococcus aureus* DNAs lost the ability to replicate when the bacterial cultures were treated with silver nanoparticles, which might lead to the damaged expression of ribosomal subunit proteins (Yamanaka et al., 2005; Kim et al., 2012). This could be true for the antifungal effect as well. Overall, our biogenic silver nanoparticles showed promising antifungal results, especially at

Table 1
Antifungal effect of the biogenic silver nanoparticles.

Nanomaterial concentrations (ppm)	*F. chlamydosporum*		*A. flavus*	
	RG	% inhibition	RG	% inhibition
0	90.00	00.00	80.75	00.00
50	80.75	10.28	66.50	17.65
100	63.75	29.17	56.50	30.03
150	52.25	41.94	44.75	44.58
200	39.00	56.67	33.00	59.13
ED_{50}	174.68		156.08	
ED_{95}	838.3		1936.6	
Slope ± SE	2.33 ± 0.11		1.76 ± 7.1	

ED_{50}: Median effective dose to kill 50% of the fungus; ED_{95}: Dose required for a desired effect in 95% of the fungal culture; R.G: radial growth; Inh. (%): percentage inhibition.

high concentrations, and may be effective against other test organisms.

5. Conclusion

In summary, a simple and feasible route for the generation of silver nanoparticles, using the fungus *P. verrucosum*, has been demonstrated. The fungus bioreduced the aqueous silver nitrate solution and stabilised the nanoparticles formed in reaction. The biogenic nanoparticles were spherical in shape and 10–12 nm in size, and could inhibit the radial growth of *F. chlamydosporum* and *A. flavus* at concentrations of 50–200 ppm. Thus, these easily bio-derived and biofunctionally useful silver nanoparticles may find applications in areas such as catalysis, medicine, and optoelectronics, among others.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this Research Group No. (RG-298).

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