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Research Article

GREEN SYNTHESIS OF SILVER ANTIMICROBIALS FOR ITS POTENTIAL APPLICATION IN CONTROL OF NOSOCOMIAL INFECTIONS

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ABSTRACT

Objective: The aim of the project was to obtain silver nanoparticles (AgNPs) with an extract obtained from *Penicillium notatum* and to functionalize paper for its use as antimicrobial.

Methods: Silver nitrate was taken as the starting material and formation of the AgNPs was monitored using ultraviolet-visible (UV-VIS) absorption spectroscopy. We have used transmission electron microscopy, zeta potential measurement and UV-VIS spectroscopy to characterize the NPs obtained. The antibacterial activity of the NP was measured by Kirby–Bauer method.

Results: Synthesized AgNPs monitored using UV-VIS spectroscopy indicated a typical surface plasmon absorption maxima of 430 nm from the UV-VIS spectrum. The average size and morphology of AgNPs were determined by differential light scattering and found in the range of 30-40 nm. The NPs generated were found to have high antimicrobial and bactericidal activity against Gram-negative as well as Gram-positive bacteria such as *Escherichia coli*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtillis*.

Conclusion: This study indicates successful green synthesis of AgNPs without the use of capping agents. Experimentation proved that AgNPs conjugated with paper can be used to design antimicrobial wipes for the hospitals. As the AgNPs have already been proved to have antimicrobial activity, the disadvantage of generating antibiotic resistant strains can be easily avoided.

Keywords: Silver nanoparticle, *Penicillium notatum*, Nosocomial infections.

INTRODUCTION

Antibiotic resistance and nosocomial infection has become a major bottleneck in public healthcare sectors. Almost every variant of bacteria has developed resistant strain to antibiotics mainly due to lateral gene transfer and plasmid exchange enabling curing of infectious disease an expensive challenge [1]. Thus, there arises the necessity to design protocols to prevent the spread of infection and at the same time be cost effective.

The use of silver has a healing metal has already been discussed vastly in charak samhita [2]. Metallic nanoparticles (NPs) have found multivariate applications in the field of biotechnology and medicine [3-6] optic lithography [7], photoelectrochemistry [8] and electronic devices [9]. The efficacy of the NPs vary based on physical parameters like size, zeta potential, crystallinity, and structure [10,11].

The Indian traditional medicine has always considered silver has a therapeutic metal with its potential application in detoxification. A number of microorganisms including algae, bacteria and fungi have been reported for the green synthesis of silver NPs (AgNPs) [12].

In this work, an extracellular production of AgNPs using *Penicillium notatum* extract was intended, and its efficiency checked as an adsorbed antimicrobial. This is the first report of using *P. notatum* as a source for the green synthesis of AgNPs and its application as an antimicrobial in adsorbed form in controlling nosocomial infections causing microbes without any plausible toxicity.

METHODS

Material

P. notatum NCIM 745 was obtained from MTCC, IMTECH, Chandigarh. Microbial growth media such as nutrient agar, potato dextrose broth (PDB) and agar and silver nitrate ($AgNO_3$) was obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Chemicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Pvt. Ltd, India.

Methods

Preparation of AgNPs

P. notatum was inoculated from mother culture on potato dextrose agar at 27°C for 3 days. This starter culture was used for propagation of the fungus in 150 ml of PDB in 500 ml Erlenmeyer flask. Subcultured media was incubated at 27°C for 72 hrs. At this stage, the fungal biomass was separated from the culture medium (PDB) by centrifugation at 8,000 rpm for 15 minutes, and the supernatant was decanted. The supernatant was sterilized by passing the suspension through a membrane filter (0.22 µ) to ensure removal of spores. Different concentrations of $AgNO_3$ solution (1-10 mM) and mycelia free media of *P. notatum* were mixed in different ratios (1:1; 1:2) and placed on a rotary shaker at room temperature for incubation in dark. The un-inoculated media was used as control. The pH and time period was also varied and standardized for maximal production of NPs. Pellet was obtained by centrifugation at 12,000 g for 20 minutes. The net protein concentration [13], citric acid [14], and ascorbic concentration [15] was measured for the supernatant before and after incubation with $AgNO_3$. NPs obtained were washed repeatedly with de-ionized water to remove any residual amount of media.

Ultraviolet-visible (UV-VIS) spectra analysis

The bioreduction of metal ions in solution was monitored by periodic sampling of aliquots (0.1 ml) of aqueous component and measuring the UV-VIS spectra of the solution in 1 mm optical path length quartz cuvettes with performed by using UV-VIS double beam spectrophotometer. (UV-VIS spectrophotometer [Systronics]). Aliquot collection was done every 30 minutes after mixing of the culture supernatant with $AgNO_3$ solution.

Characterization techniques

Dynamic light scattering (model no: HORIBA NP analyzer SZ-100) was used to analyze particles size distributions. The zeta potential of the synthesized particles was also determined (model no: HORIBA NP analyzer SZ-100). Samples for transmission electron microscopy (TEM) studies were prepared by placing drops of the AgNPs solutions on carbon-coated TEM grids.

Antibacterial assay

The antimicrobial susceptibility of AgNPs was evaluated using the disc diffusion method. Zones of inhibition were measured after 24 hrs of incubation at 37°C [16]. Six bacterial strains, *Bacillus cereus* MTCC 7351, *Escherichia coli NCIM 2642*, *Streptococcus pneumoniae* MTCC 655, *Klebsiella* sp. MTCC 10309, *Staphylococcus aureus* MTCC 7443, *Bacillus subtillis* NCIM 2329 selected based on the propensity of the species to cause nosocomial infections, were grown on Mueller–Hilton agar media for the analysis. The disk containing NPs and control (citric acid) were placed on the agar plate and then incubated at 37°C for 24 hrs. The inhibitory zones were also checked for bacteriocidal and bacteriostatic effects.

Antioxidant assay

DPPH as a radical scavenger was used to measure the antioxidant efficacy of a AgNPs. The change in color monitored at 520 nm indicates the strength of the anti-oxidative potential of the molecule being tested [17]. The DPPH solution (0.25 mM) was prepared in 95% methanol. The reaction is incubated for 30 minutes at room temperature in the dark. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 520 nm, which reflects the amount of DPPH radical remaining in the solution. Ascorbic acid was used as a reference standard and dissolved in double distilled water to make the stock solution.

Test sample was prepared by resuspending 0.2 g of AgNPs and 95% methanol was used as the blank. Percent scavenging of the DPPH free radical was measured using the following equation:

DPPH scavenging effect (%) = $(A_0 - A_T)/A_0 \times 100$

Where, A_0 was the absorbance of the control and A_T was the absorbance in the presence of the sample (AgNps dissolved in methanol) [18].

RESULTS AND DISCUSSION

Preparation of AgNPs

P. notatum (NCIM 745) was grown in PDB for a period of 3 days at 27°C. After 3 days, mycelium was pelleted by centrifugation at 8000 g for 15 minutes. Clear cell free supernatant was obtained and filtered to remove any particulate matter. It was necessary to monitor the changes in the supernatant to find the plausible cause for the formation of NPs. Hence, minor biochemical test were performed to detect the changes in the broth after incubation of fungus for 3 days. It has been observed that amino acids [19,20] citric acid [14] and ascorbic acid [15] have played a major role in capping and thus stability of the NP formation [21]. In order to determine the causative factor of NP formation, total protein content, citric acid estimation, and ascorbic acid estimation was done to monitor the change in concentration before and after incubation with $AgNO_3$. No change in protein concentration was observed although an increase in pH was observed. Hence, change in citric acid was monitored and found a very slight change in concentration of citric acid. It is proposed that the citric acid release by *P. notatum* may play a role for formation and stability of NPs [22,23]. In some of Penicillium species (*Penicillium fellutanum* and *Penicillium diversum*), proteins and some enzymes have played a major role in AgNPs formation [24,25]. Since no change in protein concentration was observed in *P. notatum*, it was certainly a variance. In addition, an incubation temperature of 5°C was needed for the formation by Kathiseran *et al*. as compared to room temperature in our experiments.

UV-VIS spectra analysis

Culture supernatant of *P. notatum* was used to synthesize AgNPs at room temperature. The supernatant and $AgNO_3$ solution was mixed in the ratio of 1:1 and the mixture was monitored for color change. The reduction of pure silver ions was observed by measuring the UV-VIS spectrum of the reaction at different time intervals taking 1 ml of the sample, compared with 1 ml of supernatant used as blank. A change in color from yellow to brown was found to occur in 1-hr, while no color change was observed in the culture supernatant without $AgNo_3$. The absorbance maxima were found to be around 438 nm. AgNPs have been found to give an absorption in a range of 400-450 nm [26].

Mie's theory of scattering postulates small spherical nanocrystals exhibit distinct surface plasmon resonance (SPR) band, whereas anisotropic particles should exhibit two or three bands, depending on their shape. According to the SPR, we can clearly suggest the AgNPs formed were spherical in shape (Fig. 1) [27].

The standardization procedure for AgNPs production indicated maximum amount was obtained when a minimal concentration of 1 mM $AgNO_3$ solution was incubated with fungal supernatant maintained at a pH 6 (Fig. 2). As per earlier reports, citric acid dissociates and enables the reduction of silver salt to metallic silver [28]. Increase in pH enable easier binding and access of citrate to ionized silver. Hence, all the experiment thereafter performed synthesized with same incubation conditions.

Characterization techniques

NPs were dried, re-suspended in distilled water and vortexed. The suspension was centrifuged at maximum speed for 20 minutes to pellet the NPs. This washing step was repeated.

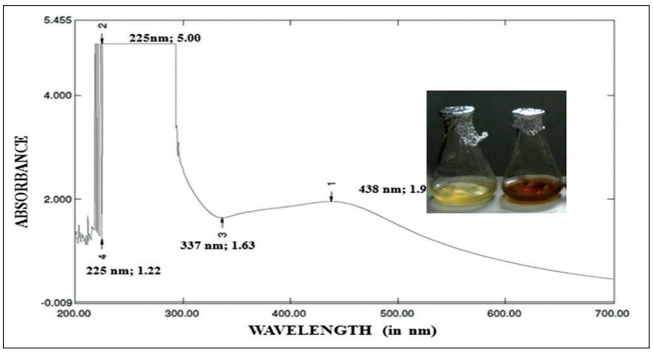

Fig. 1: Surface plasmon resonance for corresponding to formation of silver nanoparticles (AgNPs) at 438 nm. (Inset) colour change observed before and after incubation of silver nitrate and mycelia free supernatant. The peak corresponding to 438 nm is correlated to the colour change associated with AgNPs

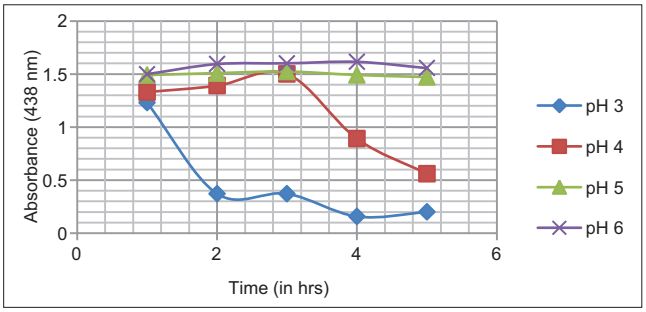

Fig. 2: Absorbance monitored on incubation of fungal supernatant with 1 mM silver nitrate solution at pH 3 (blue), pH 4 (red), pH 5 (green) and pH 6 (violet)

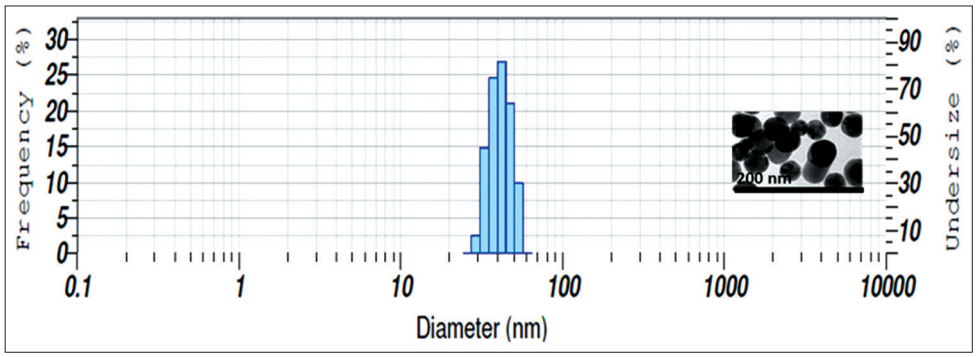

Fig. 3: Histogram for particle size distributions of silver nanoparticles (prepared from 10 mM silver nitrate solution). Inset: Transmission electron microscopy image

5-6 times to wash any residual quantity of broth on NPs. The samples were again dried and used for TEM analysis. TEM images recorded different variation sizes of AgNPs and formation of spherical NPs in the reaction solution. The maximum frequency of the NPs is in the range of 30-40 nm (Fig. 3).

No agglomeration of the particles was observed as indicated by the absence of any necking between the peaks. *Penicillium chrysogenum* and *Penicillium expansum* have generated AgNPs after a 72 hrs incubation period with a particle size above 60 nm [29]. Similarly, *Penicillium citrinum* has generated AgNPs with a size of 109 nm. Some species like an endophytic fungi from *Cucurma longa* has generated 30 nm AgNPs in the pH 6. Hemanth *et al*. have also described the formation of NPs in the range of 40-70 nm Singh *et al.* [30]. NPs generated using *P. notatum* had range 30-40 nm so it is expected to have same intensity of activity.

Zeta potential analysis of NPs

Zeta potential analysis is a technique for determining the surface charge of NPs in solution (colloids). NPs with zeta potential values >+25 mV or <−25 mV typically have high degrees of stability. Dispersions with a low zeta potential value lead to aggregation because of Van Der Waal inter-particle attractions. The synthesized AgNPs were found to have a maximum peak of zeta potential at −76.1 mV indicating a stable NP formation without any susceptibility of aggregate formation.

Microbial susceptibility assay

10 µg of AgNPs were obtained and adsorbed on sterilized paper discs. As only the concentration of citric acid varied in the supernatant, citric acid adsorbed paper was taken as the control. Antimicrobial tests were performed against *B. cereus*, *E. coli, S. pneumoniae*, *Klebsiella*, *S. aureus* and *B. subtilis* cultured in petriplates. The discs were incubated with the bacterial plates for a 24 hrs and zone of inhibition was measured (Figs. 4 and 5).

The zone of inhibition for control (citric acid) was absent as compared to a pronounced Zone of inhibition for AgNPs. It was observed that the AgNPs synthesized using *P. notatum* are more potent toward Gram-negative bacteria as compared to Gram-positive bacteria. The zone of inhibition indicated a bacteriocidal effect in case of *E. coli* and *Klebsiella* sp. Similar potency has been observed in another *Penicillium* sp. but the antimicrobial activity has been measured with respect to volume of dispersed AgNPs [31] as compared to fixed weight of NPs which has been adsorbed on paper.

Antioxidant assay

The *ex-situ* usage of DPPH was done to assay antioxidant activity demonstrated by AgNPs. Percent of inhibition for DPPH radical scavenging activity is presented. An amount of 0.2 wt% of the metal NPs was dispersed in 10 ml methanol to obtain a suspension. Then, 1 ml of metal suspension was added to 1.5 ml DPPH solution in a test tube. Assay was performed to check the scavenging potential of the AgNPs. Scavenging

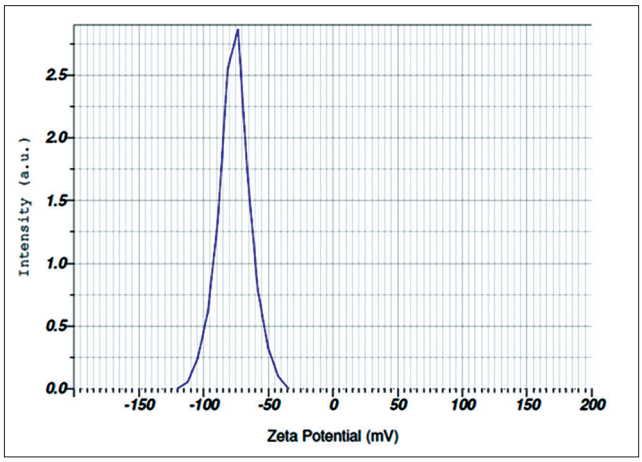

Fig. 4: Zeta potential for silver nanoparticles synthesized using *Penicillium notatum*

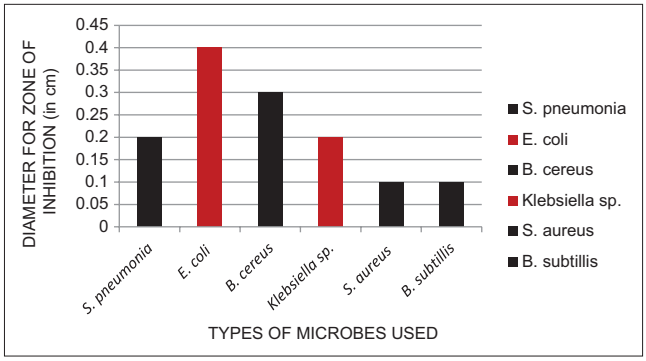

Fig. 5: Graphical representation of zone of inhibition indicated by silver nanoparticles (AgNP) over different types of bacterial species. Gram-negative bacteria (red) and Gram-positive bacteria (black). It was observed the activity the AgNP was more potent against the Gram-negative bacteria AQ4

activity of an antioxidant on DPPH radical results in the transformation to 1,1-diphenyl-2-picrylhydrazyne (DPPH-H) [32]. As part of this transition, the color of the solution turns from purple to yellow.

The extent of the color change (i.e., radical scavenging) was quantified by the decay in absorbance at 517 nm using the formula:

Control OD at 517 nm - sample OD at 517 nm/control OD at 517 nm*100.

The scavenging activity was calculated as 75.6% for AgNPs.

Based on the results, we can clearly say the NPs generated have negligible antioxidant activity [33].

CONCLUSIONS

Nosocomial infections are a major concern for patient safety and care. As per CDC/NNIS report, 2002, 20 lakhs deaths occur annually due to nosocomial infections in America alone. The major modes of transmission of the infections are through aerial or contact methods. The general misuse of antibiotics has led to antibiotic-resistant strains of various microbes [34]. It is thus required to generate a prevention measure which will have no side effect on patient care. Increasing awareness towards green chemistry and biological processes has led to a desire to develop environment-friendly approaches for the synthesis of biogenic substances using biological materials research. Metallic nanomaterials have emerged as an important methodology for the development of microbiocidals [35,36].

This study indicates successful green synthesis of AgNPs without the use of capping agents. Bioreduction of $AgNO_3$ was performed using *P. notatum* extract. In view of the data generated, it can be speculated that a citric acid produced by the fungus may be converting the cationic silver into AgNPs. Formation of nanostructures was confirmed by the development of brownish red color when an extract of *P. notatum* was added. The AgNPs were spherical in shape with no observed aggregation. AgNPs synthesized were smaller in size with no additional capping reagent for stability.

According to the CDC, the most common pathogens that cause nosocomial infections are *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli*. Five common bacteria involved in nosocomial infections were selected to check the potency of AgNPs as a preventive measure was used to check the plausibility of using AgNPs as sterilization mechanism. It was found that the NPs were maximally active against *E. coli* followed by *B. cereus* and *Klebsiella.* With this study, we could prove that AgNPs conjugated with paper can be used to design antimicrobial wipes for the hospitals. As the AgNPs have already been proved to have antimicrobial activity, the disadvantage of generating antibiotic resistant strains can be easily avoided.

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