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Biosynthesis of Silver Nanoparticles from *Penicillium spinulosum* (MTCC-4952) and Evaluation of their Antibacterial Activity in Combination with Amoxicillin and Ofloxacin

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In the recent years Nanotechnology has attracted the great attention to the researchers and pharmaceutical companies due to its immense application in the field of science and technology for the purpose of developing new nanomaterial's . In our present study, nanosilver was bio-synthesized by extracellular method from *Penicillium spinulosum* (MTCC-4952). The development of dark brown color in the l flask by addition of AgNO3 indicates the formation of silver nanoparticles (AgNPs). These nanoparticles were confirmed by UV-Vis spectrophotometer which showed absorption peak at 416nm, Fourier transform infrared (FTIR), Field emission scanning electron microscopy (FESEM) and Atomic force microscopy (AFM). Size of the Nanoparticles was around 18nm by FESEM analysis. Silver nanoparticles showed good antibacterial activity against the selected bacterial pathogens but in combined activity with Amoxicillin and Ofloxacin enhanced the antibacterial property of the antibiotics studied.

Key words: Silver nanoparticles, *Penicillium spinulosum* (MTCC4952), FESEM, FTIR, AFM,UV-VIS Spectrophotometer.

Modern age is the age of science and technology because of advancement in the living standard and health awareness but people used lot of antibacterial agents due to which those microbes are getting resistant to various antibiotics, forcing to find new antibacterial materials. Metallic nanoparticles especially nanosilver have attracted great attention of the researchers because of their remarkable antibacterial property due its small size and surface area effect[1,2,3].Silver has long been recognized as having inhibitory effect towards many bacterial strains which are commonly present in medical and industrial processes[4]. Silver and nanosilver have wide application in medical industry which includes topical ointment and creams containing silver to control the infection and healing of burns and wounds[5].

Biological method is the method of choice among physical and chemical method for the synthesis of silver nanoparticles due to cost effective, less toxic, controlled reaction rate,easily amiable and environmental friendly[6]. Different living organisms were used for the synthesis of nanoparticles like bacteria,algae, fungi and plants[7,8]. Use of fungi for the synthesis of Nanoparticles is quite amazing because of their ability to secrete large amount of enzymes which leads to the formation of metal Nanoparticles[9].

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The aim and objectives of the present study is to biosynthesize silver nanoparticles *Penicillium spinulosum* MTCC(4952) got from IMTECH, Chandigarh, India to confirmation of silver nanoparticles by UV-Vis spectroscopy followed by various microscopic examinations and to check its (silver nanoparticles) efficacy as a bactericide as well as its combined effect with Amoxicillin and Ofloxacin in order to control the growth of selected bacterial pathogens viz., *Staphylococcus aureus, Bacillus cereus, E. coli, Vibrio cholerae* and *Proteus vulgaris* was studied.

MATERIALS AND METHODS

The culture of *Penicillium spinulosum* (MTCC 4952) strain was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained in Sabouraud Dextrose Agar (SDA) medium and sub cultured from time to time to optimize its viability and virulence in the laboratory during the present study.

Synthesis of silver Nanoparticles

Penicillium spinolusum (MTCC4952) was utilized for the extracellular synthesis 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 $MgSO_4$. $7H_2O$ 0.1; $(NH_4)_2SO_4$ 1.0; yeast extract 0.6; glucose 10.0 at 25±3°C. After incubation for 3 days, 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°C 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°C, 140rpm for 72hours in dark condition.

Characterization of silver nanoparticles

The samples were observed for change of solution color and maximum absorbance was analyzed using UV- spectrophotometer. 2ml of sample supernatant was taken after 72hours and absorbance was measured by using UV-visible spectrophotometer between 300-600nm. The sample was subjected to FTIR spectroscopy analysis. Two milligram of the dried sample was taken along with potassium bromide (KBr) and pressed to form pellet. The sample was taken and put in to sample holder and FTIR graph were taken. These silver nanoparticles were further characterized by AFM which was used to determine the particle size and agglomeration of the nanoparticles.The sample used for the analysis was sonicated for 5 minutes, centrifuged at 15000 rpm and made into a thin film for AFM analysis. The two dimensionaly image of AgNPs were taken by AFM which showed the particle height and average roughness of silver nanoparticles. FESEM was used to determine the surface morphology , size and shape of the nanoparticles, for which sample was prepared by centrifugation, dried into powder form and subjected to FESEM analysis.

Antibacterial analysis

Theses biologically synthesized silver nanoparticles were checked for its antibacterial activity by using disc diffusion method[10]. The antibacterial activity of silver nanoparticles from *Penicillium spinulosum* (MTCC 4952) was used against the selected bacterial pathogenic bacteria such as *Staphylococcus aureus, Escherichia coli, Bacillus cereus, vibrio cholerae* and *Proteus vulgaris*. The combined effect of silver nanoparticles with standard antibiotic discs of Amoxicillin and Ofloxacin were used to find out the synergistic effect against the above pathogens. The zone of inhibition was measured after overnight incubation at 37^0c.

Calculation for Increase in fold area

The mean of increase in fold area were calculated by the mean surface area for the zone of inhibition of each antibiotics that were used alone and antibiotic + AgNPs separately. The increase in fold area of different pathogens for antibiotics and antibiotics + AgNPs was calculated by using this equation: $(B^2 – A^2)/A^2$, where A was the antibiotic alone and B was the antibiotic + AgNPs respectively[11].

RESULTS AND DISCUSSION

Penicillium spinulosum (MTCC 4952) biomass was used for the synthesis of silver nanoparticles. Upon addition of the 1mM $AgNO_3$ resulted in the change of colour of solution in to

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

S. No.	Pathogens	AgNPs 25µl/disc	Amoxicillin 10mcg disc (A)	Amoxicillin+ AgNPs (B)	Increase in fold area (%)
1	*Staphylococcus aureus*	14	7	19	6.36
2	*Escherichia coli*	11	10	18	2.24
3	*Bacillus cereus*	13	7	18	5.61
4	*Vibrio cholerae*	12	6	13	3.69
5	*Proteus vulgaris*	12	9	14	1.41

Increase in fold area was calculated by using the equation $(B^2-A^2)/A^2$, where A is the zone of inhibition of antibiotic and B is the zone of inhibition of antibiotic +AgNPs respectively

Table 2. Zone of inhibition (mm) of AgNPs and Ofloxacin against test pathogens in the presence and absence of silver nanoparticles

S. No.	Pathogens	AgNPs 25µl/disc	Amoxicillin 10mcg disc (A)	Amoxicillin+ AgNPs (B)	Increase in fold area (%)
1	*Staphylococcus aureus*	14	26	32	0.51
2	*Escherichia coli*	11	15	22	1.15
3	*Bacillus cereus*	13	30	38	0.60
4	*Vibrio cholerae*	12	24	32	0.77
5	*Proteus vulgaris*	12	17	30	2.11

Increase in fold area was calculated by using the equation $(B^2-A^2)/A^2$, where A is the zone of inhibition of Antibiotic and B is the zone of inhibition of antibiotic +AgNPs respectively.

brown colour indicating the formation of silver nanoparticles12 (Fig 1) to the biomass the dark brown indicates the formation of silver nanoparticles[12] (Fig 1).

Later the, Nanoparticles have been characterized by UV-Vis absorption spectroscopy done by using UV-Vis spectrophotometer. The Nanoparticles formed in the solution shows the absorption peak at 416nm [13] [Fig2].The exact mechanism for the synthesis of Nanoparticles is not yet known but some reports suggest that it is due to the electron transfer from the NADH by NADH-dependent reductase as electron carrier[14] while as some reports suggest that it is due the shuttle quinine extracellular process[15].

FTIR analysis were used for identification of the molecules, proteins and functional groups involved in the reduction of silver ions into silver

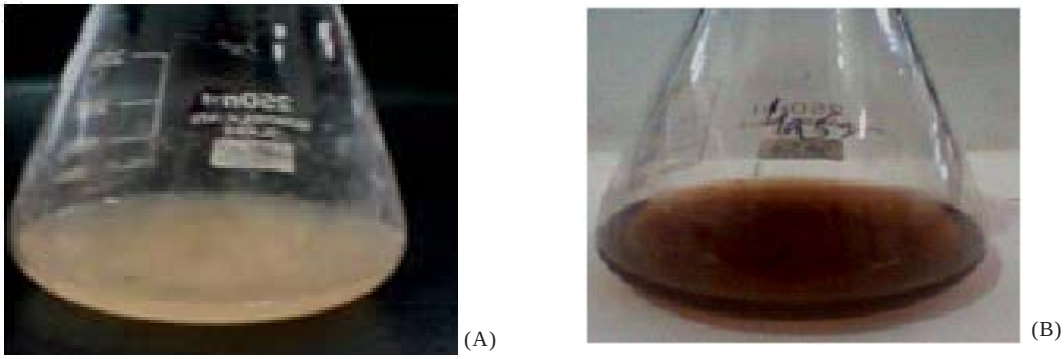

Fig. 1. Biosynthesis of Silver Nanoparticles – change of colour in the reaction (A) Before addition of $AgNO_3$, (B) After addition of $AgNO_3$

nanoparticles as capping agent is given in (Fig 3). The FTIR analysis obtained for the nanoparticles showed that the absorption peaks located at 3426 cm^{-1}(N-H stretch amide), 2926 cm^{-1}(C-H alkanes),2365 cm^{-1}(O-H stretch),1648 cm^{-1}(C-O amides) 1385cm^{-1} (CH bend of alkenes),1075cm^{-1}(C-N Stretch of amines),876 cm^{-1} (S-O stretch) and also some small peaks are there .

The particles were further characterized by AFM to determine the size, agglomeration, inhomogenity, surface roughness together. Two dimensional image of AFM showed the agglomeration and particle size as 25 nm (Fig 4)

Field emission scanning electron microscope were used to understand the size,shape and morphology of the silver nanoparticles which showed that the silver nanoparticles were well dispersed and spherical in their shapes in the range of 18.90nm (Fig 5).

The antibacterial activity of silver nanoparticles were analyzed through disc diffusion method against different pathogens viz., *Bacillus cereus Staphylococcus aureus, Proteus vulgaris, E coli,* and *Vibrio cholerae* and found satisfactory in the present study. The combined effect of silver nanoparticles with Amoxicillin(10mcg disc) and Ofloxacin (5mcg disc) were also analyzed. Each disc is impregnated with 25µl nanoparticle solution. The highest increase in fold area was found for Amoxicillin in presence of AgNPs against *Staphylococcus aureus*(6.36), followed by *Bacillus cereus* (5.61), *Vibrio cholerae* (3.69), *Escherichia coli* (2.24) and *Proteus vulgaris* (1.14). Likewise for Ofloxacin the increase fold area was observed

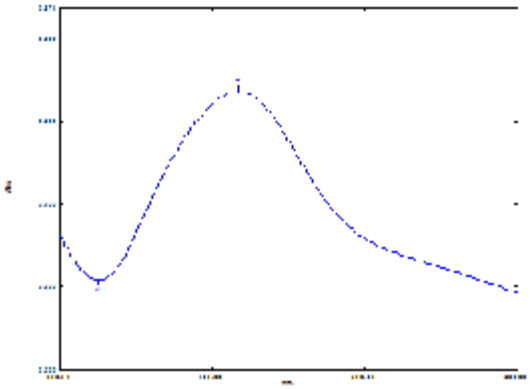

Fig. 2. UV–Vis spectrum of fungal filtrate containing silver nanoparticles synthesized from *Penicillium spinulosum* (MTCC 4952)

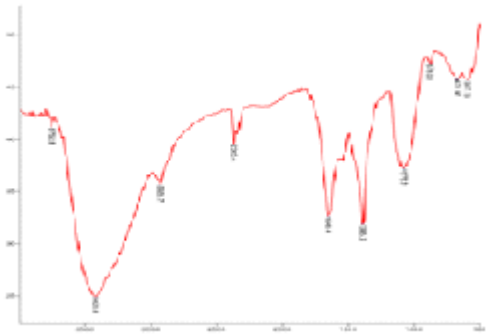

Fig. 3. FTIR spectrum of Silver Nanoparticles synthesized from *Penicillium spinulosun* (MTCC4952)

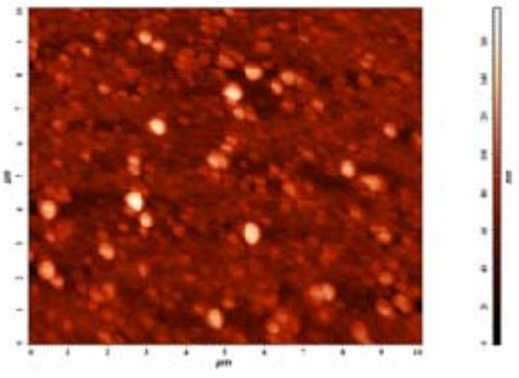

Fig. 4. 2D picture of Atomic Force Microscopy (AFM) of silver Nanoparticles synthesized from *Penicillium spinulosum* (MTTC 4952)

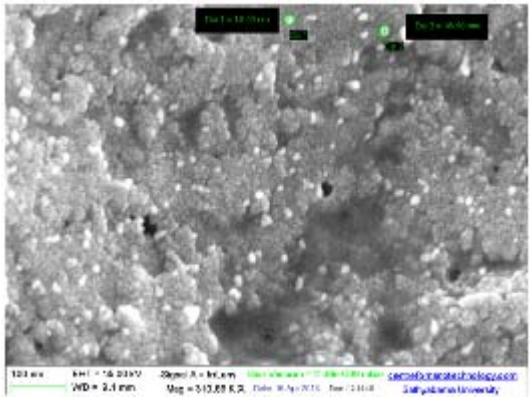

Fig. 5. Field emission Scanning electron microscope shows the particles are spherical and Size of silver nanoparticles 18.90nm. Scale bar = 100nm

highest against *Proteus vulgaris* (2.11) followed by *Escherichia coli* (1.15) *Vibrio cholerae* (0.77), *Bacillus cereus* (0.60) and *Staphylococcus aureus* (0.51) (Table 1 and Table 2). The results showed that the antibacterial activity of Amoxicillin and Ofloxacin in presence of Silver nanoparticles increases the antibacterial effect of the antibiotics which were used in the present study. Fig 6 and 7 represents the graphical representation of the combined effect of nanoparticles with the antibiotics.

From the above study it states that silver nanoparticles synthesized from the *Penicillium spinulosum* (MTCC4952) showed good antibacterial activity and its synergistic (combined) effect with Amoxicillin and Ofloxacin increases the

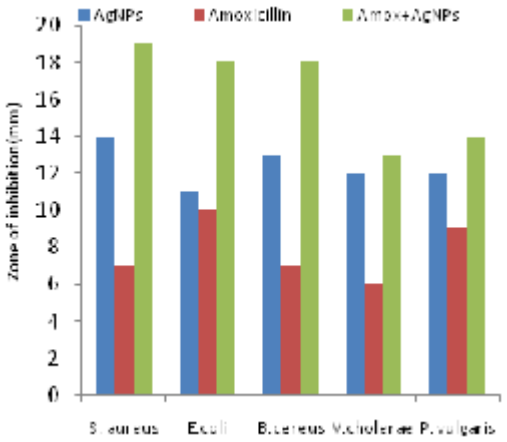

Fig. 6. Graphical representation of the combination effect of Amoxicillin with AgNPs against Selected pathogens

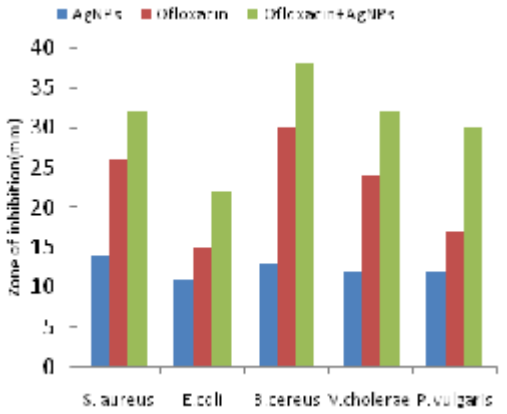

Fig. 7. Graphical representation of the combination effect of Ofloxacin with AgNPs against selected pathogens

bactericidal effect of the antibiotics which were used during the present study.

Silver nanoparticles may cause the formation of irregular pits,holes or channels in the cell membrane of the bacteria and causes release of lipopolysaccrides and proteins and also Ag produces the free radicals generated from AgNPs which are responsible for the antibacterial activity by blocking the protein synthesis by binding with the 30s ribosomal units and also blocks the DNA replication targets the phosphate back bone of the DNA strand[16,17].

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