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EXTRACELLULAR BIOSYNTHESIS OF SILVER NANOPARTICLES USING *ASPERGILLUS FLAVUS* AND THEIR ANTIMICROBIAL ACTIVITY AGAINST GRAM NEGATIVE MDR STRAINS

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ABSTRACT

Objective: To develop a reliable, ecofriendly procedure for bioreduction of silver nanoparticles by *Aspergillus flavus.* Methods: Characterization was done by using UV-VIS spectrophotometry, Infrared spectroscopy (FT-IR) and X-ray diffraction. Results: IR spectroscopy showed the potential biomolecule responsible for the reduction of silver nanoparticles and stability of the bioreduced silver nanoparticles. X-ray diffraction analysis showed that the particles were crystalline in nature with face centered cubic geometry. Conclusion: *A.flavus* was found to be a good producer of silver nanoparticles by releasing the extracellular enzyme nitrate reductase. Silver nanoparticles are known to have inhibitory and bactericidal effects against MDR strains of *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumonia*. Resistance of bacterial infections has emerged in recent years and is a major health problem. Here, we report the extra cellular biosynthesis of silver nanoparticles using the fungus, *Aspergillus flavus* and its antibacterial activities against pathogenic MDR strains.

KEYWORDS: ***Aspergillus flavus*, FT-IR, MDR strains, Silver nanoparticles, XRD.**

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INTRODUCTION

Nanotechnology is expected to be the basis of many main technological innovations in the 21st century. Research and development in this field is growing rapidly throughout the world. For the synthesis of nanoparticles, a number of chemical methods exist in the literature all these protocols involve toxic chemicals, which have been a matter of great concern for environmental reasons. Consequently, researchers in the field of nanoscale material synthesis and assembly have been eagerly looking at biological systems for an alternative[1]. The metal microbial interactions have an important role in several biotechnological applications, including the fields of bioremediation, biomineralization, bioleaching and microbial corrosion[2,3]. Recently, the utilization of biological systems has emerged as a novel method for the synthesis of metal nanoparticles. Microorganisms such as bacteria, fungi and yeast play an important role in the remediation of toxic metals through reduction of metal ions and act as interesting nanofactories[4]. These microbes are extremely good candidates in the synthesis of silver and gold nanoparticles [5-7].Drug-resistant bacteria are emerging pathogens whose resistance profiles present a major challenge for containing their spread and their impact on human health. People who become infected with drug-resistant microorganisms usually spend more time in the hospital and require a form of treatment that uses two or three different antibiotics which are less effective, more toxic, and more expensive[8]. Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter the effect. Silver has long been known for its antimicrobial properties, but its medical applications declined with the development of antibiotics. Currently, silver sulfadiazine is listed by the World Health Organization as an essential anti-infective topical medicine[9]. Since silver works as a bulk material, the use of nano-sized silver may also be appealing. Different studies have established the bactericidal effect of nanosilver against Gram negative and Gram positive bacteria, but the bactericidal mechanism of this compound has not been clearly elucidated. Morones *et al.,*[10] defined the antibacterial activity of silver nanoparticles against four types of Gram negative bacteria*, Escherichia coli, Vibrio cholera, Pseudomonas aeruginosa* and *Salmonella typhus*, and suggested that silver nanoparticles attach to the surface of the cell membrane penetrate bacteria and disturb its function by releasing silver ions[11, 12]. Other groups have worked with gram positive bacteria, such as *Staphylococcus aureus* [13, 14]. Selvakumar *et al*.,[15] also has reported the antimicrobial activity of extracellular synthesis of silver nanoparticles from *Streptomyces rochei* from marine samples.Whether silver nanoparticles are an option to confront the transmission of and infection by pathogenic drug-resistant bacteria remains to be determined. For this reason, in the current study, silver nanoparticles were synthesized by *Aspergillus flavus*. To assess the antibacterial properties of silver nanoparticles against pathogens, we challenged clinical isolates which are multidrug-resistant. The isolates used were *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsielle pneumonia*.

EXPERIMENTAL

In the present study for the isolation of fungi various soil samples were collected from Hatti gold mines, Raichur district, Karnataka, and were cultured on potato dextrose agar media for the isolation of potential strains. They were identified based on lacto phenol cotton blue staining and colony morphology and used for the biosynthesis of silver nanoparticles.

2.1. Biosynthesis of silver nanoparticles

The fungus *A. flavus* 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) at 29^0 C at 150 rpm for 72 hour[4]. After incubation, mycelia was separated by filtration, washed with sterile distilled water to remove traces of media components, resuspended in 100ml distilled water, and incubated at same position for 48 hours. The suspension was filtered through whatman filter paper no.1. The cell filtrate was challenged with $AgNO_3$ solution (1mM) and incubated at 29^0 C for reduction.

2.2.Characterization of silver nanoparticles

After addition of $AgNO_3$ to enzyme filtrate, conical flasks were kept for visual observation of color change of the cell filtrate. Now the produced silver nanoparticles were subjected to optical measurement, which was carried out by using UV-VIS spectrophotometer (T 90+ UV-VIS spectrophotometer) and scanning the spectra between 200 to 800 nm.After UV-VIS analysis the silver nanoparticles were purified by centrifugation at 10,000rpm/15min, then resuspended the pellets in sterile distilled water and again centrifuged at 10,000rpm / 10 min. The collected pellets were air dried at room temperature for IR analysis. The IR spectrum of the dried sample was recorded on Perkin Elmer one FT-IR spectrophotometer in the range 450 to 3000cm-1.The silver nanoparticles purified by centrifugation at 10,000rpm/15min, were then resuspended in sterile distilled water and again centrifuged at 10,000 rpm/10 min. The collected pellets were air dried at room temperature for obtaining powder form. X-ray diffraction technique was used to analyze the metallic nature of nanoparticles after bioreduction. The dried mixture of silver nanoparticles was collected for determining the formation of nanoparticles by X-ray diffractometer.

2.3. Antimicrobial activity

Antimicrobial activities of the synthesized silver nanoparticles were determined using the standard zone of inhibition. The molten and cooled Muller Hinton Agar (MHA) was poured in sterilized petri dishes. The plates were left overnight at 37^0C to check for any contamination. Bacterial test organisms were grown in nutrient broth for 24 hours. Bacterial lawn was prepared using each bacterial strain. Agar wells were made on MHA plates using gel puncture. Then the plates loaded with silver nanoparticles were incubated at 37^0 C. After incubation the plates were examined for evidence of zone of inhibition, which appeared as a clear area around the wells [14].

RESULTS AND DISCUSSION

3.1 UV-VIS spectra analysis

The silver nanoparticle synthesis is in lime light of modern nanotechnology. The development of biologically inspired experimental processes for the synthesis of silver nanoparticles is evolved into an important branch of Nanotechnology. The present study deals with the synthesis of silver nanoparticles by using enzyme filtrate of *A. flavus* and its antimicrobial activity against Multi Drug Resistant *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumonia.* The approach appears to be cost effective, alternative to conventional methods of assembling silver nanoparticles.Noble metal nanoparticles exhibit specific surface plasmon resonance which shows peak characteristic for each metal[16]. The characteristic surface plasmon resonance of silver nanoparticles ranges between 380 nm to 450 nm due to excitation of surface plasmon vibrations and this is responsible for the striking yellow brown color of silver nanoparticles [17].When enzyme filtrate was challenged with silver nitrate (1mM), it started to change its color from yellow to reddish brown (Fig. 1) indicating the formation of silver nanoparticles with the reduction of silver ions.

Figure 1
Silver nanoparticles production at 0-96 hrs culture filtrates

The characteristic surface plasmon absorption band was observed at 410nm after 24hours. Excitation spectra of silver nanoparticles synthesized from $AgNO_3$ was also observed and is presented (Fig. 2).

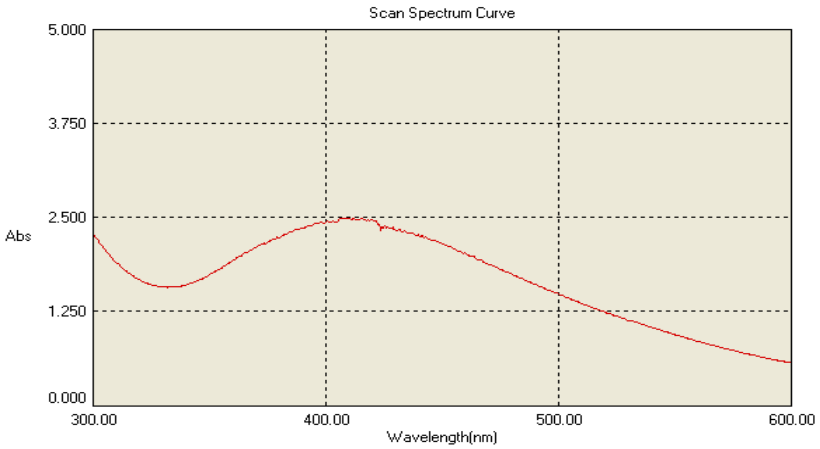

Figure 2
UV-Vis spectrum of Ag nanoparticles

3.2. IR spectroscopic analysis

The absorption bands centered at 1073, 1233, 1313, 1385, 1610, 2161, 2928 and 3290 were observed. The FT-IR measurement was utilized to study the presence of a protein molecule in the solution (Fig.3). IR measurement was carriedout to identify the potential biomolecule in enzyme filtrate responsible for the reduction of silver ions and also capping agent responsible for the stability of the bioreduced silver nanoparticles [16]. The FT-IR spectra in 1400-1700cm^{-1} region provides information about the presence of "C=O" and "N=H" groups. The peaks in the region between 3290 to 2161 were assigned to O-H stretching of alcohols and phenol compounds and aldehyde -C-H- stretching of alkanes. The peaks in the region 1610 and 1385 to 1073corresponds to N-H of primary and secondary amides and –C-N- stretching vibrations of amines or –C-O- stretching of alcohols, ethers, carboxylic acids and anhydrides. Our results correlate with Senapati *et al*, Jeeven *et al* and Vijayaraj *et al* [6, 18, 19]. IR analysis reveals the dual function of biological molecule responsible for the reduction and stabilization of AgNps in the aqueous medium [18].

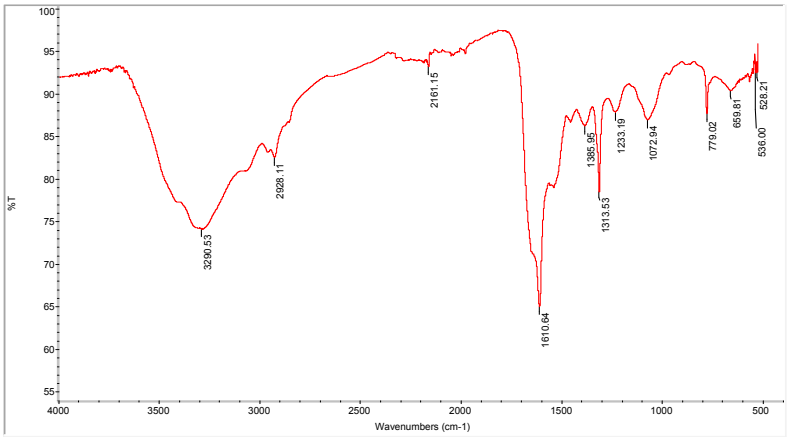

Figure 3
Infra Red Spectroscopy (FT-IR)spectrum of Ag nanoparticles

3.3. X-ray diffraction (XRD) analysis

To study the crystalline nature of AgNps produced by *A. flavus,* the XRD analysis was undertaken. The XRD pattern of the silver nitrate treated sample corresponds to that of silver nanoparticles (Fig. 4). The XRD pattern shows peaks in the whole spectrum of 2Θ values ranging from 0 to 80. A comparison of our XRD spectrum with the standard confirmed that the silver nanoparticles were in the form of nano crystals, as evidenced by the peak at 2 Θ value of 38 and integrated intensity value of (111) for silver. Our results correlate with Narasimha *et al* [20] and Prema *et al* [14].

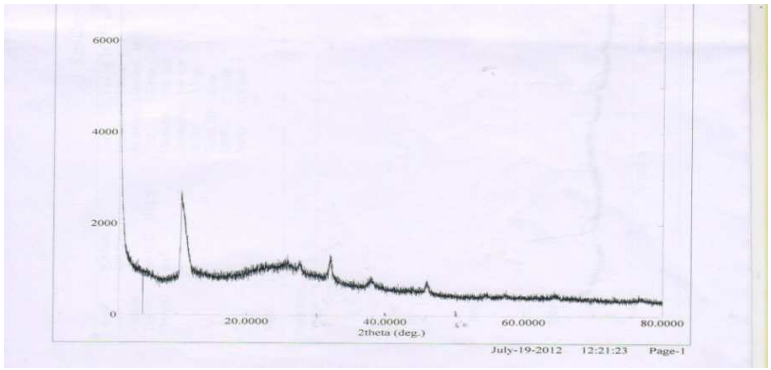

Figure 4
X-ray diffraction pattern of synthesized Ag nanoparticles

3.4. Antimicrobial Studies

Antimicrobial activity of biosynthesized silver nanoparticles was studied against the multidrug-resistant bacteria using standard zone of inhibition and are depicted in Fig-5 and Table-1. Wells were loaded with 20µl, 40µl, 60µl and 80µl of AgNps. Of the three MDR isolates *E.coli* showed maximum zone of inhibition of 16mm (80µl) while minimum zone of inhibition was 10mm (20µl). Next was *K. pneumonia* with maximum zone of inhibition of 15mm (80µl) and the minimum was 12mm (20µl), similarly *P.aeruginosa* showed maximum zone of inhibition of 15 mm (80µl) and the minimum was 10mm (20µl).

Figure 5
Inhibition zones at (20µL, 40µL, 60µL and 80µL) With P. aeruginosa, E. coli and K pneumonia

Table 1
Zones of inhibition of MDR strains

SL No	MDR strains	Zone of inhibition (mm)			
		20 µl	40 µl	60 µl	80 µl
1	*Ps.aeruginosa*	10	13	14	15
2	*E. coli*	12	13	14	16
3	*K. pneumonia*	12	13	14	15

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 is a serious concern. High prevalence of MDR infections decreases effectiveness of current treatments causing thousands of death [23]. Due to increased drug resistance, there is an urge for an alternative therapy, which has taken a turn towards silver nanoparticles. Recent, works revealed that the biosynthesized AgNps showed promising activity independently and also in combination with antibiotics [8, 21]. Similar type of work was also presented by Humberto *et al* [22] where they showed the excellent antibacterial activity of AgNps against multidrug-resistant *P. aeruginosa, E. coli, Streptococcus* sp. and *S. pyogens.*

CONCLUSION

Continuous increase in resistance to drug/antibiotics in human pathogens leads to the re-emergence of MDR pathogens. Infections caused by such pathogens require a multiple treatment, containing broad-spectrum antibiotics. In fact, these treatments are less effective, more toxic and also expensive. Nanotechnology provides a good platform to overcome the problem of resistance, with the help of the silver nanoparticles. Since ancient time, antimicrobial efficacy of silver was reported in Ayurveda and Homeopathy. The bactericidal potential can be increased by manipulating the size at nonolevel, 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 *P. aeruginosa, E. coli* and *K. pneumonia*.

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