

Enhanced antibacterial efficacy of biosynthesized AgNPs from *Penicillium glabrum* (MTCC1985) pooled with different drugs

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Abstract: Efficacy of silver nanoparticles against pathogenic microbes has been known from ancient era. Now a day, bacteria are getting resistant to varied antibiotics based on their wide adaptability nature. In the present study, silver nanoparticles (AgNPs) were bio-synthesized in-vitro by extracellular method from *Penicillium glabrum* (MTCC1985). The appearance of yellowish brown color in the conical flask suggested the formation of Ag-NPs. The supernatant of the fungus culture changed the solution into brownish color upon the completion of the 5 minute reaction. The nanoparticles were confirmed by Uv-Vis spectrophotometer, Fourier transform infrared (FTIR) analysis, Field emission scanning electron microscopy (FESEM), Atomic force microscopy (AFM). Size of the nanoparticles was measured in between 26nm to 32nm by FESM. Silver nanoparticles showed good antimicrobial activity against the selected pathogens but combined formulation with antibiotics, the biosynthesized Nanoparticles from *Penicillium glabrum* (MTCC1985) amplified the antimicrobial potency of the antibiotics studied.

From the Clinical Editor: In this study, *Penicillium glabrum* (MTCC 1985) has been used for the synthesis of silver Nanoparticles is quite fast, biocompatible, simple and free from any toxic chemicals The antibacterial efficacy of various antibiotics was found to be enhanced in the combination of silver Nanoparticles against varied human bacterial pathogens.

Key words: AgNPs, *Penicillium glabrum* (MTCC1985), FESEM, FTIR, AFM, Uv-Vis Spectrophotometer.

Introduction

Nanomaterials especially silver Nanoparticles have a lot of applications in the field of biotechnology, biomedicine, biosensors, catalyst and therapeutic areas^{1,4}. Different methods are available for the synthesis of silver nanoparticles like physical, chemical but recently development of biological method for the synthesis of Nanoparticles is simple, quite fast, eco-friendly and free from any solvent or toxic chemicals involvement in the process^{5,6}. Synthesis and characterization of nanoparticles is presently an important area of research, as selection of size and shape of nanoparticles provides an efficient control over many of the physical and chemical properties¹. Biological method for synthesizing of silver Nanoparticles could have application in the field of medicine especially as anti-carcinogenic effect, drug carrier and diagnosis purposes. Different gold and silver nanoparticles (nonmaterial) have been synthesized by the biological method using (fungi, bacteria, algae)⁷. By using biological method, controlled silver Nanoparticles has been produced which increases the interest in

this field of nanomedicine (development of drug) research^{8,9}. Different fungi have been reported for the synthesis of silver Nanoparticles¹⁰⁻¹². These fungi reduces the metal (silver and gold) extracellularly due to the presence of reductase enzyme present in the cell wall which generate stable silver and gold Nanoparticles in the deionized water^{13,14}. Silver has been used from the ancient times in the form of silver nitrate and silver sulfadiazine in order to treat various infections like wounds, burns, ophthalmic problems and also used as a disinfectant¹⁵. Development of resistance by the bacterial pathogens to the antibiotics has become a major problem in the worldwide in the recent times¹⁶. Biosynthesized Silver Nanoparticles have been used to counter these drug resistant microbes¹. This present work is an attempt to biosynthesize silver Nanoparticles from *Penicillium glabrum* (MTCC1985) procured from IMCT, Chandigarh, India by extracellular method to confirm the formation of silver Nanoparticles by UV-Vis spectroscopy followed by various microscopic characterization and to evaluate its (silver Nanoparticles) efficacy as a bactericide as well as its synergistic effect with other drugs in order to combat the growth of selected bacterial pathogens viz., *Staphylococcus aureus*, *Bacillus cereus*, *E. coli*, and *Proteus vulgaris*.

Materials and Methods

The fungal culture of *Penicillium glabrum* (MTCC 1985) strain was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The obtained fungal culture was maintained in Sabouraud's Dextrose Agar (SDA) medium and sub cultured from time to time to regulate its viability in the laboratory during the present study.

Synthesis of silver Nanoparticles

Penicillium glabrum (MTCC1985) was used for the biosynthesis of silver nanoparticles. Fungal biomass was grown aerobically in a liquid medium containing (g/L): KH_2PO_4 7.0; 2.0 K_2HPO_4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1; $(\text{NH}_4)_2\text{SO}_4$ 1.0; yeast extract 0.6; glucose 10.0 at $25 \pm 3^\circ\text{C}$. After incubation, the biomass was filtered using Whatman filter paper No.1 and extensively washed with distilled water to remove residual media components. The resulting fresh and clean biomass was taken into the Erlenmeyer flasks, containing 100ml of deionized Milli-Q water. The flask was incubated at 25°C in a shaker at 140 rpm for 72 hours. The biomass was filtered again with Whatman filter paper No.1 and the cell free extract was used in the following experiment. 1mM AgNO_3 was prepared and 50ml was added to the cell-free extract and kept in a shaking incubator at 25°C , 140rpm for 72hours in dark condition.

Characterization of silver Nanoparticles

The samples were observed for color change and maximum absorbance was analyzed using UV-spectrophotometer. 1ml of sample supernatant was taken after 24hours and absorbance was measured by using UV-visible spectrophotometer between 300-600nm. The sample was subjected to FTIR spectroscopy analysis. Three milligram of the sample was taken and pressed into the pellet. The sample was placed into the sample holder and FTIR spectra was recorded. After the synthesis, the silver Nanoparticles were further characterized by AFM which is 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. The sample used for the analysis was sonicated for 5 minutes, centrifuged and made into a thin film for AFM analysis. SEM is used to determine the surface morphology and size of the nanoparticles. For SEM sample has been prepared by centrifugation and then dried into powder form and subjected to SEM analysis.

Antibacterial Analysis

The silver nanoparticles were checked for its antibacterial activity by disc diffusion method¹⁷. The antimicrobial activity of the prepared silver nanoparticles from *Penicillium glabrum* (MTCC 1985) was tested against the pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Proteus vulgaris*. The combined formulation of silver nanoparticles with standard antibiotic discs such as Ofloxacin, Sparfloxacin, Carbicillin and Amoxicillin were used to find out the synergistic effect against the above pathogens. The zone of inhibition was measured after overnight incubation at 37°C .

Calculation for Increase in fold area

The mean of increase in fold area can be calculated by the mean surface area for the zone of inhibition of each antibiotics that has been used alone and antibiotic + AgNPs. The increase in fold area of different pathogens for antibiotics and antibiotics + AgNPs can be calculated by using this equation: $(B^2 - A^2)/A^2$, where A is the antibiotic alone and B is the antibiotic + AgNPs respectively ⁸.

Results and Discussion

The *Penicillium glabrum* (MTCC1985) was procured from the Institute of Microbial Technology (IMTECH) Chandigarh, India and employed in the present study for the biosynthesis of silver Nanoparticles. Ag-NPs were synthesized from Ag⁺ ions by treating the supernatant of *Penicillium glabrum* (MTCC1985). The appearance of a yellowish brown color in the conical flask suggested the formation of Ag-NPs^{1,10}. The supernatant of the *Penicillium glabrum* (MTCC1985) culture changed the solution to a brownish color upon the completion of the 5 minute reaction with Ag⁺ (Fig 1). The Ag-NPs were characterized by UV-Vis spectroscopy. The technique outlined above has proved to be very useful for the analysis of nanoparticles^{1,11,12,13}. As illustrated in Fig 2, Uv-Vis spectra, a strong surface plasmon resonance were centered at approximately 420nm indicated the presence of silver Nanoparticles.



Fig 1: Synthesis of silver nanoparticles from *Penicillium glabrum* (MTCC1985)
(A) Before treatment (B) After treatment of silver nitrate

The exact mechanism for the synthesis of silver Nanoparticles has not been clear yet but it has been suggested that the fungal biomass contain the NADH dependent nitrate reductase enzyme, when the silver ions comes in contact with the cell wall of the fungal biomass, the nitrate reductase secreted by the fungus causes the reduction of silver ions into silver nanoparticles^{18,19,20}.

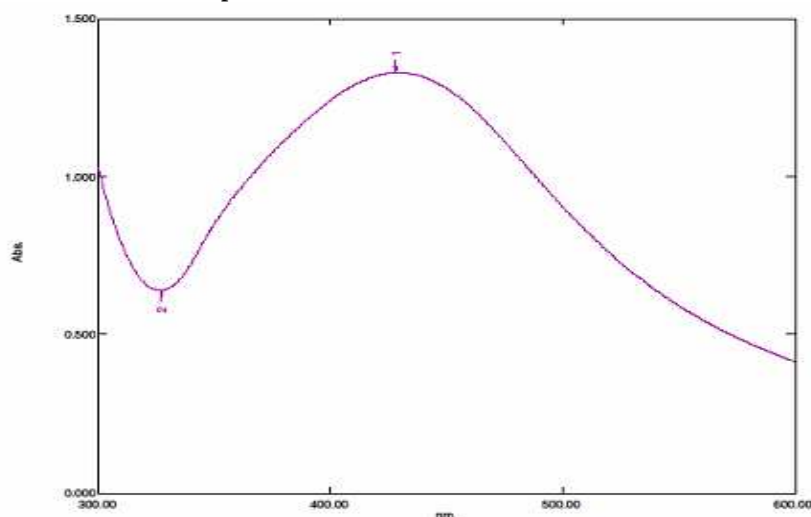


Fig. 2: Uv-Vis spectrum of silver nanoparticles synthesized from *Penicillium glabrum* (MTCC1985)

FTIR analysis was used to identify the molecules, proteins and functional groups involved in the reduction of silver ions into silver Nanoparticles (Fig 3). The FTIR analysis obtained for the Nanoparticles showed that the absorption peaks located at 3417.6 cm^{-1} (O-H stretch), 2923.8 cm^{-1} (C-H stretch), 1635.5 cm^{-1} (C=O stretch of amide), 1380.9 cm^{-1} (CH_3 bend of alkanes), 1080 cm^{-1} (C-N Stretch of aliphatic amines), 601.7 cm^{-1} (acetylenic C-H bend of alkynes).

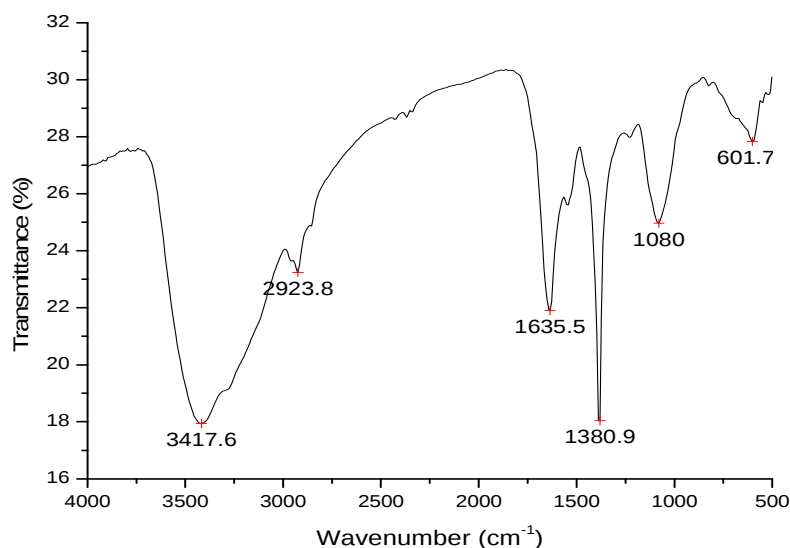


Fig. 3: FTIR analysis of Silver Nanoparticles synthesized from *Penicillium glabrum* (MTCC 1985).

Atomic Force Microscopy (AFM) analysis has been used for the determination of particle size and average roughness of the particles. Three dimensional structures of the AFM showed the particle height, average roughness and in homogeneity of the cluster formation of the surface (Fig 4). Two dimensional structure of the AFM (Fig 5) were used to determine the agglomeration. Nanoparticles size was spherical and poly dispersed whose range was in between 30 to 50nm.

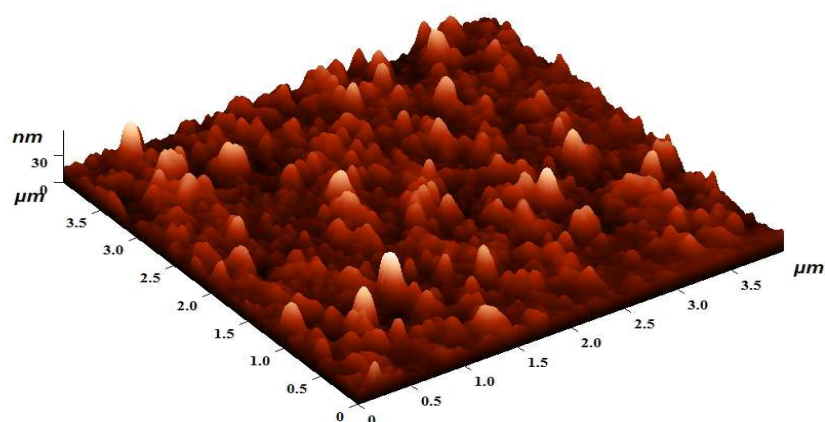


Fig 4: 3D picture of Atomic Force microscopy (AFM) shows the particle height, roughness and inhomogeneity of cluster formation of silver nanoparticles synthesized from *P. glabrum* (MTCC1985).

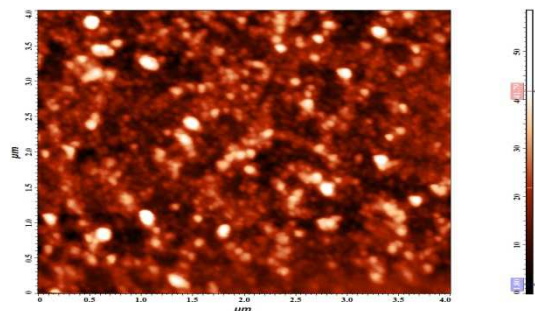


Fig 5: 2D picture of Atomic Force Microscopy (AFM) of silver Nanoparticles synthesized from *Penicillium glabrum* (MTTC1985).

Field emission Scanning electron microscopy (FESM) were used to understand the surface topology and the size of silver nanoparticles and it showed the silver Nanoparticles are spherical and well dispersed with the diameter ranges between 26 nm and 32.1nm from (Fig 6).

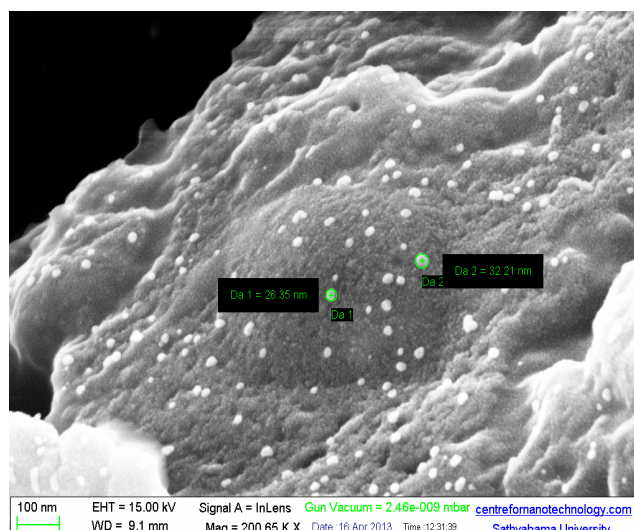


Fig. 6: Field emission Scanning electron microscope shows the well dispersed and particle size of silver nanoparticles (26.35 to 32.21 nm). Scale bar = 100nm

The antimicrobial activity of silver Nanoparticles by disc diffusion method against different clinically isolated pathogens viz., *Staphylococcus aureus*, *Bacillus cereus*, *E. coli*, and *Proteus vulgaris* were satisfactory in the present study (Fig 7). The combined formulation of silver nanoparticles with different antibiotics like carbicillin (100mcg disc) and amoxicillin (30mcg disc) showed remarkable results against all the pathogens studied (Fig 7 and Table 1) when subjected to antimicrobial analysis. The antibacterial activities of carbicillin and amoxicillin were amplified in the presence of AgNPs against the bacterial strains. The highest increase in fold area was observed for amoxicillin against *E. coli*, *Proteus vulgaris* and *Bacillus cereus* (Table 1), where as carbicillin had the highest fold area against *E. coli* (1.52%). Silver Nanoparticles showed good antimicrobial activity alone. It was found that the silver nanoparticles produced from *P. glabrum* enhanced the reaction rates of the antibiotics in a synergistic mode as well as in its own way on these clinically isolated pathogens (Fig 7). The present study carried out on enhanced antimicrobial activity of silver Nanoparticles synthesized from *P. glabrum* in combination with antibiotics was agreed with few workers but quite different from others since *Penicillium glabrum* is from the ancestral antibiotic family as a drug producer^{21, 22}.

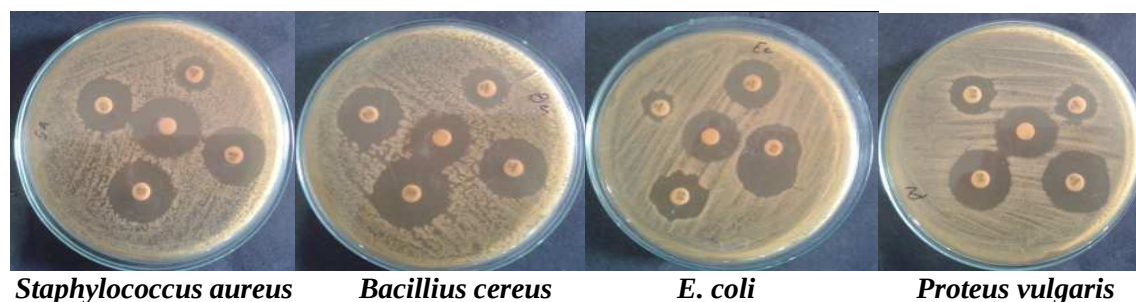


Fig 7: Synergistic antimicrobial activity of silver Nanoparticles and antibiotics on agar plates.

From the above investigation it has been found that the silver Nanoparticles synthesized from *Penicillium glabrum* (MTCC1985) along with antibiotics enhanced the antimicrobial activity against the clinically isolated pathogens. By using the biological method for the synthesis of Nanoparticles is safe, ecofriendly and can be readily used in the field of biomedicine^{1, 23}. Further investigation is required to check the exact mechanism of the biosynthesis of silver Nanoparticles from different microbes and to study its effect in-vivo including cytotoxicity studies are necessary in order to find out its biocompatibility with the animals and human beings before using it as a antimicrobial drug.

Table 1: Zone of inhibition (mm) of Carbicillin and Amoxicillin against test pathogens in presence and absence of silver nanoparticles.

Sl. No.	Pathogenic Bacteria	Carbicillin (A)	Ag NPs	Carbicillin + AgNPs (B)	Increase in fold area (%)	Amoxicillin (A)	Amoxicillin + AgNPs (B)	Increase in fold area (%)
1	<i>S. aureus</i>	21	24	27	0.65	21	28	0.77
2	<i>Bacillus cereus</i>	22	18	28	0.61	14	22	1.46
3	<i>E. coli</i>	17	20	27	1.52	12	20	1.77
4	<i>Proteus vulgaris</i>	15	20	21	0.96	14	23	1.69

Conclusion

During our study, silver Nanoparticles was bio-synthesized by extracellular method from *Penicillium glabrum* (MTCC1985) in-vitro. The appearance of yellowish brown color in the conical flask suggested the formation of AgNPs. The supernatant of the fungus culture changed the solution to a brownish color upon the completion of the 5 minute reaction. Size of the nanoparticles was measured in between 26 nm to 32 nm by FESEM. Silver nanoparticles showed good antimicrobial activity against the selected pathogens but combined formulation with antibiotics, the biosynthesized nanoparticles from *Penicillium glabrum* (MTCC 1985) amplified the antimicrobial potency of the antibiotics studied. Here it would be inferred from the current study that the synergistic use of antibiotics and silver nanoparticles together as a drug to minimize the potency and dosage against the multi drug resistant pathogens in order to cure dreaded diseases in future. But silver Nanoparticles needs animal trial to check its cytotoxicity before its availability in the market.

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