Biosynthesis and Characterization of Silver Nanoparticles from Aspergillus Terreus and its Antibacterial Efficacy against VRSA Strains

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Abstract **- The alarming growth of Vancomycin Resistant *Staphylococcus aureus* (VRSA) is the main force for the development of a new agent to restore the antibacterial activity against VRSA. In the present study Silver nanoparticles (AgNPs) were synthesized using the fungus *Aspergillus terreus*. Thus produced AgNPs were characterized by Visual observation, UV-Visible Spectrophotometer, Fourier Transform Infra Red Spectroscopy (FTIR), X-Ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). The AgNPs exhibited maximum Absorbance at 400nm under UV-Vis Spectrophotometer. FTIR confirmed the presence of protein as the stabilizing agent surrounding the silver nanoparticles. Further crystalline nature of the AgNPs was confirmed by XRD.TEM micrograph showed 20-35nm size of the AgNPs and revealed morphological characters of silver nanoparticles to be spherical. The AgNPs from *A.terreus* showed significant antibacterial activity against VRSA strains and at the same time the synergistic effect of AgNPs in conjugation with the resistant antibiotic Vancomycin showed no significant change when compared to AgNPs alone. These results clearly indicates that Vancomycin resistant *S.aureus* is susceptible to only AgNPs, which could be the best antibacterial agent against VRSA alternative to Vancomycin antibiotic.**

Key words: Aspergillus terreus, FTIR, XRD, TEM, SEM, VRSA

1. INTRODUCTION

Concern about antibiotic resistant microorganisms (such as MRSA) is causing resurgence in the search for effective non-antibiotic antimicrobials (Ellis, 2007). Therefore, interest is growing in the use of nanotechnology for the prevention and treatment of bacterial infections. Vancomycin (VAN) has been used for the treatment of MRSA infections since 1980, however, in recent years Vancomycin-resistant *S. aureus* (VRSA) has appeared, the first of which was registered in 2002 in the USA. Vancomycin and teicoplanin are glycopeptides with significant activity against Gram positive bacterial pathogens (Reynolds, 1985) and over the past two decades, Vancomycin has been considered the drug of choice for MRSA infections. Unfortunately, in 1996, the emergence of VRSA has caused additional concern. Strains of VRSA have been reported from Japan (Hiramatsu, 1997), United States (Smith, 1999 & Centre for disease control and prevention, 1999), United Kingdom (Howe et al, 1998) and Germany (Beirbaum et al, 1999). Most of these isolates appear to have developed from pre-existing MRSA infections. Assadullah et al, 2003 from India reported reduced susceptibility of Vancomycin against MRSA and Coagulase negative *S.aureus* (Assadullah et al, 2003). Subsequent isolation of VRSA isolates from other countries including Brazil (oliveira et al, 2001), India (Tiwari et al 2006), Belgium (Pierard et al, 2004) has confirmed that the emergence of these strains is a global issue. Therefore, infections caused by these resistant strains are very serious and difficult to treat.

In spite of the presence of different methods proposed to combat microbial resistance, the high prevalence of multidrug-resistant bacteria indicates an urgent requirement for new approaches to cope with this problem. High biological and chemical activity of metallic nanoparticles makes them promising agents and represent as an alternative method for coping with antibiotic resistance, and a combination of these nanoparticles with antibiotics provides an opportunity not only to increase antibacterial activity of both components but also to reduce their toxic effects.

Few studies are progressing towards the interactions between antibiotics and silver nanoparticles, and many such combinations have shown a promising enhancing effects in vitro, mainly with β-lactams (ampicillin and amoxicillin) and glycopeptides (Vancomycin) while meagre or nil reports are available on the effect of AgNPs on VRSA strains. Delivery of antibiotics via nanoparticles has advantages not only in increasing the effect of the antibiotic but also in minimizing the side effects that are associated with the use of broad-spectrum antibiotics, including those used to treat representatives of the normal microflora. These nanoparticles are of constant shape and smaller in size and

are chemically stable and take part in multivalent interactions with bacteria surface (Adil et al, 2011).

The wide use of VAN for the treatment of severe infections caused by MRSA and Methicillin Resistant Coagulase negative *S.aureus* (MRCNS) and its therapeutic failures have led to an increase in microbial resistance, relapse of infection incidence and worsening of patient's clinical conditions (Pallakota,2008;Rybak,2009;Dehority,2010 and Hazlewood,2010). Due to a lack of therapeutic options, some studies have focused on the combination of two or more antibiotics as an alternative treatment [Totsuka et al, 1999; Rochon et al, 2000; Kobayashi et al, 2005; Yamaoka et al, 2007 and Nyugen et al, 2010). Promising results in vitro and in vivo could lead to effective therapy along with a reduction of therapeutic doses, adverse effects and treatment duration (Livia et al, 2011). The aim of this study was to search for an alternative in the form of AgNPs which can act as a drug of choice for VRSA.

2. MATERIALS AND METHODS:

***2.1 Isolation and Identification of Fungus*:** Fungal cultures were isolated from soil samples collected from different areas of mining regions in Bellary, Karnataka, India. The fungal isolates were characterized on the basis of colony characteristics, microscopic appearance and were sent for confirmation to the Mycology and Plant Pathology Department, Agharkar Research Institute, Pune. The isolated fungi were grown on Potato Dextrose Agar (PDA) medium at 28˚C and stored at 4˚C for further study. Multi-drug resistant *S. aureus* strains were obtained from BRIMS Medical college and hospital, Bidar, Karnataka.

2.2 Extracellular Synthesis of Silver Nanoparticles: The fungal strains isolated from soil samples was inoculated in Malt Glucose Yeast Peptone (MGYP) broth containing yeast extract and malt extract-0.3% each, glucose-1% and peptone-0.5% at 29˚C for 72h. After incubation the biomass was filtered and then washed with sterile distilled water repeatedly to remove medium components. The biomass collected was taken into Erlenmeyer flask containing 100ml distilled water and incubated at the above said conditions. The biomass after incubation was filtered again using Whatmann filter paper no.1. The fungal filtrate was further added to aqueous solution of $AgNO_3$ (1mM) and the flasks were incubated at 29˚C for upto 120 h.

***2.3 Characterization of AgNPs*:** After incubation, the preliminary detection of AgNPs was carried out by visual observation of color change of the cell filtrate. These samples were later characterized by UV-Visible spectrophotometer (UV-1700 Pharma Spec, SHIMADZU) in the range of 300 to 700nm. The interaction between protein and AgNPs was analyzed by Fourier Transform Infra-red (FTIR) spectroscopy. The IR spectrum of the dried sample was recorded (Thermo Nicolet Nexus 670 spectrometer) in the range of 500 to 4000 cm^{-1} at resolution of $4cm^{1}$. The crystalline nature of the AgNPs from *A. terreus* was analyzed by X-ray diffraction. The diffracted intensities were recorded from 0 to 80 (2 Ɵ). The Braggs peak position and their intensities were compared with the standard JCPDS files. TEM analysis was done to determine the size and shape of the AgNPs. Scanning Electron Microscope (SEM) was used to record surface morphology of synthesized AgNPs.

2.4 Antibacterial Activity of Synthesized AgNPs:
The antibacterial activity of synthesized AgNPs was evaluated using agar-well diffusion method. In the present experiment, of the 53 *S. aureus* strains collected from hospital, only two strains were found to be Vancomycin resistant. Thus, obtained VRSA were maintained on nutrient agar slants at 4˚C. Further they were sub-cultured in nutrient broth for 24hrs at 37˚C and stored at 4˚C. Each bacterial strain was swabbed uniformly on the Mueller Hinton Agar (MHA) plates to prepare bacterial lawn into the individual plates. Wells of 6mm diameter were made onto each bacterium inoculated agar plates using gel puncture. AgNPs of different concentrations 20, 40, 60 and 80 µl were poured into the corresponding wells using sterile micropipette. The antibacterial activity was determined by clear inhibition zone obtained around the sample loaded in the well after incubating the plates at 37˚C for 24hrs.

2.5 Synergistic Effect of AgNPs with Commercial Antibiotic Discs of Vancomycin:
Synergistic effect of AgNPs along with Vancomycin was tested using agar well diffusion method. Two VRSA strains were sub-cultured in nutrient broth for 24h at 37˚C. Each strain was swabbed uniformly on MHA using sterile cotton swabs. AgNPs solutions with dipped antibiotic discs were loaded in the respective wells and the results were recorded by measuring zone diameter in mm after 24h of incubation at 37˚C.

***2.6 Assaying the Minimum Inhibitory Concentration (MIC) of AgNPs*:**
To evaluate the MIC of AgNPs and growth curve of VRSA to AgNPs, different concentrations of silver nanoparticle solution (5, 10, 15, 20, 25 and 30µl/ml) was added in LB broth, The VRSA cultures were maintained at 10^8 CFU/ml and incubated at 37˚C. The bacterial growth was determined by measuring optical density at 600nm using spectrophotometer.

3. RESULTS AND DISCUSSION

The fungal isolates screened from soil samples after morphological and microscopic examination was identified as *Aspergillus terreus* **(Fig1.a & b).** Of the 53 *S.aureus* strains collected from BRIMS medical college and hospital, two isolates were found to be VRSA and further they were sub cultured in nutrient broth for further experimental studies. The fungal biomass after 120h incubation was filtered and the filtrate was subjected to $AgNO_3$.The reaction mixture changed its color to brown within 24h **(Fig2. a & b)** indicating the formation of AgNPs.

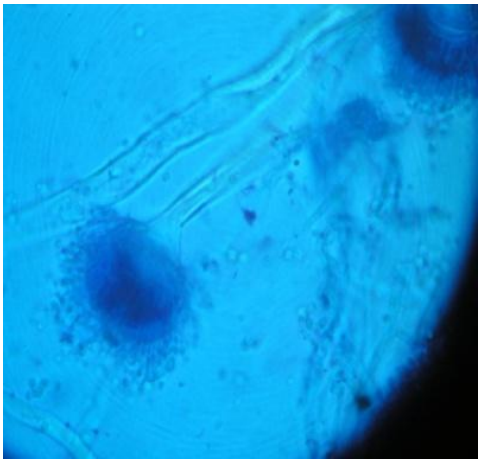
The characterization of synthesized AgNPs was done by UV-Visible Spectrophotometer and the recorded UV-absorption spectra exhibited an intense peak at 400nm **(Fig.3)** corresponding to the surface Plasmon resonance frequency of AgNPs. Patil et al (2011) reported that the biological process for the synthesis of AgNPs using *A.flavus* showed maximum absorbance at 420nm while the absorption maximum at 430nm was reported by Ranganath et al (2012) using *Lactobacillus* species. Dattu et al, 2014 studied the optimization of AgNPs from *Penicillium* sps isolated from *Curcuma longa* (Turmeric) and characterized by UV-Vis spectroscopy and maximum absorbance peak was reported at 420. Similar results were observed by Ninganagouda et al (2013) who revealed plasma resonance of AgNPs between 380 and 450 nm.

FTIR representative spectra in the region of 4000 to 500 cm^{-1} revealed the presence of different functional groups such as 3331.68-secondary amide (N-H stretch-bonded), 2922.02-alkane (C-H stretching), 1773.02-Anhydride (C=O stretching), 1638.12-Amide (C=O stretching) 1544.89-Aromatics(C-C stretching),1077.84, 1383.28 and 1229.67 primary alcohol (C-O stretching), 1192.53 & 1161.77 secondary alcohols-(C-O stretching), 830.23 & 551.69-alkene (=C-H bending) respectively **(Fig.4).** Our results correlated with Ninjanagouda et al (2013) where silver nanoparticles were produced from *A.flavus*. Monali et al,(2009) synthesisized extracellular AgNPs from the fungus *Alternaria alternate* and has shown the presence of nine bands at 1032, 1283,1356, 1383, 1453, 1513, 1583, 1629, and 1740 cm–1 respectively.

The crystalline nature of the AgNPs from *A. terreus* was analyzed by X-ray diffraction. The diffracted intensities were recorded from 0 to 80 (2 Θ). Intense peaks corresponding to (111), (200), (220) and (311) were observed as depicted in **Fig.5.** The Braggs peak position and their intensities were compared with the standard JCPDS files. Jyoti et al (2014) reported the intense peaks at 2Θ angles of 32˚, 38˚ and 43˚ corresponding to the facets (101,111 and 200) using *Rhizopus* sps. The XRD results of our AgNPs produced from *Aspergillus terreus* supported the presence of crystalline AgNPs which correlates with results of Li et al (2012) for production of AgNP from the same sps.

The TEM results have shown spherical shaped structures of AgNPs with size ranging from 20 to 35nm **(Fig.6)**. Similarly the production of AgNPs biologically from non pathogenic fungus and its synergistic antibacterial activity with the antibiotic cefazolin was studied in detail by Singh et al (2011) and the size of AgNps were reported in the range of 30 to 50nm. SEM results revealed the particles to be uniformly dispersed without agglomeration with smooth morphology **(Fig.7).** Devika et al, (2012) reported the formation of well dispersed AgNPs from the fungus *Pleurotus ostreatus* with size ranging 40-50nm .Vanmathi et al, 2012 reported spherical nanoparticles produced from *Fusarium oxysporum* in the size range 20-50nm and studied that the nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent.

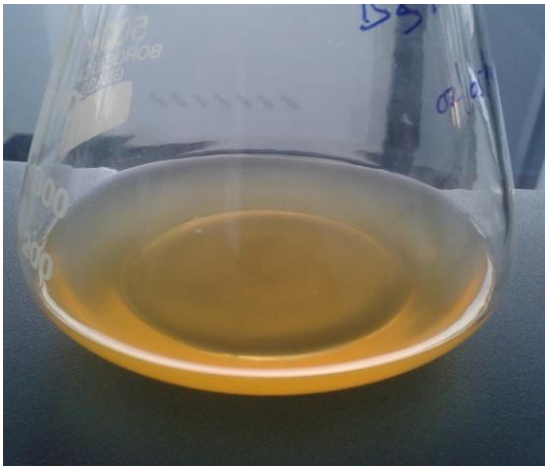
(a)



(b)

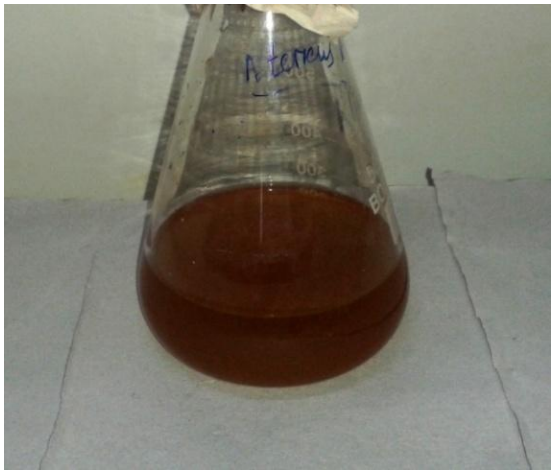
Fig1. a) *A terreus* on PDA

b) Microscopic image of *A.terreus*



(a)

Fig.2.a) Fungal filtrate of *A.terreus*



(b)

b) Brown color of AgNPs after 24hrs

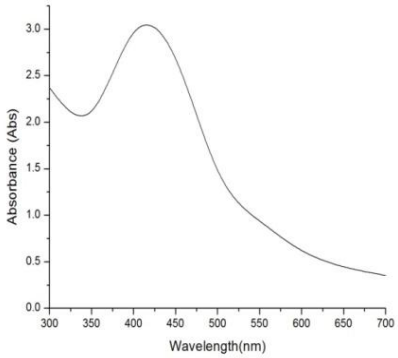


Fig 3. UV-Visible absorption spectra of AgNps

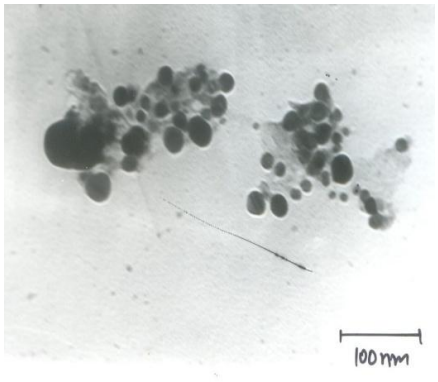


Fig.6 TEM image of AgNps from *A.terreus*

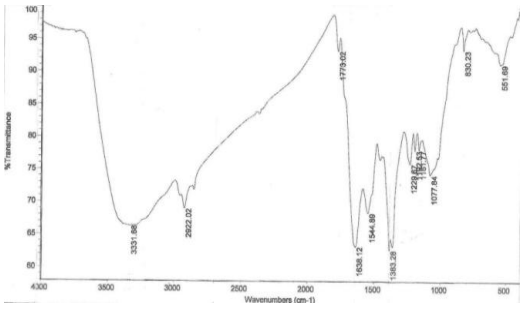


Fig 4: FTIR analysis of AgNPs

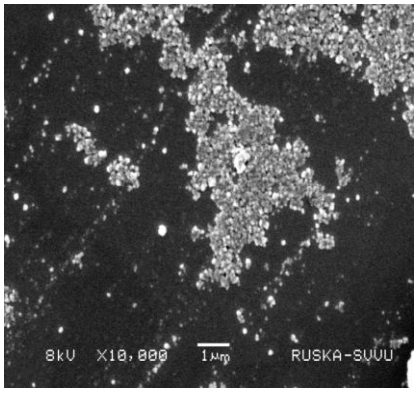


Fig.7.SEM image of AgNps from *A.terreus*

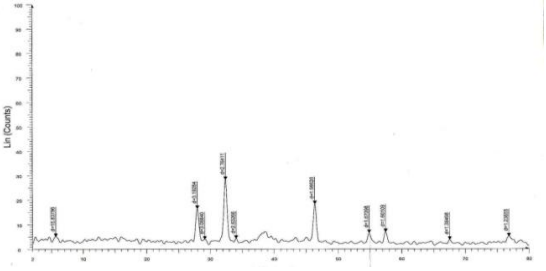


Fig 5: XRD pattern of AgNPs

Experiments were conducted to study the effect of our AgNPs produced by *A.terreus* on VSSA and VRSA strains. Studies were also extended to find out the synergistic effect of AgNPs on VSSA and VRSA with Vancomycin antibiotic. Of the 53 isolates screened only two isolates were found to be resistant to Vancomycin and five Vancomycin sensitive isolates were used for experimental studies to compare the results of the effect of AgNPs on VSSA and VRSA. The five Vancomycin sensitive isolates were-VPG1, VPG2, VPG3, VPG4 and VPG7. The concentrations of AgNPs used to study the

effect of AgNPs on VSSA were 20 to 80 µl (with the difference of 20µl).

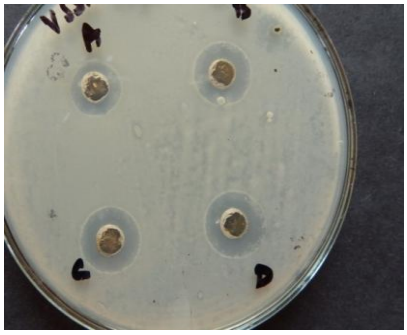
Results revealed that AgNPs were more effective on VPG1 showing maximum inhibition zone of 17mm at 80µl concentration of AgNPs. Whereas, VPG2 and VPG7 showed an inhibition zone of 16mm each at 80µl concentration, while VPG3 and VPG4 showed zone diameter of 15mm each at the above said concentration **Plate-1(Table-1).** While the results of inhibition zone with other concentrations (20-60 µl) were in between with all the isolates.

The effect of AgNPs on the two isolates of VRSA strains was studied. The results were very encouraging. The VRSA isolate (VPG5) gave the zone diameter of 17mm **(Plate-2)** at 80µl concentration, the highest zone of inhibition 20mm was observed with the VRSA isolate PVG1 **(Table-2)**. Our results indicate that AgNPs can play a very vital role as an alternative agent to antibiotics against VRSA strains. Studies were also extended not only to check the effect of AgNPs on VSSA and VRSA but also to study the synergistic effect of AgNPs on VRSA and VSSA along with the antibiotic Vancomycin. Though Vancomycin sensitive *S.aureus* isolates were checked with AgNPs, we wanted to check the effect of exposing AgNPs along with Vancomycin on VSSA strains as well as VRSA. To our astonishments, we got encouraging results. The maximum inhibition was observed with VPG2 and VPG7 showing maximum inhibition zone of 20mm at 80µl. While VPG1 and VPG3 showed 19mm and 18mm inhibition zone at 80µl concentration respectively. VPG4 showed the minimum inhibition zone of 16mm at the same concentration of AgNPs **(Table 3).** We also compared the percentage fold increase of the synergistic effect of AgNPs and Vancomycin on VSSA and VRSA strains. It was 25% maximum fold increase for VPG2 and VPG7 and 20 % with VPG3 and minimum of 11.76% and 6.66% with VPG1 and VPG4 in case with VSSA strains. When these results were compared with the % fold increase of the effect of AgNPs and Vancomycin on VRSA strains it was nil **(Table-4)** which clearly indicates that AgNPs produced by *Aspergillus terreus* can act as an effective alternative agent for the resistant antibiotics against VRSA strains.

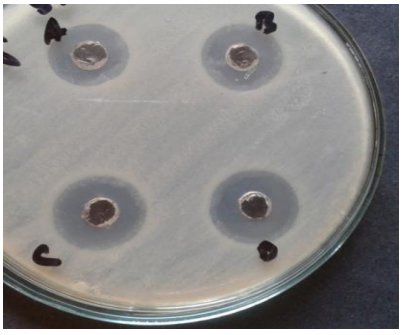
Richa Singh et al (2013) reported the highest synergy of AgNPs with Vancomycin against gram positive organisms. However, the administration of AgNPs on VRSA along with Vancomycin revealed the same results as that of AgNPs on VRSA which clearly indicates that there was no synergistic effect of Vancomycin and AgNPs except that only AgNPs have played a major role in inhibiting Vancomycin Resistant *S.aureus*. To determine the lowest concentration that inhibited visible growth totally, MIC was carried out. The MIC of AgNPs against VRSA strains was found to be 20µl. From these results it can be deduced that the biosynthesized AgNPs were found to have good antibacterial activity on the growth of bacteria. Shahverdi et al (2007) demonstrated that combining penicillin-G, Amoxicillin, Erythromycin, Clindamycin, and Vancomycin with silver nanoparticles leads to an increase in antibacterial activity of the antibiotics against *S.aureus* and *E. coli*. This finding suggests that combining antibiotics with silver nanoparticles increases antibacterial properties of antibiotics. In addition, according to Patil and coworkers, chloramphenicol loaded with nanoparticles showed substantially enhanced activity against *Salmonella typhi* (Patil et al. 2009).

As reported by Balaji et al, 2013 *S.aureus* will give fewer anchoring sites for AgNPs making them difficult to penetrate. The increase in synergistic effect may be caused by bonding reaction between antibiotic and nanosilver. The antibiotic molecule contains many active groups such as hydroxyl and amido groups which react easily with nanosilver chellation preventing DNA from unwinding.

Silver tend to have a higher affinity for phosphorous and sulphur compounds. The membrane of bacteria is well known to contain many Sulphur containing proteins which might be the preferential sites for AgNPs as the nanoparticle enters the cell wall of the bacterium they interfere with the bacterial growth signaling system by modulating the tyrosine phosphorylation putative peptide substances critical for cell viability and multiplication substances critical for cell viability and multiplication apart from forming ROS species (Shivaraj et al, 2014).

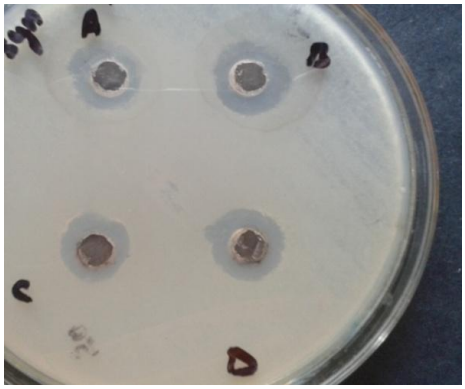


(a) Plate-1.a) Effect of AgNps on VSSA

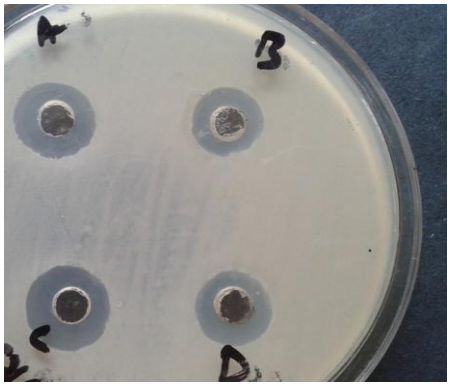


(b) Synergistic effect on VSSA

(A) 20, (B) 40, (C) 60 and (D) 80 µl respectively



(a) Plate-2.a) Effect of AgNPs on VRSA



(b) Synergistic effect on VRSA

(A) 20, (B) 40, (C) 60 and (D) 80 µl respectively

CONCLUSION

The use of microorganisms in the synthesis of silver nanoparticles emerges as an eco-friendly, economical, efficient and sustainable approach. The silver nanoparticles have been produced extracellularly by *Aspergillus terreus*. UV-Vis spectro-photometer, FTIR and XRD techniques have confirmed the reduction of $AgNO_3$ to AgNPs by the *A.terreus*. The size of AgNPs were observed in the range of 20-35nm with spherical shape and found to be uniform and dispersed. The zone of inhibition formed in the antimicrobial screening test indicated that the AgNPs synthesized in this process have the efficient antimicrobial activity againstVRSA, however synergistic effect did not showed much difference in zone of inhibition on VRSA. The biologically synthesized silver nanoparticles could be of immense use in medical field for their efficient antimicrobial function.

There is a global havoc for the development by bacteria AgNPs can prove as an alternative tool for bacteria which are resistant to bacteria as it is observed in this study that *S.aureus* is resistant to Vancomycin.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this
Paper.

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Table 1: Effect of AgNPs on VSSA strains

Sl.No	**VSSA strains**	**Zone of inhibition in mm**			
		20µl	**40 µl**	**60 µl**	**80 µl**
1	VPG1	12	15	15	17
2	VPG2	12	13	14	16
3	VPG3	12	14	14	15
4	VPG4	12	13	14	15
5	VPG7	12	14	16	16

Table: 2 Synergistic effect of AgNP and Vancomycin on VSSA strains

Sl.no	**VSSA strains**	**Zone of inhibition in mm**				**Fold increase in %**
		20µl	**40 µl**	**60 µl**	**80 µl**	
1	VPG1	17	18	19	19	11.76
2	VPG2	18	19	20	20	25
3	VPG3	14	16	17	18	20
4	VPG4	14	15	16	16	6.66
5	VPG7	12	16	18	20	25

Table: 3 Effect of AgNPs on VRSA strains

Sl.No	VRSA strains	Zone of inhibiton in mm			
		20µl	40µl	60µl	80µl
1	VPG5	14	15	16	17
2	PVG1	13	14	16	20

Table: Synergistic effect of AgNPs and Vancomycin on VRSA strains

Sl.No	VRSA strains	Zone of inhibiton in mm				
		20µl	40µl	60µl	80µl	Fold increase in %
1	VPG5	15	16	17	17	Nil
2	PVG1	13	14	15	20	Nil