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Efficacy of nanosilver from soil fungus enhancing the antiseptic activity of Ciprofloxacin against pathogenic bacteria

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ABSTRACT

During the present study, the biosynthesis of silver nanoparticles was made by using the filamentous fungus, Penicillium citrinum isolated from the soil sample of an industrial area of Chennai, Tamil Nadu, India. Fungal biomass was aerobically grown and fungal cell filtrate was challenged with1mM of $AgNo_3$ resulted in the change of color solution indicated the formation of silver nanoparticles. These nanoparticles were confirmed by UV-Spectrophotometric analysis and further analyzed by AFM study, which determined the particle size and average roughness. X ray diffraction (XRD) analysis determined the metallic nature of silver nanoparticles. The synthesized silver nanoparticles were checked for antibacterial efficacy alone and along with Ciprofloxacin which showed that these nanoparticles enhanced the antimicrobial activity of Ciprofloxacin against some bacterial pathogens.

Key words: AFM, Silver nanoparticles, UV-spectrophotometer, Ciprofloxacin

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INTRODUCTION

In day today life, pathogenic microbes are becoming resistant to various antibiotics available in the market makes the matter of concern for the medical professionals and researchers[1,2] in order to find out a solution to overcome it. So people especially form the research group are trying to find out the new antibacterial material to counter the bacterial resistance, especially metallic nanomaterials and nanoparticles, in which silver based compounds have good antibacterial activity [3, 4]. Nanoparticles have a wide range of applications, as in controlling the growth of various pathogens, bio-labeling and in the treatment of various cancers. The bactericidal property of silver nanoparticles known from ancient times and it has been demonstrated that at low concentrations it shows the efficacy. It has also been reported that Ag+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane. The interaction of Ag+ with bacteria is directly depending upon the size and shape of the nanoparticles. Now a day's different methods are available for the synthesis of nanoparticles like Physical, Chemical and biological methods. Physical and chemical methods are costly and time consuming, but biological method is considered as quick, cheap and free from any toxic chemicals. Different microorganisms have been used for the biosynthesis of nanoparticles but using fungi for the biosynthesis of nanoparticles is advanced as they produce lot of enzymes in the cell free filtrate i.e., reductase enzyme which

reduces the silver nitrate into silver nanoparticles [5, 6, 7, 8]. The biological method for biosynthesis of metal nanoparticles especially uses the fungal mycelia extract. Moreover, the time required for the completion of reaction of biosynthesis of nanoparticles using fungi ranges between approximately 24 hrs and 48 hrs, whereas maximum synthesis of AgNPs can be achieved after 24 hrs of incubation. Here in this proposed work we have worked on the extracellular biosynthesis of silver nanoparticles from *Penicillium citrinum* which is followed by microscopic characterization UV- spectrophotometric analysis and AFM analysis. These nanoparticles were checked for antibacterial activity to check the combined effect of nanoparticles with Ciprofloxacin against various pathogens.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from an industrial area of Chennai, Tamil Nadu, India. Soil samples were taken from 3 to 4cm depth with the help of sterile spatula. Soil samples were then transferred into sterile plastic bags and brought to the Biomedical and Research laboratory, Sathyabama University, Chennai and stored in a refrigerator at 4^0c up to further process.

Isolation of fungi

Isolation of soil fungi was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get the concentration which ranges from10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to SDA plates. The plates were incubated at room temperature for 5 - 8 days. The fungal isolates were sub cultured on SDA plates in order to segregate the isolated fungi into pure culture. Pure isolated fungal cultures were maintained at 4^o C for further studies.

Microscopic characterization

Penicillium citrinum was observed by the author's expertise using hand lens and the colony morphology was recorded with respect to color, shape, size and nature of colony and also by consulting laboratory manuals in the Department of Biomedical Engineering, Sathyabama University Chennai.

Biosynthesis of silver Nanoparticles

Penicillium citrinum was employed for the biosynthesis of nanosilver and fungal biomass was produced by using the fungal biomass was grown aerobically in a liquid medium containing (g/L): KH_2PO_4 7.0; 2.0 K_2HPO_4 $MgSO_4$. $7H_2O$ 0.1; $(NH_4)2SO_4$ 1.0; yeast extract 0.6; glucose 10.0 at 25 ± 3^0c. After incubation, the biomass was filtered using Whatman filter paper No.1 and extensively washed with distilled water to remove residual parts. The fresh and clean biomass was taken into an Erlenmeyer flask, containing 100ml of deionized Milli-Q water. The flask was incubated at 25^OC in a shaker incubator at 140 rpm for 72 hours. The biomass was filtered again with Whatmann filter paper No.1 and the cell free extract was used further. 1mM $AgNo_3$ was prepared and 50ml was added to the cell-free extract and kept further in the incubator at 25^OC, 140rpm for 72hours in dark condition.

Characterization of silver nanoparticles

After the three days of incubation the solution color gradually changes into yellowish and then the absorbance were measured using the UV- Spectrophotometry from 300 – 600nm quartz cuvette with control (Elico Ltd, Bangalore). After the synthesis, these silver nanoparticles were further characterized by AFM which was used to determine the particle size and agglomeration of the nanoparticles. The two dimensional and three dimensional image of AFM were taken which showed the particle height and average roughness of silver nanoparticles.These biologically synthesized silver nanoparticles was sonicated followed by centrifugation. Then sample is then dried and made a thin film on the glass slide and subjected to AFM analysis [9]. For X ray diffraction (XRD) analysis sample has been prepared by sonication of the silver nanoparticle solution which is followed by centrifugation at 10,000rpm at 15minutes.The supernatant is discarded and pellet is dried and makes it in to powder form and then subject for XRD analysis.

Bactericidal Effect

Silver nanoparticles synthesized from the *Penicillium citrinum* were evaluated for its antibacterial potency by disc diffusion method against various pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholerae* and *Proteus vulgaris* following NCCLS guidelines [10]. These nanoparticles were compared along with antibiotics, Ciprofloxacin and zone of inhibition was measured after overnight incubation. Experiments were repeated three times and standard deviation was calculated.

RESULTS AND DISCUSSION

The cell free filtrate produced from the fungal biomass of *Penicillium citrinum* were employed for the biosynthesis of silver nanoparticles. Upon addition of the silver nitrate ($AgNo_3$) to the solution, the color of the solution gradually changed into brown indicated the formation of silver nanoparticles [Fig 1] due to surface plasma vibration and the reductase enzyme released by the fungi [10,11].

A B

Fig 1: A) Before addition of $AgNo_3$ and B) After addition of $AgNO_3$

The nanoparticles were further characterized by UV–Vis Spectrophotometry which showed the absorption peak at 430nm which is specific for the silver nanoparticles (Fig 2) which suggested that the silver nanoparticles are well dispersed and uniform in size [10].

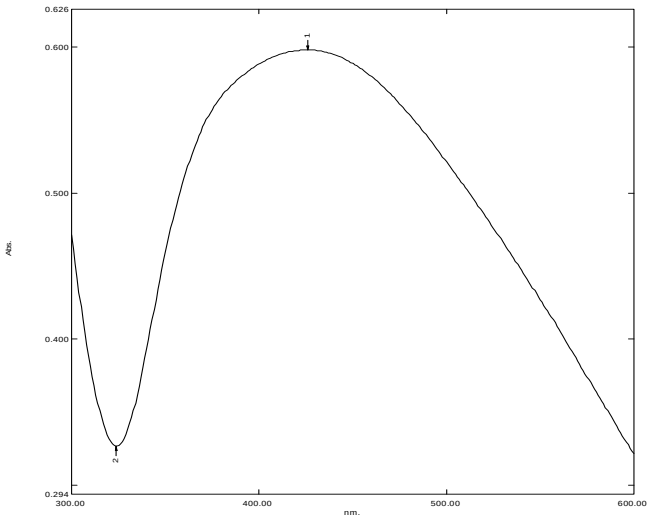

Fig 2: UV spectrophotometric analysis of AgNPs synthesized from *Penicillium citrinum*

The AFM analysis is used to determine the shape size and agglomeration of the nanoparticles. Two dimensional image of AFM showed the particles are agglomerated and spherical in shape and size was around 81nm, whereas 3D analysis showed the average roughness of the nanoparticles (Fig 3, Fig 4). It was observed that silver nanoparticles were agglomerated, spherical, polydispersed.

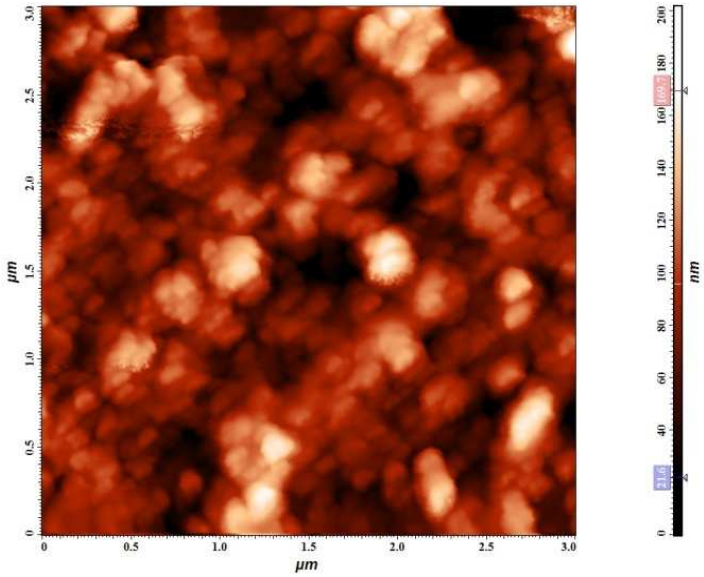

Fig 3: 2D AFM image of AgNPs synthesized from *Penicillium citrinum*

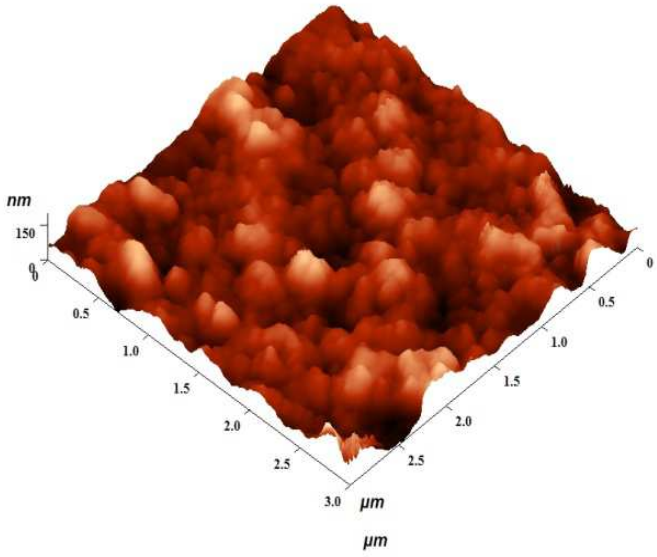

Fig 4: 3D AFM image of AgNPs synthesized from *Penicillium citrinum*

X ray diffraction analysis is used to determine the crystalline and metallic nature of nanoparticles by using X ray diffract meter. X ray diffraction showed the theta value peaks at 32, 38, 66 and 77 of silver obtained from *Penicillium citrinum.* XRD analysis showed that silver nanoparticles synthesized from *Penicillium citrinum* are face centered cubic structures[11] and also showed that these silver nanoparticles are crystalline in nature(Fig 5)[12].

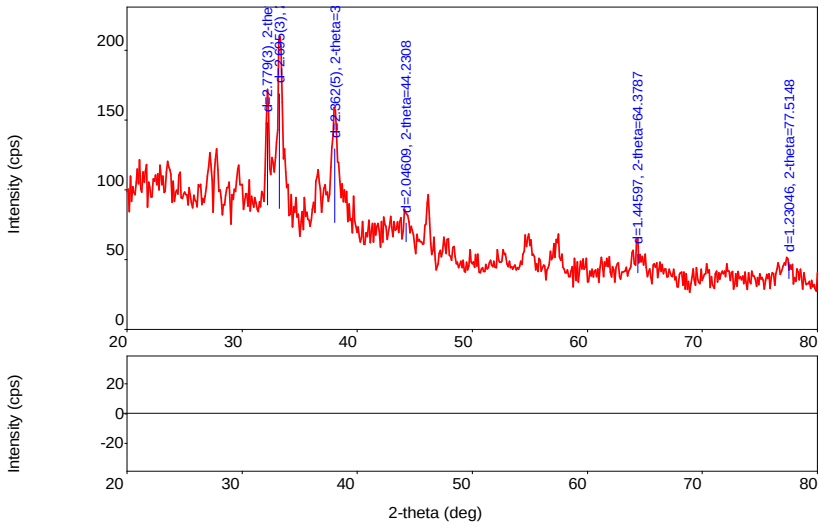

Fig 5: XRD analysis of AgNPs synthesized from *Penicillium citrinum*

These biologically synthesized silver nanoparticles were further checked for its antibacterial activity against various bacterial pathogens like, *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholerae* and *Proteus vulgaris* by disc diffusion method. Each disc was impregnated with 30µg silver nanoparticles and showed good antimicrobial activity alone and the combined effect of silver nanoparticles with Ciprofloxacin (5mcg) antibiotic available in the market. Cell free filtrate was taken as a negative control. The highest zone of inhibition was showed *Proteus vulgaris* (32mm) followed by *E. coli* (27mm), *Vibrio cholerae* (25mm) and *Staphylococcus aureus* (23mm) as shown in Table 1. Fig 6 showed the graphical representation of the combined effect of Ciprofloxacin and AgNPs. From the above experiment, it showed that silver nanoparticles alone showed good antibacterial activity and also showed that it enhances the antibacterial activity of Ciprofloxacin were studied during the experiment.

Table 1: Zone of Inhibition (mm) of AgNPs, antibiotic and its combined effect with antibiotics

Pathogens	Cell free filtrate	AgNPs 30µg/disc	Ciprofloxacin 5mcg/disc	Ciprofloxacin +AgNPs
S. aureus	7±0.21	14±0.35	19±1.65	23±0.26
P. vulgaris	8±0.27	16±0.28	25±0.56	32±0.85
E. coli	8±0.34	15±0.35	23±0.75	27±0.34
V. cholerae	7±0.63	13±1.35	22±0.52	25±0.48

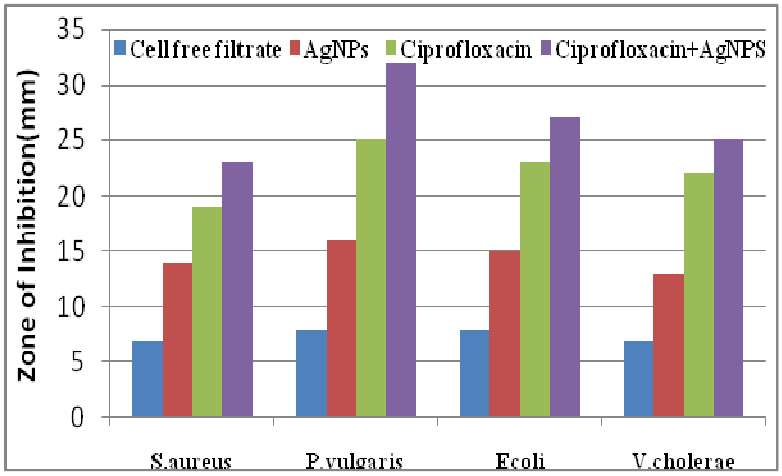

Fig 6: Graph showing combined effect of antibiotic (Ciprofloxacin) and AgNPs

CONCLUSION

During the present experiment, biological method was used for the synthesis of silver nanoparticles from *Penicillium citrinum*, which was found cheap, free from toxic chemicals, safe and environmentally friendly. The color of the solution changed from colorless to yellow an latter brown confirmed the formation of nanoparticles. UV spectrophotometer showed the strong peak at 430nm. AFM showed that nanoparticles are spherical in shape and having the size 81nm. XRD analysis showed that particles are in nano-size and are also confirmed the metallic nature of nanoparticles and face centered cubic. These nanoparticles showed good bactericidal activity alone and also enhanced the antibacterial activity of Ciprofloxacin in a combined form. The synergistic mode of action of silver nanoparticles with the antibiotics proved here to be a suitable drug against the bacteria studied herewith.

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