

Extracellular Biosynthesis of Silver Nanoparticles Using Fungi *Penicillium diversum* and Their Antimicrobial Activity Studies

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Published online: 11 September 2012
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Abstract In this present investigation, we report a simple, cost-effective, and eco-friendly method of synthesizing colloidal silver nanoparticles by using fungi *Penicillium diversum*. UV–visible spectroscopy studies were carried out to quantify the formation of silver nanoparticles. The X-ray diffraction pattern suggests the crystallinity of silver nanoparticles. Atomic force microscopy and transmission electron microscopy images show that the silver nanoparticles are polydispersed and are in a size range of 5 to 45 nm with an average size of 20 nm. From the Fourier transform infrared spectroscopy, we presume that the reductase enzyme present in the fungal extract may be responsible for the reduction and stabilization of the silver nanoparticles. The resultant silver nanoparticles showed effective antimicrobial activity against *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, and the clinical isolate of *Paratyphina*.

Keywords Extracellular biosynthesis · Silver nanoparticles · *Penicillium diversum* · Morphological study · Antimicrobial activity

1 Introduction

Biosynthesis is a simple, eco-friendly green approach for synthesizing nanomaterials using biological components, primarily prokaryotes and eukaryotes. Microorganisms play

a direct or an indirect role in several biological activities [1]. This interaction of metals and microbes is natural and continuous since the inception of life which provides an exciting area for research [2]. Nanoparticles of noble metals have been synthesized by several methods like biological and chemical routes [3]. When the size of a material is reduced to the nanometer scale, the large surface area compared to volume becomes dominant leading to new characteristic properties [4]. The synthesis of noble metal nanoparticles has attracted increasing attention due to their new and different characteristics as compared with those of the macroscopic condensed phase [5]. As a result, nanoparticles are used for many of applications in various fields, such as: medicine, biotechnology, optics, microelectronics, catalysis, information storage, and energy conversions [6].

Silver nanoparticles (AgNP) have a larger surface area and higher dispersion owing to their very small size (<20 nm) [7]. Moreover, colloidal silver solutions carry increased interest for investigation due to their cytotoxicity properties and for applications in pharmacology [8], medicine, food industry, water purification, etc. [9]. The ability of some microorganisms such as bacteria and fungi which control the synthesis of metallic nanoparticles may be employed in the search for new nano materials. In contrast, extracellular synthesis of nanoparticles occurs in *Thermomonospora* sp. [10], which reduces Ag^+ ions. The metabolic activity of microorganisms can lead to the precipitation of nanoparticles in the external environment of a cell and the fungi and are extremely good candidates for such processes. The extracellular syntheses of Ag and AuNP by the fungus *Colletotrichum* sp. [11] or *Aspergillus fumigatus* have been reported [12]. A novel biological method for synthesis of AgNP using *Vericillum* was proposed by Mukherjee et al. [13]. This extracellular enzyme shows excellent redox properties, and it can act as an electron shuttle in metal reduction. It was evident that electron shuttles or other reducing agents (e.g., hydroquinones) released by microorganisms are capable of reducing ions to nanoparticles. The

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increasing demand for metal nanoparticles has encouraged the development of new eco-friendly routes. These include employing important microorganisms like *Fusarium semitectum* [14], *Cladosporium cladosporioides* [15]; mushrooms like *Volvariella volvacea* [16] and *Pleurotus florida* [17]; and plants like neem [18], alfalfa [19], guava [20], clove [21], etc.

The usage of Ag as an antimicrobial agent in clinical settings has a long history. Ag therapy has various beneficial effects such as (a) multilevel antibacterial effect on cells which considerably reduces the chances of developing resistance, (b) effectiveness against multidrug-resistant organisms, and (c) low systemic toxicity. However, silver compounds that are used for topical applications, viz., AgNO₃ and Ag sulfadiazine, may get neutralized by anions (chloride, bicarbonate, and protein) in body fluids or cause cosmetic abnormality like argyria (blue–gray coloration) upon prolonged use may arrest the healing process owing to fibroblast and epithelial cell toxicity. Despite these shortcomings, Ag sulfadiazine is the most popular topical antimicrobial silver delivery system in use due to the nonavailability of safer alternatives. Hence, such functionalized AgNP-based antimicrobial formulations may become better alternatives for the said purpose [22].

In this paper, we focused our attention on the preparation and characterization of extracellular biosynthesis of silver nanoparticles using *Penicillium diversum* and the study of their antimicrobial effects. The treatment of an aqueous solution of AgNO₃ with *P. diversum* extract resulted in the rapid formation of stable nanoparticles. The growth of nanoparticles is monitored by UV–vis spectrophotometer and complemented with their characterization using transmission electron microscopy (TEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR). The possible mechanism and difference in reduction time for the formation of AgNP during the synthesis are discussed. Furthermore, this green biogenic approach for the production of silver nanoparticles is a rapid and simple alternative to chemical synthesis that can deliver effective antimicrobial agents.

2 Experimental

2.1 Materials and Methods

2.1.1 Extracellular Biosynthesis of Silver Nanoparticles

The fungus culture, *P. diversum*, was obtained from IMTEC, Chandigarh; all chemicals used were of analytical grade. *P. diversum* was grown in potato dextrose broth (potato starch, 4 g/l; dextrose, 20 g/l). The pH was adjusted approximately to enable the inhibition of bacterial growth. The flasks were incubated in an environmental shaker at 200 rpm at 25 °C.

Silver nitrate (AgNO₃), 10^{−3}M final concentration, was mixed with 50 ml of the cell filtrate in a 250-ml Erlenmeyer

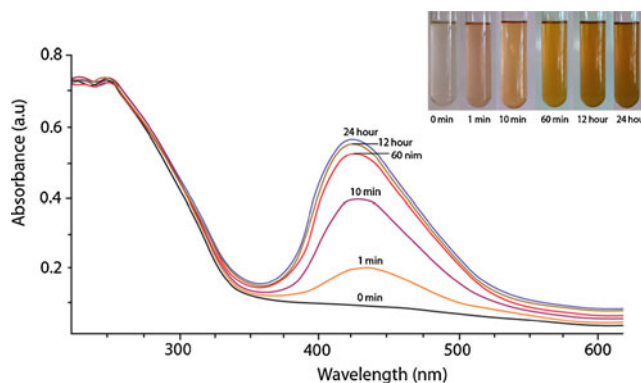


Fig. 1 UV–visible spectra indicating AgNP synthesis recorded as a function of time ($\lambda_{\text{max}} = 413 \text{ nm}$ for AuNP). For brevity, data corresponding to all spectra are not plotted

flask and agitated at 25 °C in the dark. The control (without the Ag⁺ ion, only biomass) was also run along with the experimental flask. A change in color was observed in the silver nitrate solution incubated with *P. diversum*, which is a preliminary confirmation of the formation of AgNP.

2.1.2 Purification of Silver Nanoparticles

The functionalized AgNP powder sample was prepared by centrifuging the synthesized silver nanoparticle solution at 10,000 rpm for 20 min. The solid residue formed is then washed with sterile deionized water three times to get rid of the unattached biological impurities on the surface of the nanoparticles. The resultant residue is then dried completely, and the powder obtained is functionalized AgNP devoid of any organic impurities which are not involved in the reduction or capping process.

2.2 Characterizations

2.2.1 Spectral Studies

After the addition of the extracellular Ag solution of *P. diversum* to AgNO₃ 10^{−3}M solution (1:10 in ratio), UV–vis studies were performed on an ECIL 5704SS UV–visible spectrophotometer operated at a resolution of 1 nm. To determine the probable biomolecules responsible for the reduction of the Ag ions and for the confirmation of the bio-moiety covering the AgNP,

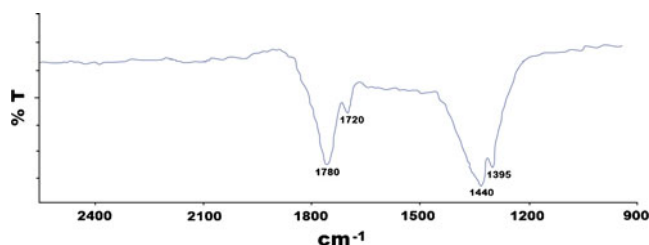


Fig. 2 FTIR spectra of bio-functionalized AgNP synthesized using extracellular extract of the fungi *P. diversum* with absorption peaks located at about 1,395, 1,440, 1,720, and 1,780 cm^{−1}

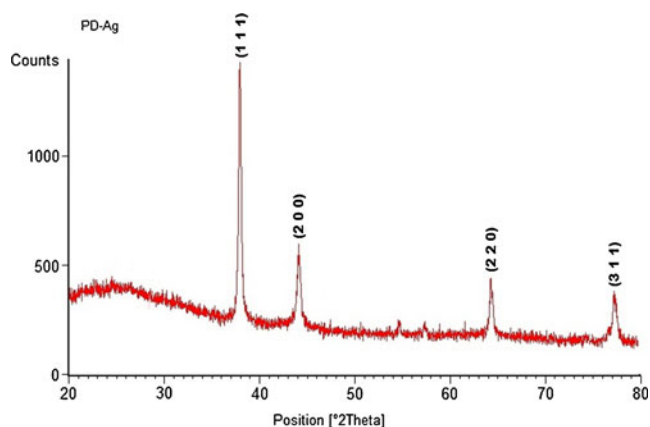


Fig. 3 XRD pattern with peaks at the corresponding diffraction signals of (1 1 1), (2 0 0), (2 2 0), and (3 1 1) facets of silver

Fourier transform infrared radiation (FTIR) spectroscopy was deployed. The samples were dried in an oven overnight at 60 °C, ground with KBr powder, made into pellet form, and analyzed on a PerkinElmer spectrum one model in the diffuse reflectance mode operating at a resolution of 4 cm⁻¹. XRD measurements of synthesized AgNP powder on a glass substrate were recorded in a wide range of Bragg angles 2θ at a scanning rate of 20°/min, and this is carried out on a Philips Model PW 1830 instrument which was operated at a voltage of 40 kV and a current of 30 mA with CuK α radiation ($\lambda=1.5405$ Å) as target.

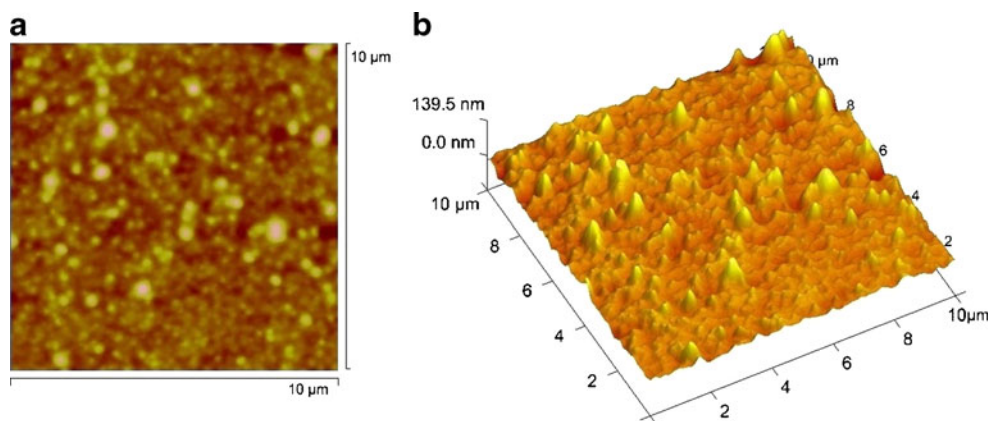
2.2.2 Morphological Studies

The images of AFM were collected under ambient conditions on a Veeco-Innova scanning probe microscope using etched Si-nano probe tips (RTESPA-M) contact mode. TEM characterization was performed by TECHNAI G20- 163 STWIN (200KV) machine in an image mode at a resolution of 50 nm scale.

2.2.3 Antimicrobial Activity

Antimicrobial activity was carried out at Biogenics, Hubli, under the controlled conditions using Biosafety levels; the

Fig. 4 a Image analysis showing AFM images of the silver nanoparticles with sizes approximately from 10 to 50 nm and with roughly spherical shapes. The nanoparticles are dispersed without any clustering. **b** AFM image showing the 3D view of the silver nanoparticles



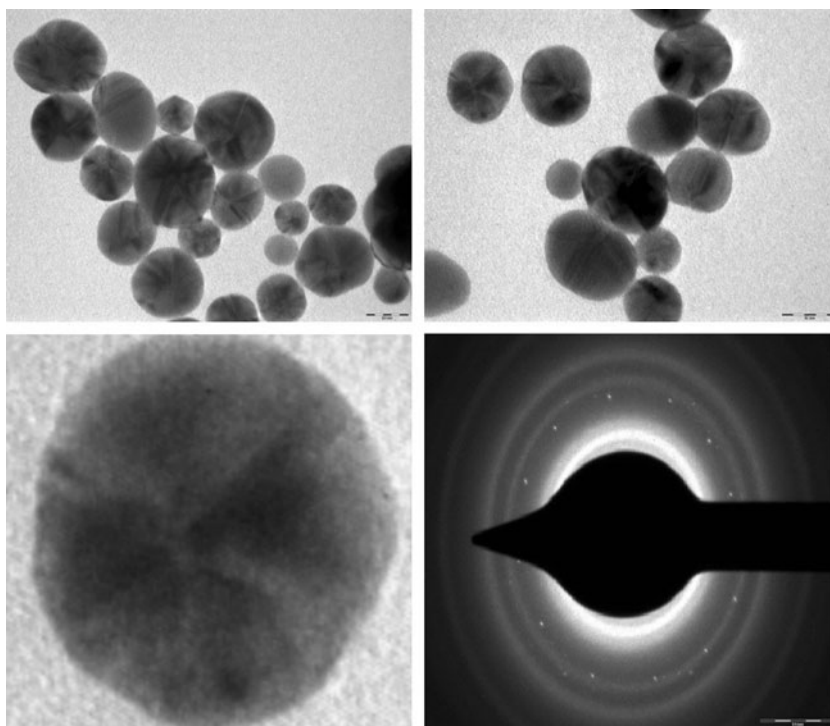
pathogenicity of the microbes were analyzed as per the guidelines provided by the Centers for Disease Control and Prevention. The antibacterial activities of silver nanoparticles were investigated by disk diffusion method, and after solidification, bacterial cultures were swabbed on these plates. The sterile disk was dipped in a functionalized AgNP solution (5 μg/ml), placed on the agar plate, and kept for incubation at 37 °C for 24 h. A zone of inhibition was measured. The results of the investigation showed that silver nanoparticles synthesized from the fungus *P. diversum* possess discrete antibacterial activity against clinically isolated pathogenic bacteria at a concentration of 5 μg/ml. Each plate was inoculated with 18-h-old cultures (100 μl) and spread evenly on the plate. After 20 min, the wells were filled with the compound (2.5, 10, and 20 μg/ml). All the plates were incubated at 37 °C for 48 h, and the diameter of the inhibition zone was noted.

3 Results and Discussion

Figure 1 shows the UV–vis spectra of the AgNO₃ solutions with *P. diversum*. The characteristic surface plasmon absorption peak at 450 nm was observed at 1 h, 447 nm at 12 h, and 445 nm after 24 h for having attained the maximum intensity. The homogeneous spherical silver nanoparticles are known to produce the surface plasmon resonance band at 413 nm. In the present system, the maximum absorbance peak was observed at 450 nm instead of 413 nm, which might be due to the reduction in the particle size, and the band was extensive with an absorption tail in the longer wavelengths, which could be in principle due to the size and shape distribution of the particles [22]. The result indicated that the silver ions are reduced to silver nanoparticles after treatment with the *P. diversum* extracellular solution for 24 h, the quantity of silver nanoparticles increased with the time of exposure, and the shape of the particles might have been deviated from the ideal spherical geometry.

FTIR measurements were carried out to identify the possible biomolecules responsible for the reduction of the silver

Fig. 5 Well-distributed AgNP with occasional aggregation are mainly spherical in shape and in the size range of 5–30 nm



ions and their capping with biomass. Figure 2 represents FTIR spectra of nanoparticles with absorption peaks located at about 1,395, 1,440, 1,720, and 1,780 cm^{-1} . The absorption peak at around 1,395 cm^{-1} can be assigned as absorption peaks of C–OH stretching vibrations of the alcoholic group [23]. The bands at 1,440, 1,720, and 1,780 cm^{-1} relate to the bonds or functional groups C=O, aliphatic –C–O, and C–C, respectively were derived from carboxylic compounds. These peaks propose the presence of proteins on the surface of AgNP [24]. The differences in the peak locations indicate that the proteins responsible for synthesis of silver nanoparticles are diverse in nature. These protein molecules act as surface-coating molecules which prevent the internal agglomeration of the particles. Consequently, the nanoparticles are stabilized in nanocolloidal solutions.

The XRD pattern in Fig. 3 with peaks shows at the corresponding diffraction signals of (1 1 1), (2 0 0), (2 2 0), and (3 1 1) facets of silver. The lattice constant calculated from this pattern was 4.0872 Å, a value in agreement with the literature report ($a=4.086$ Å, Joint Committee on Powder Diffraction Standards JCPDS file no. 04–0783). The broadening of the peaks was observed due to the effect of particle size. The noise observed in the graph is due to the presence of various crystalline biological macromolecules like proteins or enzymes. The result reveals that the silver nanoparticles formed are crystalline in nature. The size and morphology studies of the silver nanoparticles were done using AFM, SEM, and TEM. The particle size has been measured using image analysis; Fig. 4a shows AFM images of the silver nanoparticles with sizes approximately from 10 to 50 nm and with

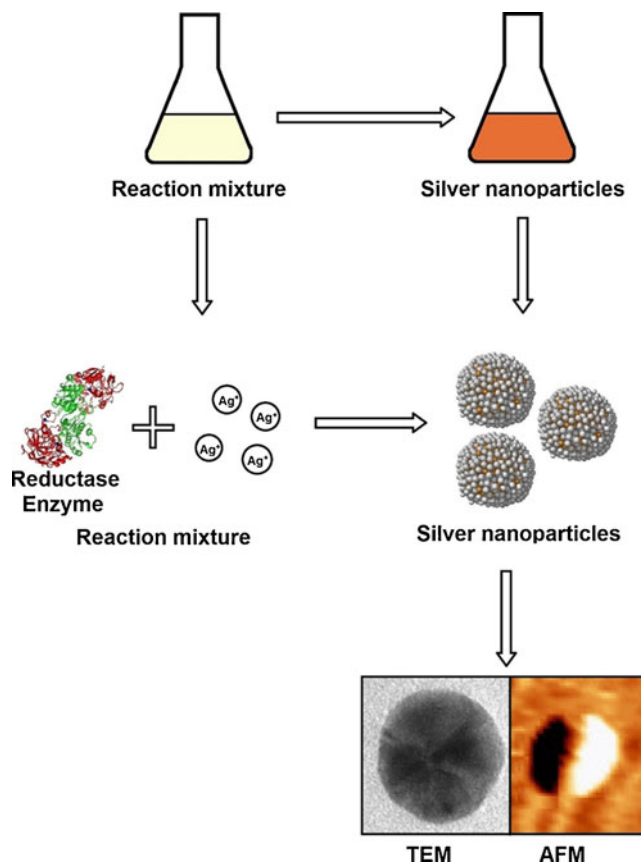
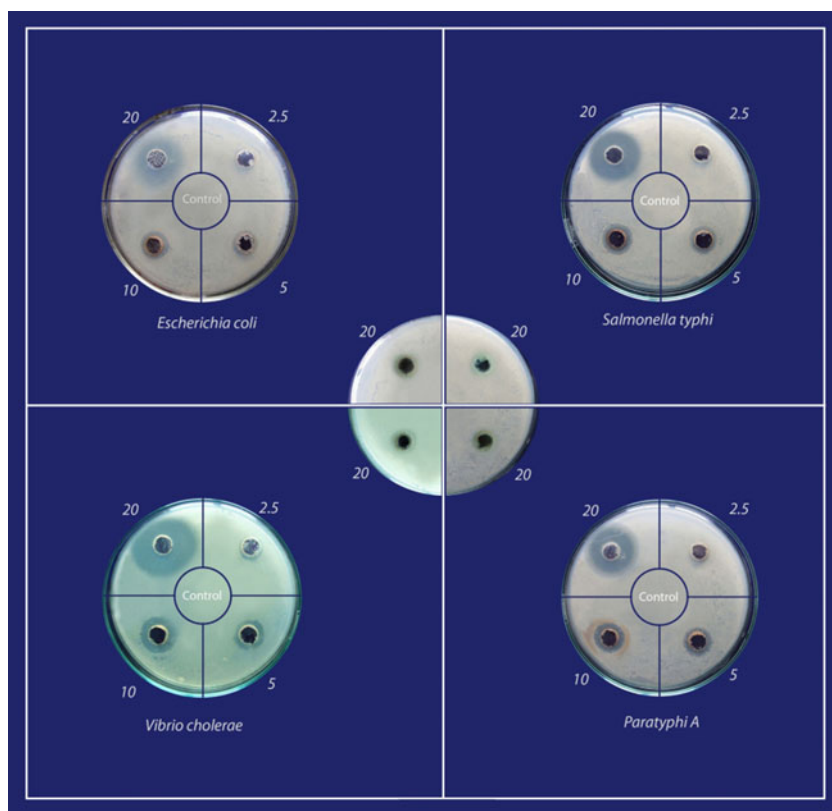


Fig. 6 The hypothetical pathway of the synthesis of AgNP biosynthesized using extracellular extract of the fungi *P. diversum*

Fig. 7 Antimicrobial activity of synthesized AgNP



roughly spherical shapes. The nanoparticles are dispersed without any clustering. The AFM image in Fig. 4b shows the 3D view of the silver nanoparticles. TEM measurements confirm the spherical shape and sizes of AgNP. As shown in Fig. 5, well-distributed AgNP with occasional aggregation are mainly spherical in shape and in the size range of 5–30 nm. Figure 5 shows the diffraction ring, as can be seen from the electron diffraction pattern recorded from the AgNP, and confirm with nanocrystalline silver. The hypothetical pathway of the synthesis of AgNP is shown in Fig. 6, it is assumed that reductase enzymes present in the fungal extract are responsible for the reduction process. After the reduction process, there exists a protein layer on the nanoparticles (confirmed by FTIR studies). TEM and AFM images of a single silver nanoparticle are also shown in the same figure. The exact mechanism and chemical component of the bioreduction process is under study. Figure 7 shows the antimicrobial activity of synthesized AgNP. Four different pathogens are chosen for the present study namely *E. coli* (ATCC 25922), *Salmonella typhi* (ATCC 10749), *Vibrio cholerae* (ATCC 39315), and *Paratyphi A* (ATCC 9150). These pathogens are treated with different concentrations of functionalized AgNP viz. 2.5, 5, 10, and 20 $\mu\text{g/ml}$.

From the MIC results, it is observed that with the 5- $\mu\text{g/ml}$ concentration, we can see the inhibition zones of size. In *V. cholerae* with 2.5- $\mu\text{g/ml}$ concentration, only 0.1 cm inhibition was observed. All the organisms showed maximum inhibition

zones with 20- $\mu\text{g/ml}$ concentrations (shown in Table 1). The reason for the good antibacterial activity of functionalized AgNP can be considered as two kinds of possibilities. One is that the surface negative charge of protein surface interferes with microbial absorption, which has also negative charge, on the surface of the AgNP. The other is that the remaining or dissolved Ag^+ in AgNP solution interacts directly with the negative charge of the protein shell. This electrostatic effect prevents the interaction between the Ag^+ ion and the microbe, and this may be the probable mechanism of good antimicrobial action [17]. The inset of Fig. 7 shows the antibacterial activity of the *P. diversum* extract. It is observed that the only extracellular aqueous extract of the microorganism used for reduction of Ag^+ ions to AgNP even at the highest concentration, i.e., 20 $\mu\text{g/ml}$, does not show any antimicrobial effect. Hence, it is clear that the antimicrobial activity is only because of functionalized AgNP.

Table 1 MIC results

Organisms	Inhibition zones (cm)			
	2.5 μg	5 μg	10 μg	20 μg
<i>Escherichia coli</i>	0	0.2	0.4	1.5
<i>Salmonella typhi</i>	0	0.2	0.5	1.6
<i>Vibrio cholerae</i>	0.1	0.3	0.7	1.9
<i>Paratyphi A</i>	0	0.3	0.6	1.6

4 Conclusions

AgNP were synthesized by biomass of *P. diversum* strain and were evaluated against pathogenic bacteria drug-resistant *E. coli*. The nanoparticles were synthesized extracellularly by *P. diversum* strain adopting standard conditions, and the synthesized purified particles were characterized by using UV–vis spectroscopy, FTIR, XRD, AFM, and TEM. UV–vis spectroscopy revealed the formation of AgNP with the range between 5 and 45 nm, and the same is confirmed by AFM as well as TEM. The antibacterial activity with AgNP shows the distinct effect and minimum inhibitory concentration (MIC) at a different concentration of 5 µg/ml for *E. coli*. In our current investigation, the biological synthesis of AgNP by non-pathogenic strains of the fungus *P. diversum* and the synthesized AgNP were found to be most active against the clinically isolated human pathogenic bacteria. The results proved that the thus obtained AgNPs showed maximum activity at the least concentration, which proved these functionalized AgNP are novel antibacterial agents.

Acknowledgments The authors are grateful to UGC, Major Research Project (F. No. 33-307/2007 (SR)), DAE-BRNS Project (No.2009/34/14/BRNS), and VGST (SMYSR-D38/7), Bangalore, for financial assistance. We also acknowledge help from SAIF, IIT Mumbai for TEM measurements, and Biogenics, Hubli, for antimicrobial studies. We thank Shri. Jagannathrao M. Deshpande, father of author Raghunandan Deshpande, for editing work.

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