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Antibacterial Activity of Silver Nanoparticles from *Rhizopus spp* Against Gram Negative *E.coli*-MDR Strains

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Development of reliable and eco-friendly process for synthesis of metallic nanoparticles is an important step in the field of nanotechnology. One of the options to achieve this objective is to use natural biological systems. In this work we have investigated extracellular biosynthesis of silver nanoparticles (AgNPs) using cell-free extract of *Rhizopus spp*.. Formation of AgNPs was indicated by the change in the colour of the cell-free extract from yellow to dark brown under static condition after 48 hrs of incubation. Characterization of AgNPs was carried out by UV-vis Spectroscopy which gave sharp plasmon resonance peak at 414 nm corresponding to spherical shaped nanoparticles. Transmission electron microscopy (TEM) micrograph showed formation of well-dispersed AgNPs in the range of 27 nm. X-ray diffraction (XRD)-spectrum of the AgNPs exhibited 2¸ values corresponding to nanocrystal. These biosynthesized AgNPs were used to study their antimicrobial activity against Multi-drug resistant (MDR) *E.coli* strains, by Agar diffusion method. Zone of inhibition was measured. Our results suggest that, silver nanoparticles are effective against MDR *E.coli*, which makes them a potential candidate for use in pharmaceutical products and medical devices that may help to prevent the transmission of drug-resistant pathogens in different clinical environments.

Key words: *Rhizopus* spp, Silver naoparticles, TEM, XRD, MDR strains.

Nanoscale silver plays an important role in understanding and manipulating biological processes which will be the central theme to present biomedical and biological issues. The extremely small size of nano particles having a large surface area relative to their volume allows them to easily interact with other particles and increases their antibacterial efficiency. 'Green synthesis' is a process of synthesis and assembly of nanoparticles and has been used for a series of special production processes. This process, benefits from the development of clean, non-toxic and environmentally acceptable procedures which involve organisms ranging from bacteria to fungi and even plants. Fungi have significantly higher productivity when used for nanoparticles biosynthesis owing to their much higher protein secretion[1]. Nanoparticles produced by a biogenic enzymatic process are far superior in several ways, to those particles produced by chemical methods[2]. Synthesis of AgNPs has been investigated utilizing many ubiquitous fungal species including *Trichoderma*, *Fusarium* and *Penicillium*[3-4].

The synthesis of AgNPs were extensively studied by using chemical and physical methods, but the development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. Biological synthesis process provides a wide range of environmentally acceptable methodology, low cost production and

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minimum time required with wide range of applications. Microbial source to produce AgNPs is of great interest in recent days. However, use of Eukaryotes, especially fungi, is potentially exciting since they secrete large amounts of proteins, thus increasing productivity [7], and are simple to deal within the laboratory. Moreover, the process can be easily scaled up, economically viable with the possibility of easily covering large surface areas by suitable growth of mycelia. Extra cellular synthesis of nanoparticles[5] could be highly advantageous from the point of view of synthesis in large quantities and easy downstream processing[6].

Silver nanoparticles were synthesized using *Rhizopus spp*, in the present work. The nano silver was formed within 24 hour of silver ions coming in contact with the cell-free filtrate. Further, these AgNPs were evaluated for their antimicrobial activity against Gram negative *E.coli* MDR strains.

MATERIALS AND METHODS

Isolation and Identification of *Rhizopus spp*

The fungi isolated from different soil sample collected from various agricultural fields of Gulbarga region, Karnataka, India. Soil samples were used as inoculum for serial dilution technique and plated on PDA. The plates were incubated at 28^0 C for 4 days. Individual fungal colonies were picked and further purified by sub-culturing on Potato Dextrose Agar (PDA) media. They were identified based on lacto phenol cotton blue staining and identification of the fungal isolate was carried out by morphological and microscopic observations.

Biosynthesis of silver nanoparticles

The fungi *Rhizopus spp.* was grown in 250 ml Erlenmeyer flasks containing 100 ml MGYP broth (Malt extract 0.3%, yeast extract 0.3%, Glucose 1.0%, Peptone 0.5% and pH 6.0)[8] at 30^0 C at static condition for 72 hrs[9]. After incubation, mycelia was separated by filtration, washed with sterile distilled water to remove traces of media components, resuspended in 100 ml distilled water, and incubated at same position for 48 hrs. The suspension was filtered through what man filter paper no.1. The cell filtrate was challenged with $AgNO_3$ solution (1 mM) and incubated at 30^0 C for reduction.

Characterization of Silver Nanoparticles

UV–visible spectroscopic analysis

The AgNP's nanoparticles were characterized by UV–visible spectroscopy. After addition of $AgNO_3$ to the filtrate, conical flasks were kept for visual observation of color change from yellow to brown. The AgNPs were subjected to UV-Vis spectrophotometer (T 90+ UV-Vis spectrophotometer) to detect nanoparticles. The absorption range was 400–450 nm[10] After UV-Vis analysis the nanoparticles were purified by centrifugation at 10,000 rpm/15min, then the pellets were resuspended in sterile distilled water and again centrifuged at 10,000 rpm / 10 min. In metal nano particles such as in silver, the conduction band and valence band lie very close to each other in which electrons move freely. These free electrons give rise to a surface plasmon resonance (SPR) absorption band[11-14], occurring due to the collective oscillation of electrons of silver nano particles in resonance with the light wave[15].

Transmission Electron Microscopy (TEM)

Samples for transmission electron microscopy (TEM) (Hitachi-H-7500) were prepared by drop-coating the AgNPs solution into the carbon-coated copper grid, which shows the size, shape and morphology of the particles.

X-Ray Diffraction

Silver nanoparticle solution, thus obtained was purified by repeated cetrifugation at 10,000 rpm/15min, then resuspended the pellets in sterile distilled water and again centrifuged at 10,000 rpm / 10 min. The dried mixture of AgNP's was collected to test the formation of AgNP's by an X'Pert Pro X-ray diffractometer opterated at the voltage of 40 Kv and a current of 30 Ma with Cu K α Radiation in a θ-2 configuration.

Analysis of the antibacterial activity of silver nanoparticles

The silver nanoparticles synthesized was tested for antimicrobial activity by agar well-diffusion method against six isolates of MDR-*E.coli* collected from Khwaja Bande Nawaz Hospital, Gulbarga. The pure bacterial cultures were subcultured on nutrient agar. Three to four similar colonies were selected and transferred to nutrient broth, incubated at 37°C for 6-8 hrs till light to moderate turbidity develops. A sterile cotton swab is dipped into the turbid bacterial culture and the soaked swab is rotated firmly against the

upper inside wall of the tube to express excess fluid. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60° angle between each streaking. The inoculum was allowed to dry for 30 minutes. Wells of 6mm diameter were made on agar plates with gel-puncture[16]. Using micropipette, 20 µl and 40 µl of AgNP's solution were poured onto each well in the plates. After incubation at 37° C for 24 hrs, the different levels of zone of inhibition of bacteria were measured and recorded.

RESULTS AND DISCUSSIONS

Isolation and Identification of *Rhizopus spp*

A variety of fungal cultures were isolated from soil, have been screened for their ability to extracellular reduction of silver ions to form nanoparticles[17]. The *Rhizopus spp*. was isolated from soil samples collected from different regions of Gulbarga**.** The *Rhizopus spp.* was identified based on morphology, spore size, shape & structure (Fig.1). Promising results were obtained using the fungal isolate, *Rhizopus* VJ-6.

Biosynthesis and characterization of Silver nanoparticles

Synthesis and application of nanoparticles is in the limelight in modern nanotechnology. In the present study, among three isolates of *Rhizopus spp.* One potential isolate VJ-6 was further employed for the biosynthesis of silver nanoparticles. The present investigation demonstrates the formation of the silver nanoparticles by the reduction of the aqueous Ag-metal ions during exposure to the cell-free extract of *Rhizopus* VJ-6. The biosynthesis of AgNPs by *Rhizopus* VJ-6 was primarily confirmed by the change of the reaction mixture from yellow to brown colour (Fig. 2) indicating the production of silver nanoparticles ($Ag+ \rightarrow Ag0$). The characteristic brown colour due to the excitation of plasmon vibrations in the nanoparticles provides a convenient signature of their formation[18]. It is reported that reduction of Ag+ to Ag0 occurs through nitrate dependent reductase quinone process, leading to the formation of 10-30 nm nanoparticles stabilized by proteins secreted by the fungus[19,4].

Table 1. Antibiotic sensitivity tests of *E.coli* isolates

S.No	Antibiotics	Test of pathogenic bacterial strains					
		E.coli 1	*E.coli* 2	*E.coli* 3	*E.coli* 4	*E.coli* 5	*E.coli* 6
1	Amikacin	S	S	S	S	S	S
2	Ampicillin	S	S	R	R	S	R
3	Cefepime	R	R	S	R	R	R
4	Ceftazidime	R	R	S	R	R	S
5	Cefuroxime	S	R	R	S	R	S
6	Ciprofloxacin	R	R	R	R	S	S
7	Gentamycin	R	S	R	S	R	R
8	Imipenem	S	S	S	R	S	R
9	Meropenem	S	S	S	R	S	R
10	Norfloxacin	R	S	R	S	R	R
11	Phosphinothricin tripeptide	R	R	S	R	S	S
12	Ticarcillin/Clavulanic acid	S	R	R	R	S	R
13	Trimephoprim Sulfamethoxaxole	S	R	S	S	R	R
14	Vancomycin	S	S	R	S	R	S

Table 2. Zones of inhibition of MDR strains

S. No	MDR Strains	Zone of inhibition in mm	
		(20¼l)	(40¼l)
1.	E.coli-1	19	20
2.	E.coli-2	18	19
3.	E.coli-3	21	22
4.	E.coli-4	18	19
5.	E.coli-5	14	15
6.	E.coli-6	17	18

UV-Vis Spectroscopy

The bioreduction of $AgNO_3$ ions in solution was monitored by periodic sampling of aliquots of aqueous component & measuring UV-

Vis spectra. The cell-free filtrate solution exposed to $AgNO_3$ ions shows a distinct absorption at around 414 nm which corresponds to SPR of silver nanoparticles (Fig. 3). The SPR band in AgNPs remains close to 414 nm suggesting nanoparticles were dispersed in aqueous solution with no evidence of aggregation. The AgNPs formed were highly stable even few weeks after the reaction. The AgNPs were characterized and confirmed by TEM analysis. Reports of Shivaraj *et al* (2013)[20] revealed surface plasmon resonance of AgNPs between 380 to 450. While reports of Afreen Banu *et al*(2011)[21] reported a peak of 422 nm. Mukherjee *et al*(2007)[22] reported an intense peak at 410 nm. It is reported that the absorption spectrum of spherical silver nanoparticles present a maximum between 420 nm and 450 nm[17].

TEM (Transmission Electron Microscopy)

TEM has provided insight in the morphology and size details of the AgNPs. TEM picture of the silver nanoparticles produced by *Rhizopus spp.*(VJ-6) after 48 hr is depicted in (Fig. 4). The AgNPs formed were predominately spherical and polydispersed with diameter in the range of 10 nm-100 nm shape and well dispersed, The size ranged between 27 to 50 nm. Nanoparticles formed were highly stable due to capping agents such as proteins, and were not in direct contact even within the aggregates indicating stabilization of the nanoparticles by a capping agent[19]. It is suggested that the biological molecules such as proteins could possible perform the function of stabilizing the AgNPs[21]. Our results correlated with the TEM results of Sunkar *et al* (2013)[23]. wherein he reported the size of the nanoparticles between 25-50nm. Similar results were obtained by Kuber C Bhainssa[24] and Verma[25].

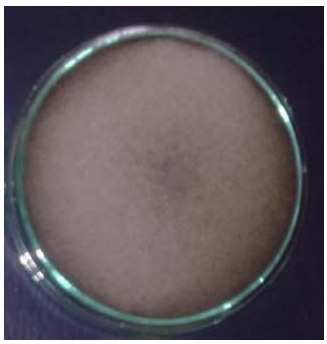

Fig. 1. *Rhizopus spp.* On PDA plate

X-Ray Diffraction

The x-ray diffraction patterns obtained for the silver nanoparticles synthesized using *Rhizopus* VJ-6 is shown in Fig. 6. For the crystalline nature of the AgNPs, intense XRD peaks were observed corresponding to the (101), (111), (200) planes at 2 θ angles of 32^0, 38^0, and 43^0 which are indexed as crystalline silver face-centered cubic (fcc) phase[30]. The peaks were compared with the X-ray diffraction database. The XRD database results of our sample from Rhizopus VJ-6 supported the presence of silver nanoparticles which correlates with the result of Guangquan Li *et al* , Manjunath sangappan *et al*[31,32].

Antimicrobial assay

The resistance of microorganisms to various existing antibiotics has risen in the recent past[29-31]. This change led to the quest of novel

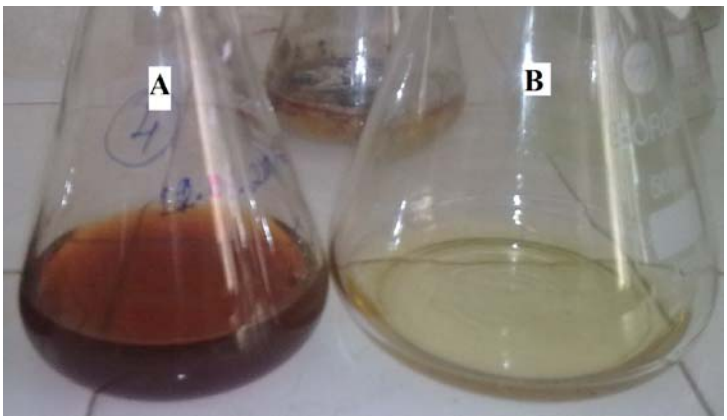

Fig 2. (a) Cell- free extract of *Rhizopus spp(Vj-6*) without, AgNO3 as control, (b)After reaction with 1mM AgNO3 solution (Aqueous)

antimicrobials from various sources. Silver and its derivatives are widely used in medicine for a long time in the treatment of infections. Thus it is fitting to investigate the antibacterial activity of the synthesized nanoparticles[33].

MDR *E.coli* isolates selected for the study

MDR –*E.coli* isolates were selected based on the resistance showed by them against more than three antibiotics (Table 1, Fig. 7.). Antimicrobial activity of biosynthesized silver nanoparticles was studied against the multidrug resistant bacteria using standard zone of inhibition and are depicted in Table-2 , Fig-8. Wells were loaded with 20 µl and 40 µl of AgNps. Of the six MDR isolates *E.coli-3* showed maximum zone of inhibition of 22 mm with 40 µl of AgNP's, while the same was 21 mm with 20 µl of AgNPs. *E.coli-1* showed maximum inhibition zone of 20 mm with 40 µl of AgNPs, while *E.coli-2* and *E.coli-4* showed 19 mm zone of inhibition each with 40 µl concentration of AgNPs. The least zone of inhibition of 15 mm was observed with *E.coli-5* at the same concentration *E.coli-6* showed 18mm zone of inhibition with 40 µl of AgNPs.

Antibacterial activity of silver ions is well known. Ionic silver strongly interacts with thiol group of vital enzymes and inactivate them[34]. Experimental evidence shows that DNA loses its replication ability once the bacteria have been treated with silver ions[34]. Most importantly silver attacks a broad range of targets in the microbes, so it is difficult for them to develop resistance against silver, this would require developing a host of mutations to protect themselves[35]. Nanosilver is known to be nontoxic to human cells to the tune of 350 mg/day[35]. Due to its strong toxicity to a wide range of microorganisms, silver based compounds have been used extensively in many bactericidal applications[36-39]. AgNPs also exhibit antiviral activity towards HIV infected cells[40], because of such wide range of applications, a simple eco-friendly, cost effective with a narrow particle size are more effective antibacterial agents with high surface volume so that a large proportion

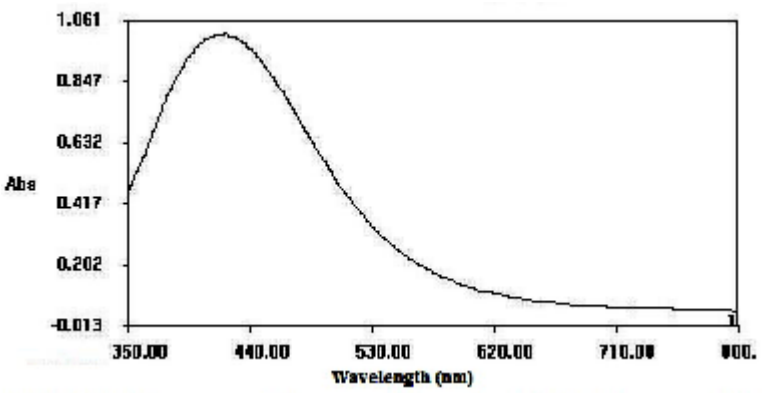

Fig. 3. UV–Visible spectrum of silver nanoparticles synthesized using *Rhizopus* spp.(VJ-6)

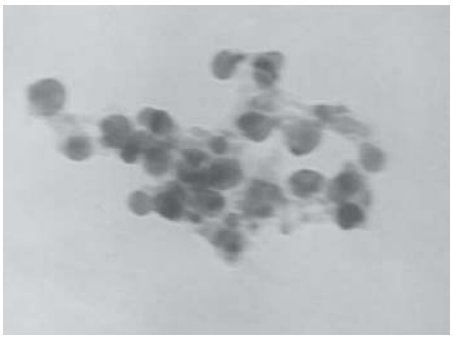

Fig. 4. TEM image of Silver nanoparticles

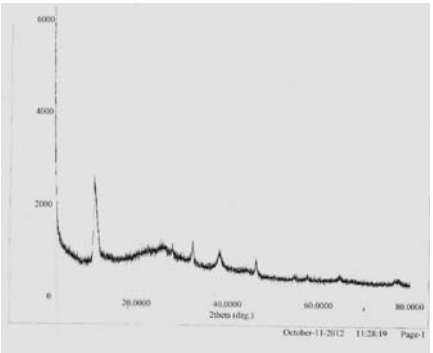

Fig. 5. X-ray diffraction pattern of synthesized Ag NP's

of silver atoms are in direct contact with their environment[41].

Re-emergence of MDR strains is facilitated by drug and/or antibiotic resistance, which is acquired way of these microbes for their survival and multiplication in uncomfortable environments. The worldwide escalation of bacterial resistance to conventional antibiotics and high prevalence of MDR infections decreases effectiveness of current treatments causing serious threat to human race. Nanotechnology has provided a good platform to overcome the problem of resistance with the help of silver nanoparticles. Recent, works revealed that the biosynthesized AgNps showed promising activity independently and also in combination with antibiotics[6,42]. Similar type of work was also presented by Humberto *et al*[43] where they showed the excellent antibacterial activity of AgNps against multidrug-resistant *P. aeruginosa, E. coli, Streptococcus* sp. and *S.pyogens.*

Since ancient time, antimicrobial efficacy of silver was reported in Ayurveda and Homeopathy. The bactericidal potential can be

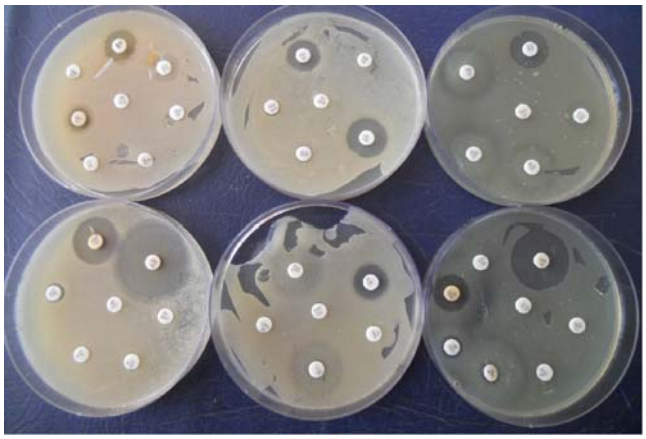

Fig. 6. *E.coli* MDR strains showing resistance against more than three antibiotics

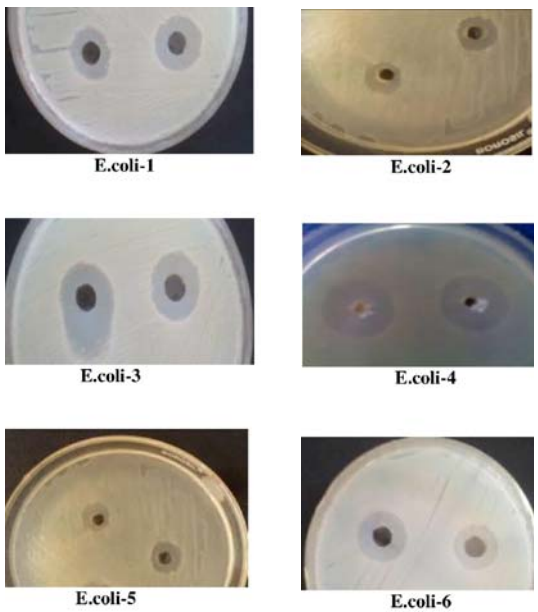

Fig. 7. Antimicrobial activity of AgNP's against *E.coli* isolates

increased by manipulating the size at nano level, leading to increased surface area to volume ratio and also by changing the chemical and physical properties. Therefore, silver nanoparticles having bactericidal potential against the multidrug-resistant strains

CONCLUSION

In the present study we have reported a simple biological, extracellular, economical & green approach for synthesizing silver nanoparticles using *Rhizopus* VJ-6. The particles were characterized by UV-Vis spectra, TEM and XRD studies. The size of the particles was found to be 27 nm. These particles with large surface area showed greater antimicrobial activity against MDR-*E.coli* strains. Our results suggest that, silver nanoparticles are effective against MDR-isolates of *E.coli*, which makes them a potential candidate for use in pharmaceutical products and medical

devices that may help to prevent the transmission of drug-resistant pathogens in different clinical environments.

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