BIOSYNTHESIS AND EFFECT OF SILVER NANOPARTICLES ON THE EFFICACY OF ANTIBIOTICS AGAINST PATHOGENIC BACTERIA

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ABSTRACT

Silver is known for its antimicrobial effects from ancient times and silver nanoparticles are gaining importance due to increased structural integrity as well as unique chemical, optical, mechanical, electronic, magnetic and biomedical properties compared to the large particles of bulk materials. The aims of the present study were to exploit the moulds for the biosynthesis of silver nanoparticles and to evaluate its antibacterial activity singly and in combination with the antibiotics. The results indicate that silver nanoparticles can be synthesised through eco-friendly and low cost protocols from *Penicillium janthinellum.* The UV-visible and FTIR-spectroscopy confirmed the formation Silver nanoparticles. Field emission scanning electron microscopy (FESEM) and Atomic force microscopy (AFM) analysis were used to understand the surface topology and morphology of silver nanoparticles and it showed the silver nanoparticles are spherical and well dispersed. The bactericidal effect of AgNPs was carried singly and in combination with antibiotics (Amoxicillin) for comparative study. The results showed the enhanced efficacy in AgNPs in combination with Amoxicillin against selected bacterial pathogens. Thus we conclude that the combined mode of antibiosis in between synthesised nanoparticle and antibiotics are more effective against pathogenic bacteria as compared with the antibiotics alone, which could become an alternative remedies to cope up with antibiotics resistance.

KEYWORDS: *Penicillium janthinellum* AgNPs; UV-visible spectroscopy, AFM, FESEM; Amoxicillin

1. INTRODUCTION

The biosynthesis of nanoparticles, as a representative intersection of nanotechnology and biotechnology, has received increasing attention due to the growing need to develop environmentally benign technologies in material sciences. Although there are many synthetic technologies are well documented, the search for suitable biomaterials for the biosynthesis of (physical and chemical) nanoparticles continues among researchers worldwide. Early this decade, the potential of various microbes have been exploited for the synthesis of metal nanoparticles. The fabrication of reliable green chemistry processes for the synthesis of nanomaterials as an important aspect of nanotechnology have been suggested and examined by various researchers as nano-factories (Mukherjee, 2001; Ahmad, 2002; Sastry, 2003; Shankar, 2003; Shankar, et al, 2004, Rai, 2006). Metal nanoparticles such as Ag, Au, Pt and Pd have been synthesized by from various microorganisms such as bacteria (Husseiny, 2007), fungi (Ahmad, 2003) and plants (Sharma, 2007). Since then, various microorganisms and plants have been employed for the synthesis of nanoparticles. Among these, silver is playing a significant role in the field of biomedical science due to its attractive physiochemical properties. It has been demonstrated that the highly reactive metal oxide of silver nanoparticles exhibit excellent bactericidal activity (Stoimenov, 2002).

The strong toxicity of silver against wide range of microorganisms is well known and silver nanoparticles have been recently shown to be a promising antimicrobial material. Sondi and Salopek-Sondi, 2004; studied the antimicrobial activity of silver nanoparticles against *Escherichia coli* as a model of Gram-negative bacteria. Another aspect of the present investigation is antibacterial efficacy of biologically synthesised nanoparticles silver nanoparticles. Due to the prevalence and increase of microbial organisms resistant to multiple antibiotics and the continuing emphasis on health care costs, many researchers have tried to develop new, effective antimicrobial reagents, free of resistance and cost-effective. Such problems and needs have led to the resurgence in the use of silver-based antiseptics that may be linked to broad-spectrum activity and far lower propensity to induce microbial resistance than antibiotics (Jones, 2004). In this study, the AgNPs have been synthesised from *Penicillium janthinellum* and to investigate synergistic effect of AgNPs combined with Amoxicillin against four bacteria.

2. MATERIALS AND METHODS

2.1. Isolation: The *Penicillium* species was isolated from the vegetable market of Chennai. The mould is commonly associated with soil, decaying organic matter, and as storage rots or pathogens of fruits and vegetables. The fungi was isolated on Sabouraud's Dextrose agar media by following the microbiology isolation guidelines and incubated for 3-7 days in the Microbiology Laboratory, Department of Biomedical University, and Chennai-60011. After identification, the fungus was sub-cultured and stored in refrigerator at 4°C up to further studies.

2.2. Microscopic and colony characterization: The fungal isolate was observed by the author expertise using hand lens and the colony morphology was recorded with respect to color, shape, size and nature of colony and also by using laboratory manuals and was identified as *Penicillium janthinellum.*

2.3. Synthesis of silver nanoparticles: The fungal mould *Penicillium janthinellum* was used for the biosynthesis of silver nanoparticles as per our earlier methodology (Nayak, 20 14). Fungal biomass was grown aerobically in a specific 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 for 72 hours at 25^0C. After incubation, the biomass was filtered using Whatman filter paper No.1 and extensively washed with distilled water three times to remove any media components. The resulting fresh and clean biomass was taken into the Erlenmeyer flasks, containing 100ml of deionised Milli-Q water. The flask was incubated at 25^Oc in a shaker at 140 rpm for 72 hours. Then the

biomass was filtered again with Whatman filter paper No.1 and the cell free extract was used in the following experiment. 1mM of $AgNO_3$ was prepared and 50ml was added to the cell- free–extract and kept in a shaking incubator at 25^{o}c and 140 rpm for 72 hours in dark condition. The samples were observed for color change and maximum absorbance was analyzed using UV-Vis spectrophotometer. After the synthesis of silver Nanoparticles, it was characterized by AFM which is used to determine the particle size and agglomeration of the nanoparticles through 2D and 3D dimensional images of the agglomeration. The sample used for the analysis was sonicated for 5 minutes, centrifuged and made into a thin film for AFM analysis. FESEM is used to determine the surface morphology and size of the nanoparticles. For FESEM analysis the synthesised AgNPs were centrifuged at 15000 for 30 minutes and then dried into powder form which were subjected to FESEM analysis.

2.4. Bactericidal activity: The antibacterial activity of AgNPs and Amoxicillin was evaluated by using Kirby-Bauer method against gram positive and gram negative organisms viz *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Proteus vulgaris* following NCCLS guidelines (Bauer, 1996). The bacteria were grown on nutrient broth/agar medium (Qualigens, India). The nutrient agar plates were inoculated with the fresh cultures of test pathogens allowed for overnight growth at 37^{o}c.The standard antibiotic disks/ Strile disks were purchased from HiMedia (Mumbai, India). Sterile (6mm) disk were impregnated along with the antibiotics (Amoxicillin) were placed on the bacterial lawn in agar plates.To evaluate the efficacy of AgNPs singly and in combined form, 10µl of freshly prepared AgNPs (1mg/ml Stock solution) were added to sterile disk, while as standard disks (Amoxicillin 10mcg/disk) on agar plates inoculated with pathogens were impregnated with 10µg solution of AgNPs and incubated at 37^{o}C for 18-24hrs.The Standard disks Amoxicillin were used as positive control and Cell free filtrate was used as negative control. The zone of inhibition was measured after 18-24h of incubation and compared with the control. The experiments were repeated in tree times.

2.5. Analysis increase in fold area: The increased fold area was calculated from the mean surface area of inhibition zone of each antibiotic tested as Amoxicillin and Amoxicillin +AgNPs (Shahverdi, 2007). The increase in fold area was of Amoxicillin was calculated by equation $(B^2-A^2)/A^2$, Were A and B were Zone of inhibitions for Amoxicillin+ AgNPs, respectively.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Nanoparticles: The extracellular biosynthesis of Silver nanoparticles using *Penicillium janthinellum* was confirmed primarily by observed from the change of color from light brown into dark brown(Fig-1), indicating the formation of silver nanoparticles which were confirmed through UV-visible spectroscopy showing absorption peak at 430nm (Fig-2). The appearance of dark brown color in the reaction vessels suggested the formation of AgNPs, which is a specific wave length for the synthesis of AgNPs (Sastry, 2003).

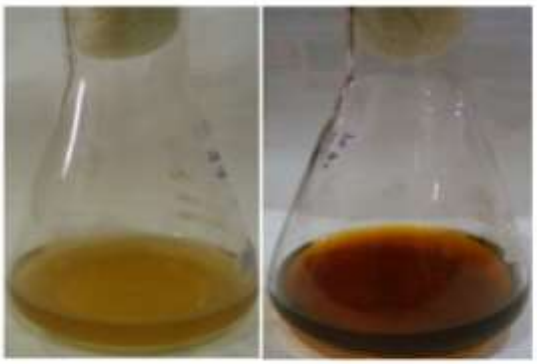

Fig 1: Synthesis of silver Nanoparticles from *Penicillium janthinellum*
(a)Before addition of silver nitrate (b) After addition of silver nitrate

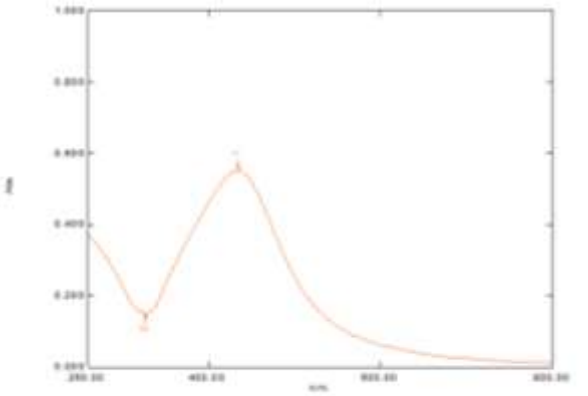

Fig 2: Confirmation of Silver Nanoparticles by UV-Vis Spectrophotometer

The size of nanoparticles was determined via different methods which showed variations due to various physical principles or methods. The average roughness and topography of synthesised silver nanoparticles were evaluated by analysing the AFM images which were showing Average Roughness, 26.58-40.75nm (Fig-3a and Fig-3b). The top view of synthesized nanoparticles in 3D structure indicates linear trends in roughness and particle in homogeneity of AgNPs, which indicates the formation of smother layers. Based on the FESEM analysis, it was found that the nanoparticles distributed uniformly and were dispersed densely and have smooth & rough surfaces. The nanoparticles appearance showed that they were morphologically spherical to ovate in structure with having average dimensional size in the range of 25-40nm (Fig-4), (Nayak, 2014; Nanda and Shahnaz, 2014).

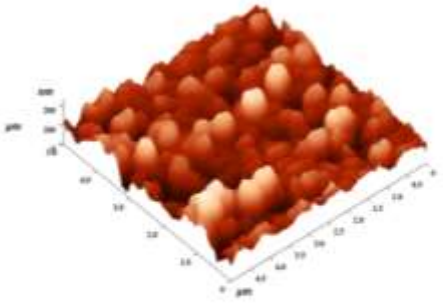

Fig3a: 3D micrograph of Atomic Force microscopy (AFM) shows particle height and roughness of silver nanoparticles synthesized from *Penicillium janthinellum*

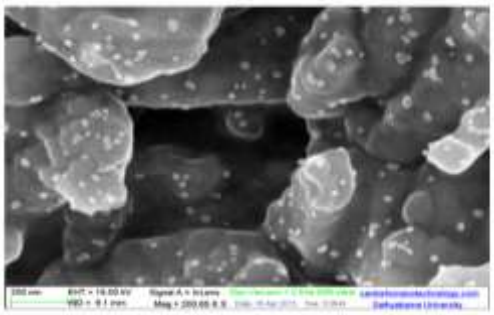

Fig 4: FESEM micrographs of silver nanoparticles synthesized from *Penicillium janthinellum*. (Scale bar 200nm).

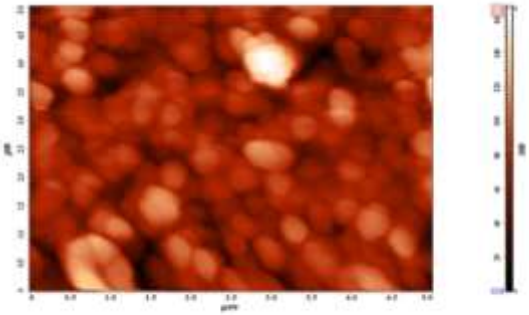

Fig 3b: 2D micrograph of Atomic Force Microscopy (AFM) of silver nanoparticles synthesized from *Penicillium janthinellum*

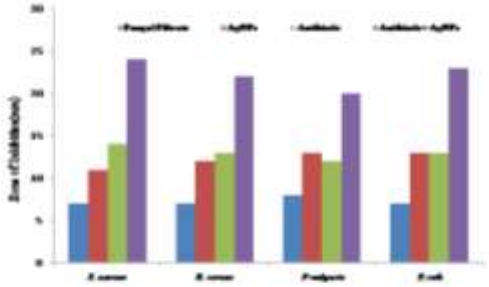

Fig.5: Graphical representation of synergistic effect of Amoxicillin (10mcg/Disk and AgNPs (10µg/Disk) against test pathogens

3.2. Bactericidal activity.

To confirm the potential combined effect AgNPs with Amoxicillin against test pathogens viz. while *Bacillus cereus* , *Staphylococcus aureus, Proteus vulgaris and Escherichia coli* were treated with biologically synthesised AgNPs at a concentration of 10µg/ml. The colloidal solution containing silver nanoparticles were centrifuged at 12,000 rpm for 15 min. The precipitate was then heat dried at 37ºC in a hot air oven to get into the powder form of the silver nanoparticles and used further at different concentrations (w/v).The comparative analysis AgNPs along with Amoxicillin was found more significant with regards to the zone of inhibitions (Fig-5). The diameters of zone of inhibitions (in mm) around the Amoxicillin with and without AgNPs are shown in the (Table- 1). The combined effect of AgNPs with Amoxicillin showed good activity against test clinical pathogens. The increase in fold area was calculated as per the earlier reports (Shahverdi 2007), which showed highest in *Escherichia coli* (2.13), followed by Staphylococcus *aureus* (1.89), *Bacillus cereus* (1.89) and *Proteus vulgaris* (1.77). Thus Amoxicillin (10mcg/Disk) along with AgNPs (10µl/Disk) showed enhanced effect on test pathogens, while as antibacterial results had shown that AgNPs synthesised to possess discrete antibacterial activity against clinically isolated pathogens.

Table-1: Comparison of antibacterial Activity of AgNPs (10µg/Disk) alone and in combination with antibiotics (Amoxicillin10mcg) and the filtrate against pathogenic bacteria.

Pathogens	Fungal Filtrate	AgNPs	Antibiotic (A)	Antibiotic + AgNPs (B)	#Increase in fold area
	Zone of inhibition (mm)				
Staphylococcus aureus	07±0.56	11±0.10	14±0.67	24±0.41	1.94
Bacillus cereus	07±0.43	12±0.81	13±0.72	22±0.59	1.89
Proteus vulgaris	08±0.33	13±0.43	12±0.40	20±0.73	1.77
Escherichia coli	07±0.45	13±0.37	13±0.49	23±0.64	2.13

#Increase in fold area was calculated as $(B^2-A^2)/A^2$, where A and B are the inhibition Zones for Amoxicillin and Amoxicillin+ AgNPs.

4. CONCLUSION:

To our knowledge the present study is the first report on the synthesis of AgNPs using mould *Penicillium janthinellum isolated* from vegetable market**.** The preset study we concludes the development of reliable, economical, simple and eco-friendly processes for the synthesis of AgNPs. The AgNPs synthesis was confirmed through UV-visible spectroscopy, AFM, and FESEM which confirms the nanoparticle synthesis. The biosynthesised AgNPs could effectively inhibit the growth of various clinical pathogens. However in addition to antibacterial effect, the synthesised AgNPs tested in combined with antibiotics has showed enhanced bactericidal activity, which could be alternative remedies to control the Multi-drug resistance human pathogens which has become the major threat to the global health care. Thus we conclude that the nanomaterials are the leading requirements in the field biomedical research in the direction of finding new drugs with potentiality, but further studies are also required to understand the cellular and mechanism behind the biosynthesis of nanoparticles and their mode of action on pathogens

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