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ORIGINAL ARTICLE

Biofabrication, characterization and antibacterial efficacy of extracellular silver nanoparticles using novel fungal strain of *Penicillium atramentosum* KM

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Abstract The biofabricated silver nanoparticles are extensively used in environmental, biotechnological and biomedical applications. The synthesis of SNPs has been carried out by using the filtrate extract of novel fungal strain *Penicillium atramentosum* KM. To undertake this study, *P. atramentosum* KM extract was exposed to silver nitrate and the obtained SNPs were thoroughly analyzed using physicochemical characterization tools such as UV–visible spectroscopy (UV–vis), Fourier transformation infrared (FTIR), X-ray diffraction (XRD) and transmission electron microscopy (TEM). As evident from the FTIR spectra plausibly the protein components of fungal extract caused the reduction of silver nitrate. The SNPs showed a characteristic UV–visible peak at 420 nm with an average size of 5–25 nm. The XRD record exhibited the characteristic peaks of 111, 200, 220 and 311 nanoparticles signifying that these nanoparticles were crystalline in nature. Parametric optimization showed maximum absorbance of 420 nm at pH 7, 25 °C with 3 mM silver nitrate, concentration ratio of fungal extract and silver nitrate was 5:5 in 72 h. The synthesized SNPs showed antimicrobial activity against bacterial strains.

1. Introduction

Nanotechnology is referred to as the term for fabrication, characterization, manipulative and application of structures by controlling shape and size at nano scale. Nanotechnology is one of the most fascinating research areas in modern materials science and the synthesis of nanoparticles is gaining importance all over the world. The nanoparticles have significantly enriched physical, chemical, and biological properties due to their nano-scaled size (1–100 nm). SNPs have a very large surface area which typically results in greater biochemical

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reactivity, catalytic activity and atomic behavior compared to larger particles of the same chemical composition [1]. The synthesis of SNPs has received considerable attention due to their potential applications in catalysis [2], plasmonics [3], optoelectronics [4], biological sensor [5] antimicrobial activities [6], DNA sequencing [7], and surface-enhanced Raman scattering (SERS) [8]. Nanoparticles have delivered solution to various problems like climate change and pollution control [9], clean water technology [10], energy generation [11], information storage [12] and biomedical applications [13]. The synthesis of nanoparticles has a significant breakthrough in the field of nanotechnology in the last 10 years and proves its potentials by new and varied applications. The nanoparticles are synthesized by many physical and chemical methods which are time consuming, costly and toxic for the environment. The physical methods such as lithography and laser ablation and chemical methods start with silver salt precursor (dissolved in solvent) reduced in a chemical reaction and the nanoparticles are formed through nucleation and growth. The development of environmental friendly and sustainable techniques for the production of SNPs to be used in medical field is a big challenge. Various reports showed that it can be synthesized by bacteria [14], fungi [15,16] and plants [17–19]. It is found that fungi are more suitable than bacteria for the use of the extracellular synthesis of SNPs as the fungi could form large biomass which facilitates handling. Some of the most commonly used fungi for fabrication of SNPs are *Aspergillus flavus* NJP08 [15], *Penicillium* sp. [16], *Fusarium oxysporum* [20], *Penicillium purpurogenum* NPMF [21], and *Fusarium acuminatum* [22].

In our previous study, the SNPs were successfully synthesized by using plant extract of *Mangifera indica* and *Psidium guajava* [18,19]. Considering the significance of biosynthesized nanomaterials, the present study has been designed to undertake the extracellular biosynthesis of SNPs by using the novel fungal *P. atramentosum* KM and to optimize various cultural conditions for maximum yield of SNPs.

2. Materials and methods

2.1. Microorganisms

The fungal strain used in the present investigation was *P. atramentosum* KM previously which had been originally isolated from tannery effluent [23]. The culture was grown and maintained on Potato Dextrose Agar Plates at 30 °C and preserved at 4 °C, respectively.

2.2. Preparation of cell filtrate

P. atramentosum KM spores were inoculated into liquid Potato Dextrose broth and incubated at 25 °C for 96 h in an orbital shaker at a constant speed of 120 rpm. The biomass was harvested by using Whatman filter paper No. 1 and washed twice with sterile double distilled water to remove any medium component. Ten grams of biomass was taken in a 250 mL conical flask and 100 mL of double deionized water was added. Then the mixer was agitated at 120 rpm and incubated at 30 °C for 72 h. After incubation, the fungal filtrate was obtained by passing through Whatman filter paper No. 1. The fungal extract was ready to use or preserved at 4 °C for future use.

2.3. Biosynthesis of silver nanoparticles

The aqueous solution of silver nitrate (Analytical grade, Hi Media Laboratories Pvt., Mumbai, India) was prepared in double deionized water in a sterile amber color bottle and kept at room temperature for 24 h. For the reduction of silver ions, fungal extract was mixed with aqueous solution of $AgNO_3$ in 250 mL Erlenmeyer flask and incubated at 25 °C on an orbital shaker at a constant speed of 120 rpm in the dark. The control consisting of silver nitrate solution mixed with sterile distilled water was also run along with the experimental flask.

2.4. Optimization of process parameter for silver nanoparticle synthesis

The different parameters were optimized for the synthesis of SNPs including concentration of silver nitrate, concentration ratio of fungal extract and silver nitrate, time, temperature and pH which had been identified as factors which affect the productivity of SNPs. The concentration of silver nitrate was optimized using different concentrations of $AgNO_3$ such as 1, 2, 3, 4 and 5 mM, respectively. The concentration ratio of fungal extract and silver precursor in range of 1:9, 2:8, 3:7, 4:6, and 5:5 was used to find out the optimum ratio. The effect of time on reaction was studied by using different time intervals from 0 to 96 h of incubation. The temperature of the reaction was investigated by incubating at 5, 15, 25, 35 and 45 °C, where the reaction temperature was maintained using a water bath. The effect of pH on the synthesis of SNPs was evaluated by suspending $AgNO_3$ at pH of 2, 5, 7, 9 and 11. The pH was maintained with help of 0.1 N HCl and 0.1 N NaOH. The various parameters for optimization synthesis of SNPs were monitored by using a UV–Visible spectrophotometer (Systronic-115, India) with a resolution of 1.0 nm between 300 and 600 nm.

2.5. Characterization of synthesized silver nanoparticles

2.5.1. FTIR spectroscopy analysis

The sample was washed twice with double distilled water followed by ethanol to remove all filtrate contents and the remaining unconverted silver ions by centrifugation at 10,000 rpm for 10 min to get purified SNPs in suspension before characterizations. The FTIR spectrum of SNPs was obtained from Thermo Scientific Nicolet (iS50, India) FTIR with resolution at 4.000 from 400.1569 to 4000.1221 range.

2.5.2. X-ray diffraction measurements

The purified suspension of SNPs was freeze dried to obtain dry powder. Finally, X-ray diffraction (XRD) patterns of the dried SNPs were analyzed by an X'Pert Pro PANalytical X-ray diffractometer instrument with X'Pert high score plus software operating at a voltage of 45 kV and a current of 40 mA with Cu Kα radiation.

2.5.3. Transmission electron microscopy

For transmission electron microscope (TEM) measurements, a drop of 10 fold diluted sample of the synthesized SNPs was placed on the carbon coated copper grids and allowed the water to evaporate before loading them onto a specimen

holder. TEM observations were performed on an H-7500 electron microscope (Hitachi, Japan) operated at an accelerating voltage of 120 kV.

2.5.4. Antimicrobial activity

The antimicrobial activities of synthesized SNPs were determined by using the agar well diffusion method against pathogenic bacteria. All the test cultures were procured from the Microbial Type Culture Collection Center (MTCC), Chandigarh, India. The test cultures, e.g., *Bacillus cereus* (MTCC-1305), *Staphylococcus aureus* (MTCC-3160), *Micrococcus luteus* (MTCC-1809), *Salmonella typhimurium* (MTCC-1253), *Aeromonas hydrophila* (MTCC-1739) and *Enterobacter aerogenes* (MTCC-2823) were maintained at

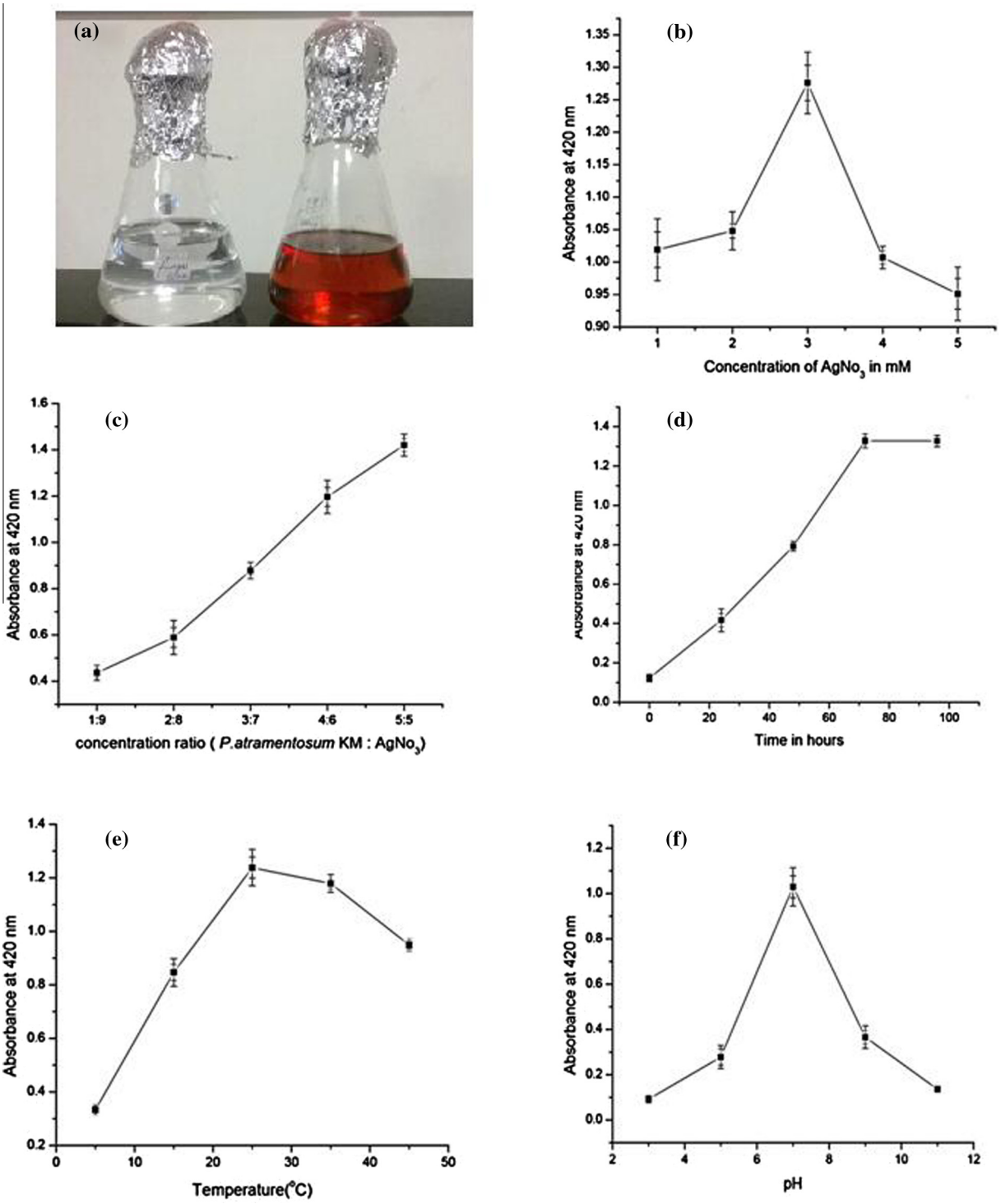

Figure 1 (a) Erlenmeyer flask containing cell-free filtrate of *P. atramentosum* KM without (A) and with (B) silver nitrate solution (3 mM) after 72 h of reaction. (b) Effect of concentration of silver nitrate ($AgNO_3$). (c) Effect of concentration ratio of *P. atramentosum* KM extract and silver nitrate. (d) Effect of time. (e) Effect of temperature. (f) Effect of pH on production of silver nanoparticles.

4 °C on nutrient agar until use. Then the SNPs were poured into each well on all the plates with the help of micropipette (20 µl). The plates were incubated at 37 °C for 24 h in upright position and zone of inhibition was measured.

2.5.5. Statistical analysis

Each experiment was carried out in triplicate and results are presented as the mean ± standard deviation (SD) in the respective figures.

3. Results

On exposure to colorless $AgNO_3$ solutions, *P. atramentosum* KM formed dark brown colored solutions that indicated the formation of SNPs (Fig. 1a). UV–Vis absorption spectrum of SNPs formed in the reaction mixture exhibited an absorbance peak at 420 nm. 3 mM concentration of silver nitrate gave sharp and characteristic absorption spectra, whereas the peak got shifted at 1, 2, 4 and 5 mM concentrations (Fig. 1b). The ratio of 5 mL of fungal extract along with 5 mL of silver nitrate showed a characteristic peak at 420 nm, whereas the sharp peak was not obtained at concentration ratios of 1:9, 2:8, 3:7, and 4:6 (Fig. 1c). The incubation period of 72 h was found to be suitable for SNPs' synthesis (Fig. 1d). The bioreduction of silver ions at 25 °C of incubation temperature showed optimum activity and the results are presented in Fig. 1e. Similarly when reaction mixture incubated at different pHs indicated a sharp peak at pH 7 whereas both acidic and basic pHs hamper the reduction of silver ion into SNPs (Fig. 1f).

The FTIR spectrum of SNPs showed three distinct peaks, 462.86, 1643.82 and 3360.72 cm^{-1} (Fig. 2). The XRD patterns of the synthesized SNPs are as shown in the Fig 3 and showed peak in the whole spectrum of 2θ values ranging from 20 to 80. Typical bright-field TEM images of the synthesized SNPs are shown in Fig. 4 and it confirmed a spherical shape with an average size of 5–25 nm.

When tested by agar well diffusion method, the SNPs showed significant antibacterial efficacy against bacterial pathogens (Fig. 5). The SNPs showed highest antimicrobial activity against gram positive *B. cereus* and *S. aureus* with the zone of inhibition of 23 mm and 22 mm respectively whereas Gram negative bacterium *A. hydrophila* showed a zone of inhibition of 22.5 mm.

4. Discussion

Recently SNPs have attained great interest because of their unique physical and chemical properties. Extracellular secretion of the microorganisms offers the advantage of obtaining large quantities in a relatively pure state, free from other cellular proteins associated with the organism with relatively simpler downstream processing. Formation of SNPs could be easily monitored by change in color from colorless to dark brown. The generation of dark brown color is due to the surface plasmon resonance (SPR) exhibited by the SNPs [24].

It is well known that the morphology and size of metal nanoparticles produced from metallic precursor in solution depend on various reaction conditions such as concentration of metal ion, ratio of metallic salt/reducing agent, time, temperature and pH [31].

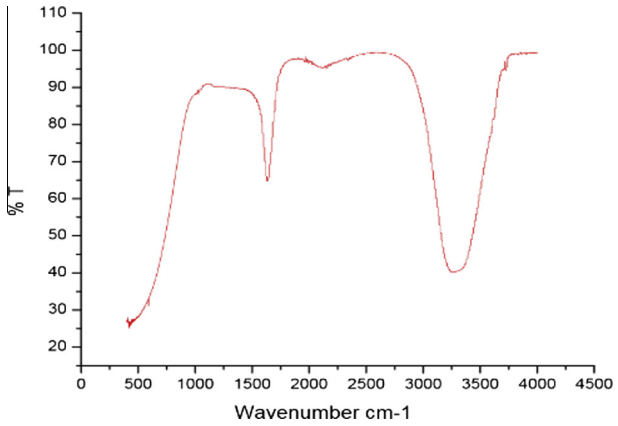

Figure 2 FTIR spectrum of synthesized silver nanoparticles.

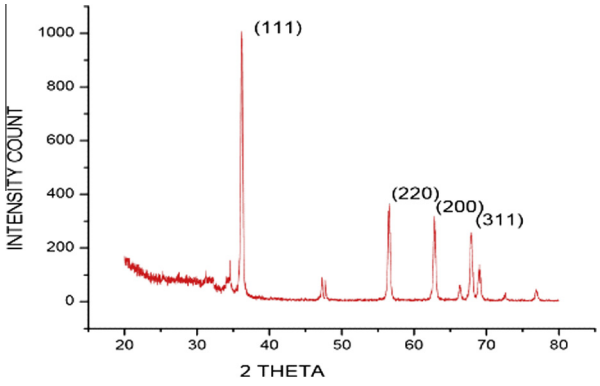

Figure 3 X-ray diffraction pattern of synthesized silver nanoparticles.

The results from our investigation indicated that 3 mM concentration of silver nitrate is suitable for the synthesis of SNPs. Similarly, Kalishwaralal et al. [14] found that 3 mM substrate concentration is suitable for the synthesis of SNPs by using *Bacillus licheniformis*. Our results were contradictory with those of Singh et al. [16] who reported 1 mM optimum substrate concentration for SNPs' synthesis by using *Penicillum* sp. It could be explained by the fact that they used substrate up to 2 mM concentration only. By a gradual increase in concentration of silver nitrate to 3 mM, the nanoparticle production was increased, however, by further increasing to 5 mM, the production decreased. It might be interpreted that further increase in concentration above 3 mM could have toxic effects on *P. atramentosum* KM extract.

The optimization study of concentration ratio of fungal extract and silver nitrate led to the enhanced SNPs' synthesis. Vaidyanathan et al. [30] had also reported that equal concentration ratio of bacterial extract and silver nitrate was optimum for SNPs' synthesis by using the organism *B. licheniformis*. The synthesis was found to be dependent on the enzymatic activity. On increasing the concentration ratio of fungal extract and silver nitrate up to 5:5 the enzyme activity for converting all silver ions to SNPs increased. It might be due to the fact that the more enzyme is available, the more quickly the substrate can be converted into product.

Time is a significant factor affecting SNPs' production. Incubation period of 72 h was considered to be enough for

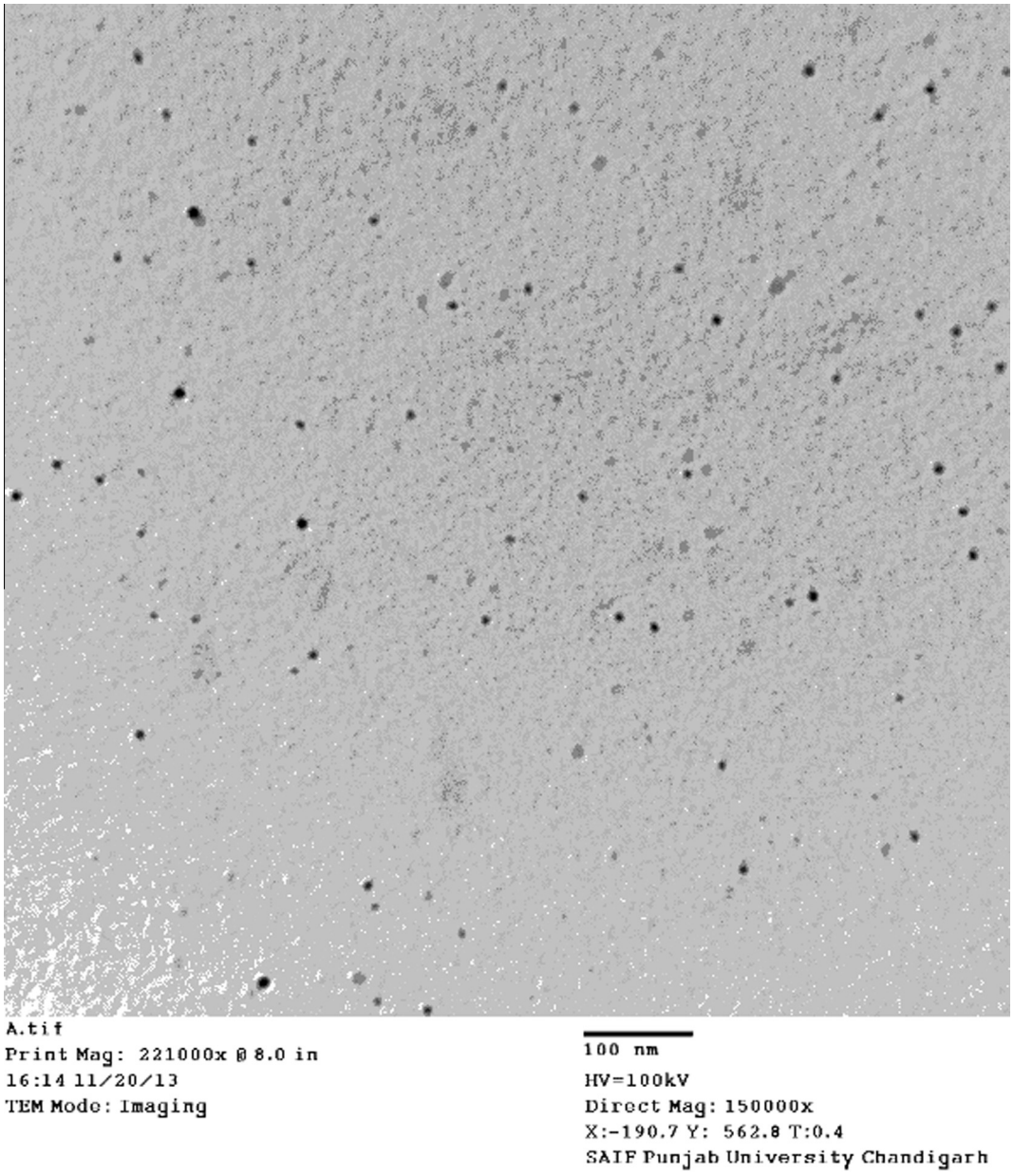

Figure 4 TEM micrograph showing silver nanoparticles.

the maximum production of SNPs. Our findings were in agreement with the results obtained by Jain et al. [15] and Bhainsa et al. [29] who reported 72 h of incubation period by using fungal extracts of *Aspergillus flavus* and *Aspergillus fumigates*, respectively. The reduction of silver ions to SNPs' dependence on the time could be associated with the reaction time of the enzymes' exist in the fungal extracts.

Incubation temperature of 25 °C showed higher yield of SNPs. Singh et al. [16] and Mukherjee et al. [32] also reported similar results by using endophytic fungi *Penicillium* sp. and *Verticillium*. The reduction of silver ions to SNPs' dependence on the temperature could be associated with the enzymes stability exists in the fungal extract. At low and high temperatures, broadening of absorbance was observed indicative of enzyme denaturation.

The enzymes secreted by the fungus, *P. atramentosum* KM was very stable at neutral pH while at higher pH and lower pH, the enzyme did not show any functional activity for the synthesis of SNPs. It could be evident from our results where neutral pH was found to be suitable for SNPs' synthesis. Several authors also reported similar results by using endophytic fungi *Penicillium* sp., *A. flavus* and *Rhizopus stolonifer* [15,16,34].

It is evident from FTIR measurements that the possible interactions between silver and bioactive molecules may be responsible for the synthesis and stabilization of SNPs with the capping agent available in the fungal filtrate. The SNPs are stable and well dispersed. The FTIR spectrum of SNPs showed a peak at 3360.72 cm^{-1} which refers to the stretching vibrations of primary amines while the peak at 1636.17 cm^{-1}

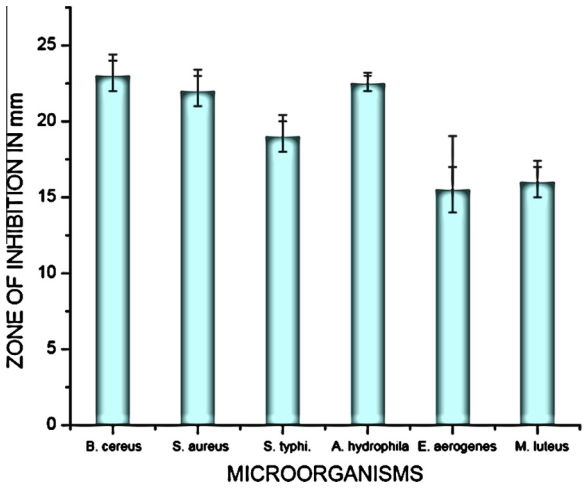

Figure 5 Antimicrobial activities of silver nanoparticles against bacteria. All values represented in the table are average of results of three separately conducted experiments.

is due to the carbonyl stretch vibrations in the amide linkages of proteins and 548.38 cm^{-1} is the fingerprint. The carbonyl groups of amino acid residues and peptides have a strong ability to bind to silver [25]. The proteins present over the SNPs' surface act as capping agent [26]. The results were in accordance with those of Sastry et al. [27] and Sanghi and Verma [28] who reported that functional groups such as —C—, O—C—, —C—O— and —C═C— are derived from proteins of the fungal extract and acted as capping ligands of the nanoparticles.

XRD pattern spectra clearly indicated the pure silver crystalline nature. The data obtained matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS file No. 04-0783). The XRD spectrum confirmed that the silver particles formed in these experimental procedures were in the form of nano size, it was evident by the peaks at 2θ values of 36.259 corresponding to (1 1 1) plane for silver, respectively. The full width at half maximum (FWHM) values measured for 1 1 1 plane of reflection were used with the Debye–Scherrer's equation $d = 0.9\lambda/\beta\cos\theta$. Our results indicated that the synthesized SNPs by *P. atramentosum* KM were in the form of nanocrystals. Similar results have also been reported when extracts of other fungi such as *Fusarium oxysporum* and *Fusarium semitectum* were employed [20,25].

TEM images of the synthesized SNPs exhibited spherical shape and an average size is 5–25 nm. SNPs in size ranges of 5–60 nm and 5–25 nm were reported for *Fusarium* spp. [20,25] and *Aspergillus* spp. [24,29], respectively. Furthermore the nanoparticles synthesized by bioreduction displayed highly effective activity against human pathogenic bacteria. SNPs as nanometer scale silver provide an extremely large surface area for better contact with bacteria thus they get attached to the cell membrane, increase permeability of the cell membranes and finally result in cell death. Antibacterial activity may be due to loss of activity of DNA replication. It has been hypothesized that the expression of ribosomal subunit proteins as well as some other cellular proteins for ATP synthesis becomes inactivated [6,16,33]. Our findings of antimicrobial activity corroborated with the earlier report of Fayaz et al. [33] wherein they investigated antimicrobial activity of SNPs against *Salmonella typhi*, *Escherichia coli*, *S. aureus*, and *M. luteus*.

5. Conclusion

The present investigation reported a simple, rapid and eco-friendly route to synthesize SNPs by fungal extract *P. atramentosum* KM. The results clearly indicated that the optimization process played a crucial role in the silver precursor reduction. SNPs in size range of 5–25 nm were synthesized that showed potent antimicrobial activity against various bacterial species like *S. aureus*, *B. cereus* and *A. hydrophila*. Hence it is worth mentioning that *P. atramentosum* KM strain is by far the most appropriate fungal strain reported in the literature and has immense potential to be considered for industrial biofabrication of SNPs.

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