

# Studies on biosynthesis of silver nanoparticles using *Rhizopus sp.* and its antibacterial efficacy on *E. coli* MDR strains

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## ABSTRACT

Nanotechnology is a field that is burgeoning day by day, making an impact in all spheres of human life. Biological methods of synthesis have paved way for the “greener synthesis” of nanoparticles and these have proven to be better methods due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization. In this context 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 cellfree 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 429 nm corresponding to spherical shaped nanoparticles. Transmission electron microscopy (TEM) micrograph showed formation of well-dispersed AgNPs in the range of 25-50 nm. Scanning electron microscopy (SEM) showed the particles to be uniformly dispersed without agglomeration with smooth morphology. EDS showed the presence of elemental silver at 3kev. X-ray diffraction (XRD)-spectrum of the AgNPs exhibited  $2\theta$  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. Synthesis of nanosized particles with antibacterial properties, which are called "nanoantibiotics", is of great interest in the development of new pharmaceutical products.

**Keywords:** *Rhizopus spp*; Silver Nanoparticles; TEM; SEM; EDS; XRD; Multi drug resistance (MDR)

## 1. INTRODUCTION

Nanotechnology is emerged as a fastest growing field with numerous applications in science and technology for manufacturing new materials. Nanotechnology is defined as the design, characterization and application of structures, devices and systems by controlling shape and size at 1 to 100 nm (Albrecht et al 2006). Modern era is of nanomedicine owing to their various therapeutic applications with more efficacies and lesser side effects.

The popularity is due to their potential for achieving specific process and selectivity in pharmacological action. Silver nanoparticles (AgNPs) are being used increasingly in wound dressings, catheters, and various household products due to their antimicrobial activity

(Bhattacharya and Mukherjee (2008). AgNPs formed by chemical and physical methods are very expensive and also include the use of toxic chemicals. Moreover it must be available at lower cost for their effective utilization; thus, there is a need for an environmentally and economically feasible way to synthesize these nanoparticles.

Biological synthesis of AgNPs is possible with help of bacteria, fungi, and plant extracts. Biological synthesis of silver nanoparticles is a bottom-up method that includes reduction/oxidation reactions. The microbial enzymes or phytochemicals with antioxidant or reducing properties act on the corresponding compounds and give the anticipated nanoparticles (Kalishwaralal et al 2008, Parashar et al 2009). Fungi can produce larger amounts of nanoparticles as compared to bacteria as they can secrete larger amounts of proteins which directly translate to higher yield of nanoparticles. the mechanism involve in synthesis of AgNPs by fungi is trapping of  $\text{Ag}^+$  ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system.

Silver nanoparticles can have strong antibacterial activity towards both gram positive and gram negative bacteria (Sadhasivam et al 2010, Nanda and Saravanan 2009). Several studies propose that the antimicrobial activity of AgNPs as due to the slow release of silver ions which react with thiol groups of proteins or interfere with DNA replication (Feng et al 2000). Also silver nanoparticles may get attached to the cell membrane surface which inturn can damage or disturb the functions of the cell leading to bacterial death (Kim et al 2007, Kumar et al 2007, Melaiye et al 2005). This makes studies on synthesis of AgNPs much important.

## **2. MATERIALS AND METHODS**

### **2. 1. Isolation and identification of Fungi**

Soil samples were collected from Gulbarga district, Karnataka for the isolation of fungal strains and cultured on potato dextrose agar (PDA) medium. Isolates were identified based on colony morphology and lacto phenol cotton blue staining. Isolates were sub cultured and preserved for further work.

### **2. 2. Biosynthesis of Silver Nanoparticles**

The fungus, *Rhizopus spp.* was grown in 250 ml Erlenmeyer flasks containing 100 ml of MGYB broth (Malt extract 0.3 %, Glucose 1 %, Yeast extract 0.3 % and Peptone 0.5 %) at 29 °C for 72 hr., then mycelia was separated, washed using distilled water and repeated 2-3 times to remove any traces of previous medium content. Then mycelia was transferred to fresh Erlenmeyer flask containing 100 ml distilled water, kept for incubation at afore said conditions for 48 hr. The suspension was filtered with the help of Whatman's filter paper No. 1, as obtained filtrate was challenged with 1 mM  $\text{AgNO}_3$  at 29 °C for reduction under static condition.

### **2. 3. Characterization of Silver Nanoparticles**

Synthesized silver nanoparticles were characterized using UV-Vis spectroscopy (T 90+ UV-VIS spectrophotometer) shows specific surface plasmon resonance peak.

Transmission Electron Microscopy (TEM - Hitachi H 7500 ID, Japan) reveals the size and shape of nanoparticles. Scanning electron microscopy (SEM – JEOL Model JSM-6390 LV) was employed to determine the shape and surface morphology of the nanoparticles.

Presence of elemental silver was detected by employing Energy Dispersive Spectroscopy (EDS-JEOL Model JED - 2300), X-Ray diffractometer (XRD) provides the crystalline nature of the particles.

#### 2. 4. Determination of antibacterial activity by well diffusion method

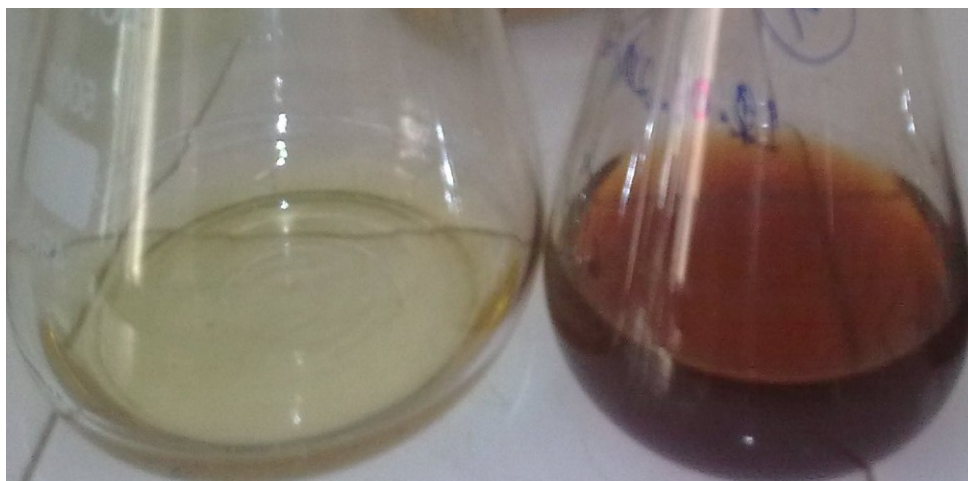
The AgNPs synthesized from the selected isolate was tested for its antibacterial activity against pathogenic bacteria such as *E. coli* 1, *E. coli* 2 by standard well diffusion method in Mullor Hinton Agar (MHA) plates (Saravanan et al 2011). Pure cultures of bacterial pathogens were grown in Nutrient broth at 37 °C for 18-24 hours. Wells were made on the Mullor- Hinton agar plates using a gel puncture and the plates were inoculated by swabbing the bacterial pathogens to create a confluent lawn of bacterial growth. 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.

### 3. RESULTS AND DISCUSSION

A variety of fungal cultures were isolated from soil, have been screened for their ability to extracellular reduction of silver ions to form nanoparticles. 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-2.

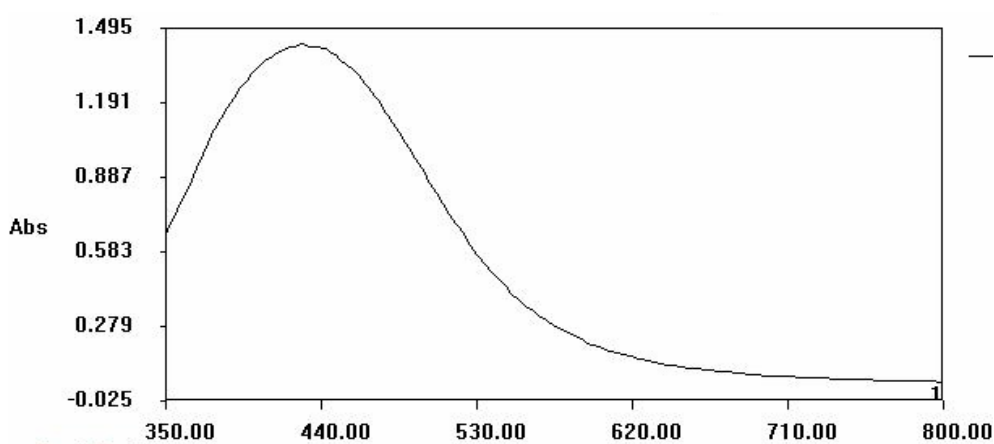


Fig. 1. *Rhizopus* sp.



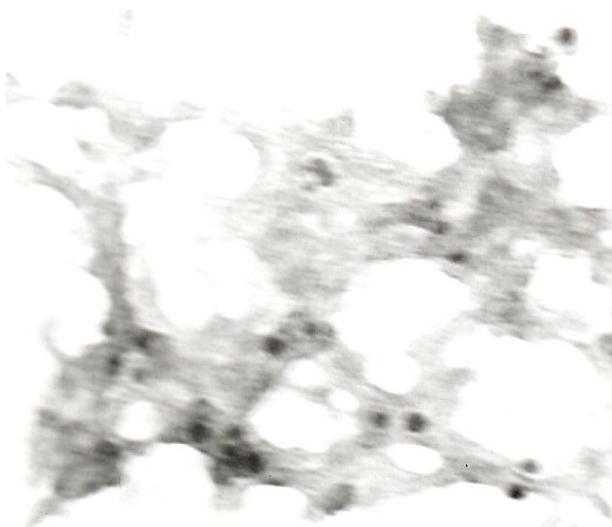
**Figure 2.** Colour change observed in cell free fungal filtrate of *Rhizopus sp.* after exposure to silver nitrate.

In the present study, the potential isolate *Rhizopus* VJ-2 was further employed for the biosynthesis of silver nanoparticles. 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-2. The biosynthesis of AgNPs by *Rhizopus* VJ-2 was primarily confirmed by the change of the reaction mixture from yellow to brown colour (Fig. 2) indicating the production of silver nanoparticles. The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation. The reduction of  $\text{AgNO}_3$  ions in solution was monitored by periodic sampling of aliquots of aqueous component & measuring UV Vis spectrometer. The cell-free filtrate solution exposed to  $\text{AgNO}_3$  ions shows a distinct absorption at around 429 nm which can be seen in Fig. 3. The AgNPs formed were highly stable even few weeks after the reaction. Reports of Shivaraj et al (2013) revealed surface plasmon resonance of AgNPs between 380 to 450. While reports of Afreen Banu et al (2011) reported a peak of 422 nm. Mukherjee et al (2008) reported an intense peak at 410 nm.

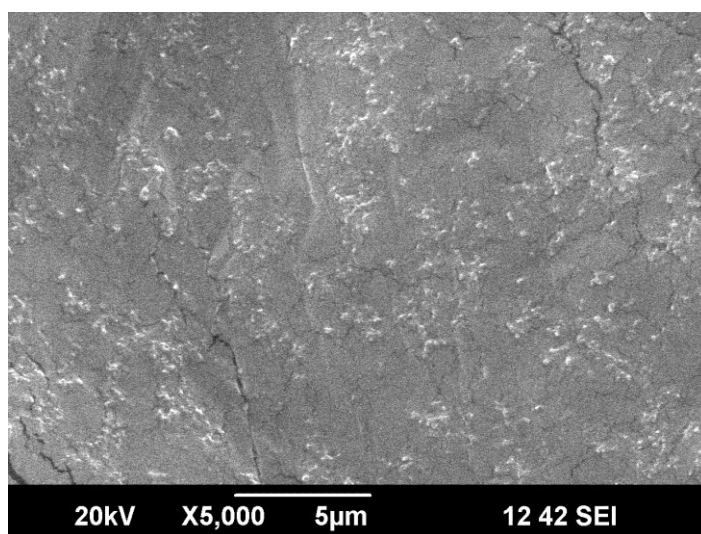


**Fig. 3.** UV-Visible spectrum of silver nanoparticles synthesized using *Rhizopus sp.*

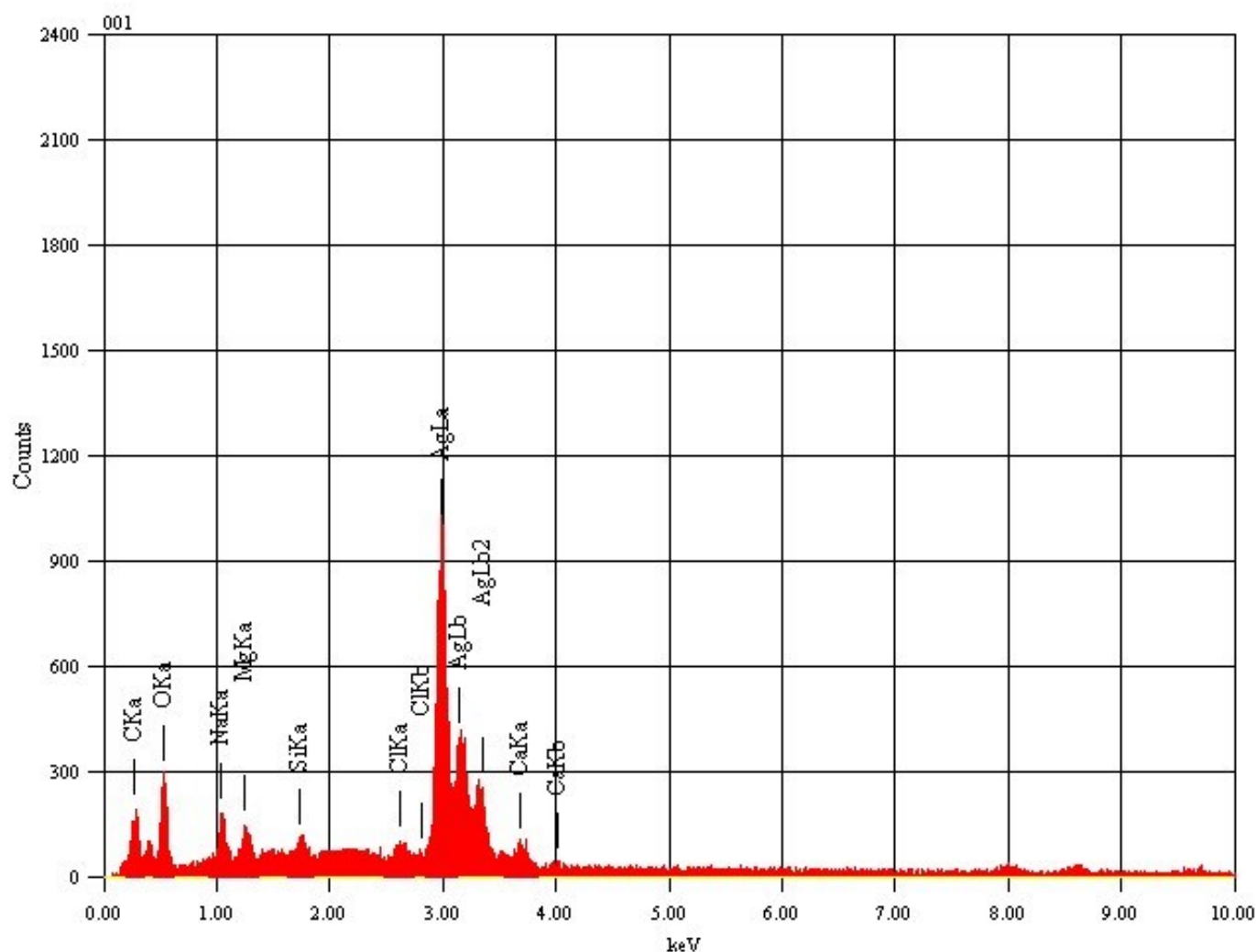
The TEM technique used to visualize size and shape of the biosynthesized silver nanoparticles have shown spherical shaped structures with size ranging between 25 to 50 nm presented in Fig. 4 and even Dattu Singh et al (2013) reported the size of the nanoparticles range about 25 nm. All the particles were well separated and no agglomeration was noticed. SEM results revealed the particles to be uniformly dispersed without agglomeration with smooth morphology (Fig. 5). EDS analysis, gives the optical absorption peak approximately at 3keV which is typical of the absorption of metallic silver nanocrystallites the other signals of Na, Si and K might be due to the excitation of enzymes which left unreduced. Varshney et al (2009) reported strong signals from the silver atoms in the nanoparticles of their studied fungi while weaker signals from C, O, S, P, Na, Mg and Ca atoms were also recorded (Fig 6).



**Fig. 4.** TEM image of Silver nanoparticles.



**Fig 5.** SEM image of Silver nanoparticles.



**Fig. 6.** EDS spectrum of synthesized AgNPs.

The crystalline nature of the AgNPs from *Rhizopus* VJ-2 was analyzed by X-ray diffraction. The diffracted intensities were recorded from 0 to 80 ( $2\theta$ ). Intense peaks corresponding to (111), (200), (220) and (311) are indexed as crystalline silver face-centered cubic (fcc) phase. The peaks were compared with the X-ray diffraction database (Fig. 7).

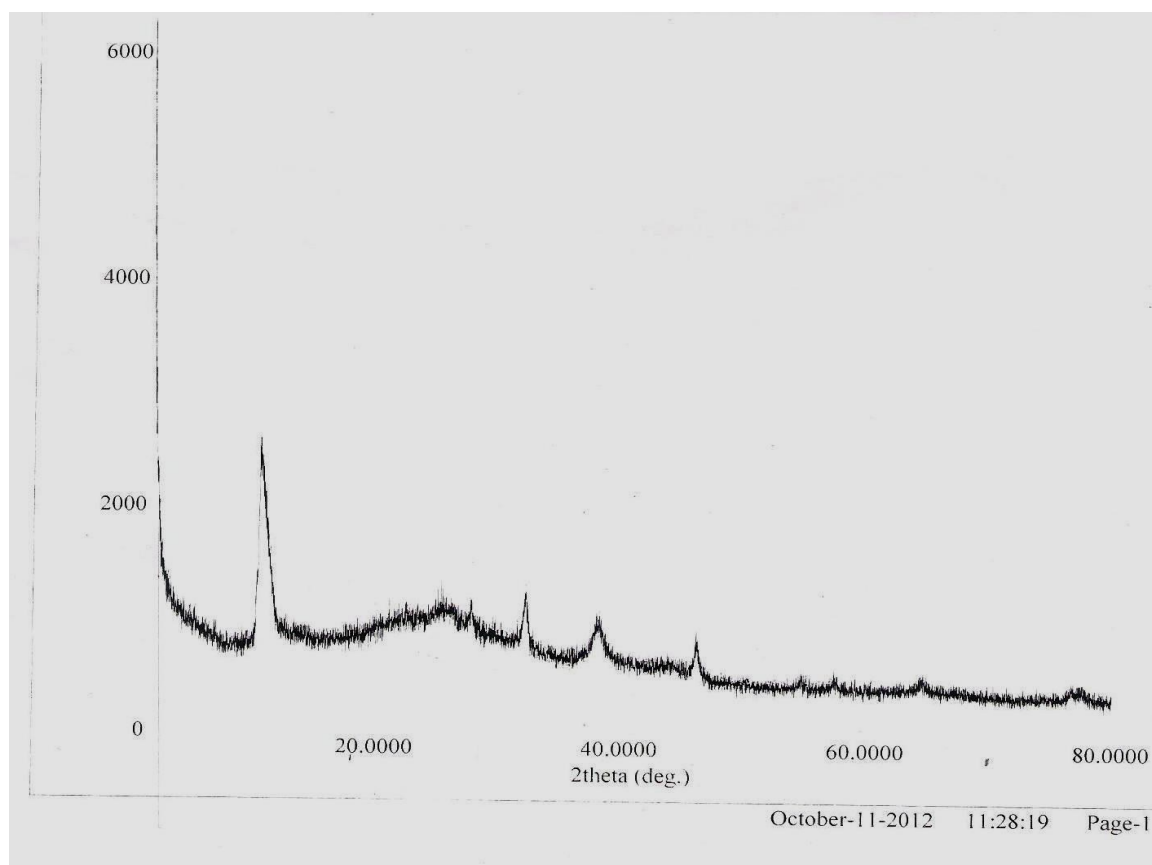
In our experiment the biosynthesized nanosilver showed excellent antibacterial activity against multidrug resistant *E.coli* isolated from clinical laboratories Gulbarga, Karnataka. Two strains of *E. coli* (*E. coli* 1 and *E. coli* 2) were selected for the study.

Agar diffusion method was employed to study the antibacterial effect. Biologically synthesized nanosilver showed zone of inhibition (mm) of about 19 mm at 20  $\mu$ l concentration AgNPs and 20 mm at 40  $\mu$ l concentration AgNPs for strains of *E. coli*-1, about 22 mm at 20  $\mu$ l concentration AgNPs and 23 mm at 40  $\mu$ l concentration AgNPs for *E.coli*- 2 respectively (Fig. 8 and Table 1).

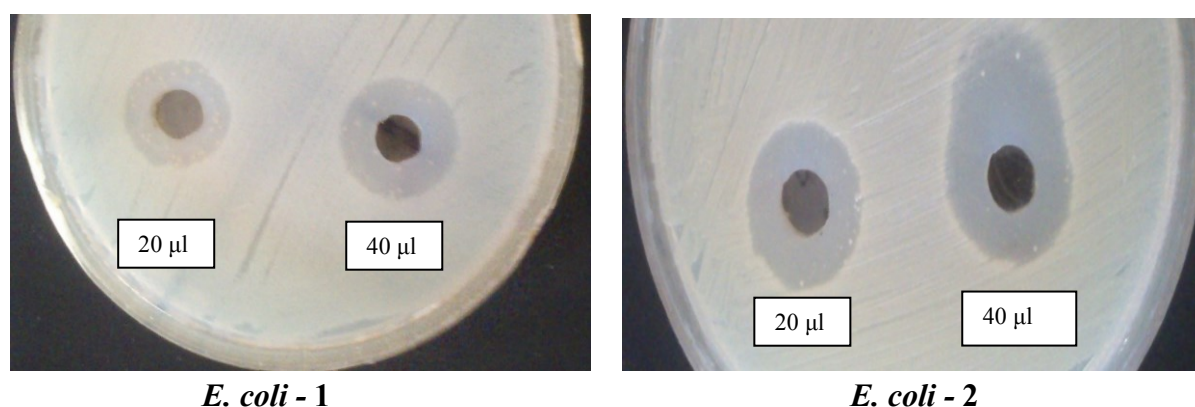
Here we report the efficacy of mycogenic metal nanosilver to kill MDR strains which is difficult through the conventional chemotherapy. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles.



The gram negative bacteria have a layer of lipopolysaccharide at the exterior, followed by a thin layer of peptidoglycan. Negative charges on the lipopolysaccharides are attracted towards positive charges available on silver nanoparticles and disturb its power function such as permeability and respiration.



**Fig 7.** X-ray diffraction pattern of synthesized AgNP's.



**Fig. 8.** Antimicrobial activity of AgNP's against ESβL *E. coli* isolates.

**Table 1.** Zones of inhibition of MDR *E. coli* strains treated with various concentration of AgNPs.

Sl. No	MDR Strains	Zone of inhibition in mm	
		(20 $\mu$ l)	(40 $\mu$ l)
1.	<i>E. coli</i> - 1	19	20
2.	<i>E. coli</i> - 2	22	23

#### 4. CONCLUSION

In this study we introduce a simple, fast, and economic biological procedure to synthesize silver nanoparticles using filamentous fungi *Rhizopus sp.* Silver nanoparticles were characterized using UV-Vis spectroscopy, TEM, SEM, EDS and XRD techniques. The size of the nanoparticles was found to be in the range of 25-50 nm. Our results suggest that, silver nanoparticles are effective against MDR-isolates of *E. coli* with maximum zone of inhibition. Silver and its derivatives are widely used in medicine for a long time in the treatment of infections.

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