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FUNGAL STRAIN GUIDED BOTTOM-UP SYNTHESIS OF SILVER NANOPARTICLES AND ELUCIDATION OF ITS ANTI-BACTERIAL ACTIVITY

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ABSTRACT

Green synthesis of silver nanoparticles (AgNPs) were accomplished using biological source of fungal strain Rhizopus oryzae PR7. UV-Vis spectroscopy measurement of prepared AgNPs revealed a characteristic peak at ~420 nm. FT-IR spectroscopy indicated an inverse peak of ~330 cm^{-1}, 1450 cm^{-1}. The effect of enzyme concentration over the relative production of AgNPs has been also studied. The rate kinetics for the production of AgNPs showed that maximum conversion to its nano form was achieved within 60 mins. Anti-bacterial studies of prepared AgNPs performed over various Gram positive and Gram-negative target microorganisms revealed the effectiveness of Nano silver particles as antibacterial agents.

KEY WORDS

AgNP; Antibacterial; FT-IR, Rhizopus oryzae.

INTRODUCTION

Nanoparticles are the materials in the range of 1-100 nm scale [1]. Silver nanoparticles are nano-meter scaled materials produced by different chemical and green mediated routes [2]. It has been reported earlier that silver nanoparticles of different shapes have been synthesized employing chemical route has elicited potential anti-microbial activities [3-5]. Biological methods of nanoparticles synthesis using microorganism [6–8], enzyme [9], and plant or plant extract [10] have been suggested as possible eco-friendly alternatives to chemical and physical methods.

Fungi are easy microorganisms to manipulate as they grow in mycelial form; they are more resistant facing adverse conditions and provide a cost-effective large-scale production [11]. For these reasons, fungi appear to be interesting microorganisms for the green synthesis of silver nanoparticles. Green route mediated silver nanoparticles are well known for their antibacterial activity against different bacterial species due to their intrinsic toxicity against the organism [12-15].

In this study, we used culture broth of *Rhizopus oryzae* and compared their synthesis of silver nanoparticles by monitoring the conversion using UV-visible spectroscopy. We also investigated the effects of reaction conditions such as reaction time, enzyme concentration, $AgNO_3$ concentration etc. of the silver nanoparticles and also the antibacterial activity of synthesized silver nano particle.

MATERIALS AND METHODS

Microorganism: *Rhizopus oryzae,* PR7 MTCC 9642 was isolated from the decaying vegetation enriched soil of India. The strain was identified by and deposited to Microbial Type Culture Collection, Chandigarh, India.

Cultivation of microorganism: The strain was cultivated in 100ml Erlenmeyer flasks each containing 20mL dextrose agar medium composed of (gL^{-1}): peptone 0.9;

$(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4 \cdot H_2O$ 0.1 and dextrose 0.5 at pH 6 and temperature 37◦C for 48 hrs in static condition.

Synthesis of silver nanoparticles

In vitro synthesis: Culture broth was centrifuged and filtered with 0.22µm syringe filter. The filtrate (1-2 ml) was added with aqueous 1 mM $AgNO_3$ solution (10 ml) in an Erlenmeyer flask and incubated at room temperature. As a result, a brown-yellow solution was formed, indicating the formation of silver nanoparticles. It showed that aqueous silver ions could be reduced by aqueous extract of plant parts to generate extremely stable silver nanoparticles in water.

In vivo synthesis: The mycelial mat formed after 48 hrs of growth was immersed in 1 mM $AgNO_3$ solution and kept for 12 hrs.

Characterization of silver nanoparticle

UV-Visible spectra analysis: The formation of AgNPs was scanned at a range of 300-500 nm using a UV-Vis spectrophotometer (Shimadzu, Japan) taking 1mM silver nitrate solution as blank.

FT-IR analysis: Thin properly pressed sample disc were placed in Fourier Transform Infra-Red (FTIR) for the analysis of the nanoparticles. FT-IR spectrum in the range 4500–500 cm^{-1} at a resolution of $4cm^{-1}$ was measured in a Shimadzu made Fourier Transform Infrared Spectrophotometer (IRPrestige-21).

SEM analysis: The structure and average size of the produced silver nanoparticles, were analysed by Scanning Electron Microscopy. For SEM, paraformaldehyde–glutaraldehyde fixed, and totally dehydrated specimens were sputter coated with gold palladium under vacuum and observed and photographed in a scanning electron microscope (FEI Quanta-200 MK 2). Both the fugal mycelia and the silver nanoparticles were studied.

Determination of the rate kinetics: The effect of time on the production of nano silver particle by the reductase enzyme present in the fungal culture broth was determined by treating the culture broth with silver nitrate solution for various time period (20-120 min).

Determination of the role of enzyme concentration: the effect of enzyme concentration was studied by allowing the conversion of particulate silver into its nano form by various concentration (0.2-2.5 ml) of reductase enzyme presumed to be present in the fungal culture broth.

Determination of antibacterial activities: The bacterial strains, namely *Bacillus subtilis*, *Klebsiella variicola, Staphylococcus aureus and Pseudomonas aeruginosa*, *Escherichia coli* E111, were grown separately on spread plate containing basal medium (BM) composed of (g/l): peptone 0.9; $(NH_4)_2HPO_4$ 0.1; KCl 0.1; $MgSO_4$, $7H_2O$ 0.1 and glucose 0.5 (pH 7.0), incubated at 30ºC for 42 hours. Each of the indicator bacterial strain was grown separately. Each petri-plate was divided into two halves and at each half, one well was prepared through scooping out of the agar from the plate. To observe the effects of the synthesized nanoparticles on the indicator bacteria, nano silver particle suspension synthesized from a natural source (50 µl) was poured into the "experimental" well, and 50 µl of culture filtrate was pipette into the control wells. The same method was applied and repeated for all the four bacterial agar plates. The petri-plates were incubated for 24 hours [13]. The presence of any transparent colony or the zone of inhibition or 'halo' around the experimental well denoted bacterial lysis. The halo diameter and well diameter was measured and the ratio between the two indicates the level of antibacterial activity. All the experiments were done thrice and are statistically significant.

RESULTS AND DISCUSSION

Synthesis of silver nano particles: Reduction of silver ion into silver particles by various biomolecules present in the fungal culture extract could be followed by a striking colour change from almost colourless to dark brown as shown in Fig 1, due to excitation of surface Plasmon resonance vibrations in the particles [14].

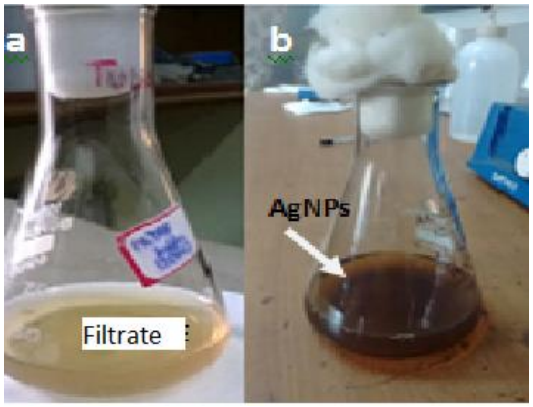


Fig. 1 Change in colour after the synthesis of silver nano paticle (a)fungal culture filtrate and (b)AgNPs (dark brown, shown by arrow)

UV-Vis spectroscopy: Characterization of biosynthesized AgNPs was accomplished using UV-Vis spectroscopy showed a strong broad peak at ~425 nm (Fig 2).

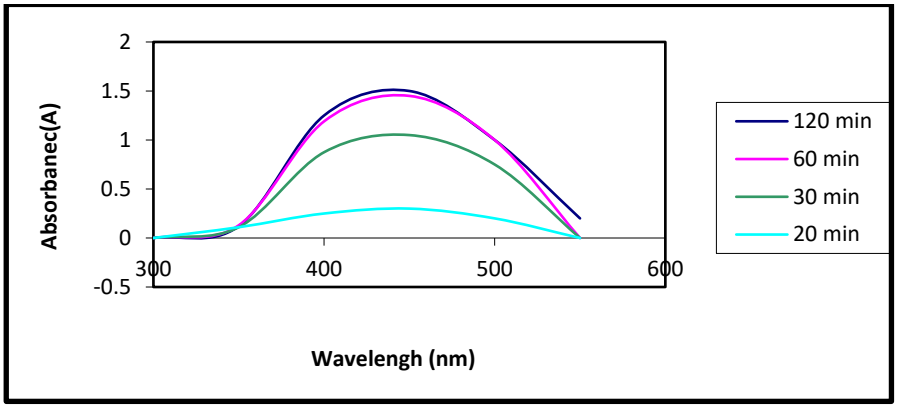


Fig.2 UV–Vis spectral analysis of Ag NPs using culture extract of *Rhizopus oryzae*

Photomicrographic Study: Scanning electron micrograph revealed the mycelial structure of the working strain after synthesis of silver nano particles (Fig 3) and the synthesized particles with a size ranging from 20-70 nm (Fig 4).

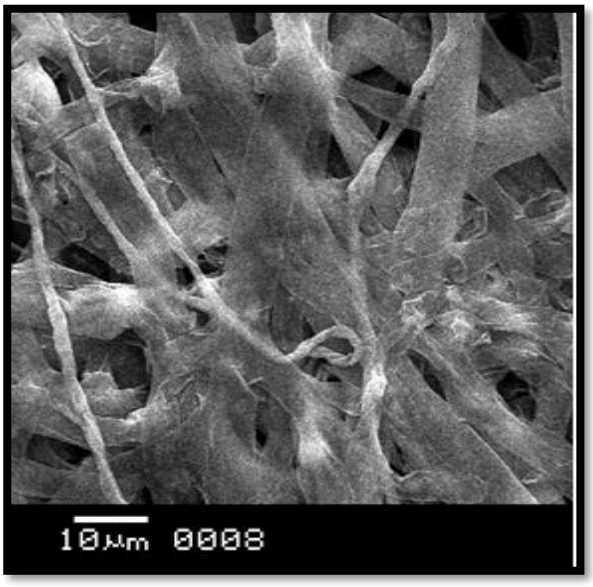


Fig 3. SEM of the mycelial structure of *Rhizopus oryzae* after synthesis of silver nano particles.

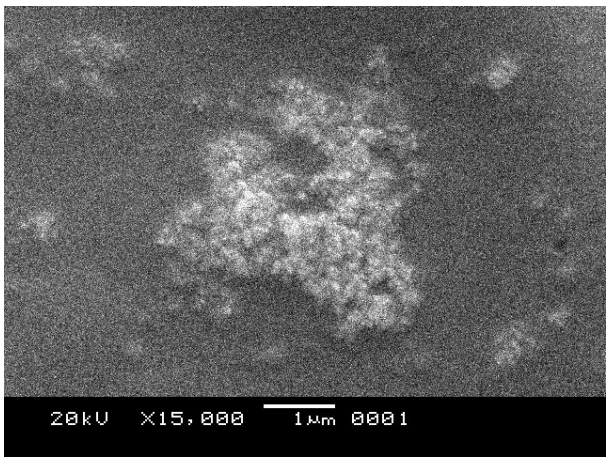


Fig 4. SEM of the nano silver particles synthesised by the enzyme of *Rhizopus oryzae*.

FTIR spectroscopy: The FT-IR spectrum obtained for silver nanoparticles obtained by the reduction of $AgNO_3$ with fungal culture extract indicated strong transmission peaks at ~3300 cm^{-1} (Fig. 5) which was due to –OH vibrations and 2300 cm^{-1} and also due to C=N stretching. The other peaks namely at 1650 cm^{-1} and 1400 cm^{-1} were due to N=C stretching and C=O vibrations respectively. Peaks of 1200 cm^{-1} and 800 cm^{-1} were mainly due to propionates and $(CH_3)_2$ **[7]** groups respectively. It was found that the reaction time and amount of culture filtrate played a significant role in strong absorption peaks at 3,309 and 3,421 cm^{-1} resulting from stretching of the –NH band of amino groups or is indicative of bonded –OH hydroxyl group. The absorption peaks at about 2,897 cm−1 could be assigned to stretching vibrations of $-CH_2$ and CH_3 functional groups. The peaks at 1,712 and 1,581 cm−1 indicate the fingerprint region of CO, C-O, and O-H groups. The intense band at 1,068 cm−1 could be assigned to the C-N stretching vibrations of aliphatic amines. The FT-IR spectrum also showed bands at 1,581 and 1,446 cm^{-1} identified as amide I and amide II, which appeared due to carbonyl (C=O) and amine (−NH) stretching vibrations in the amide linkages of the proteins, respectively. The absorption band at 1,446 cm−1 **[7],** could be attributed due to the methylene scissoring vibrations from the proteins.

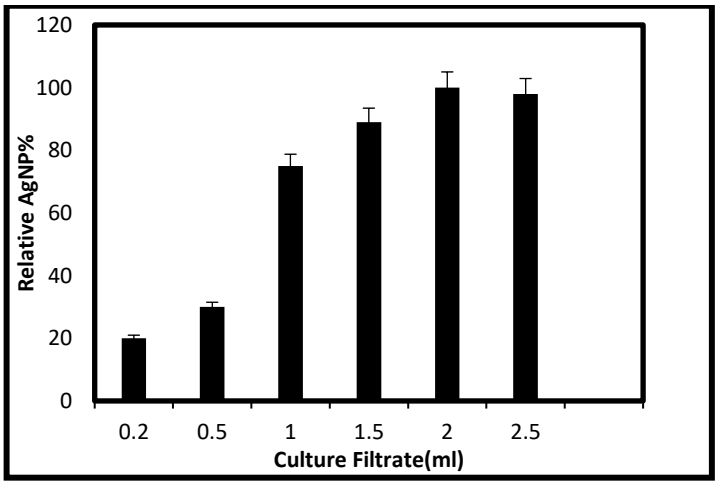


Fig 5. Effect of concentration of fungal culture filtrate (reductase) on the production of of silver nano particles.

Effect of time and enzyme concentration on nano silver synthesis: Maximum conversion of particulate silver into the nano form was achieved within 60 min, after which no such prominent change could be recorded (Fig 2). It was found that 2 ml of culture broth was found to be most suitable for the production of nano silver particle under optimized condition, above which amount of AgNP formation became reduced possibly due to enzyme saturation (Fig 6).

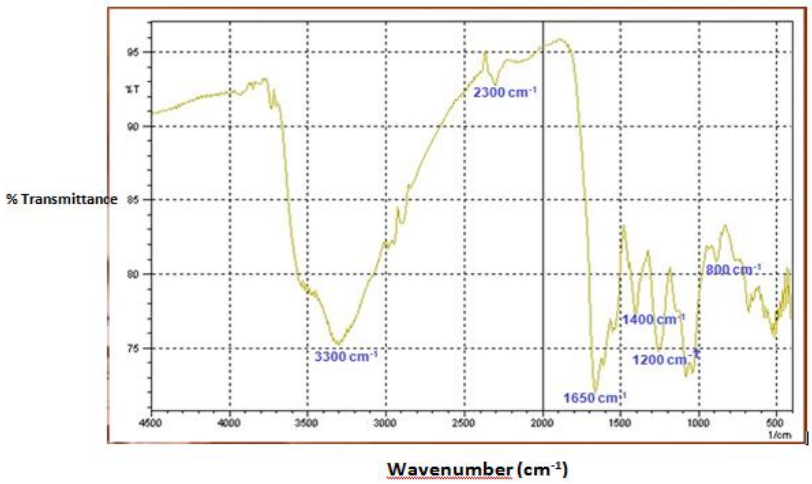
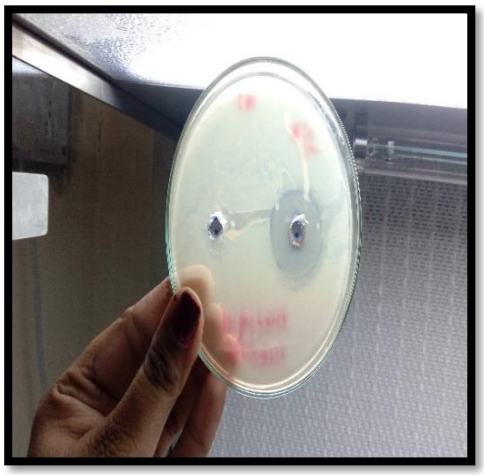
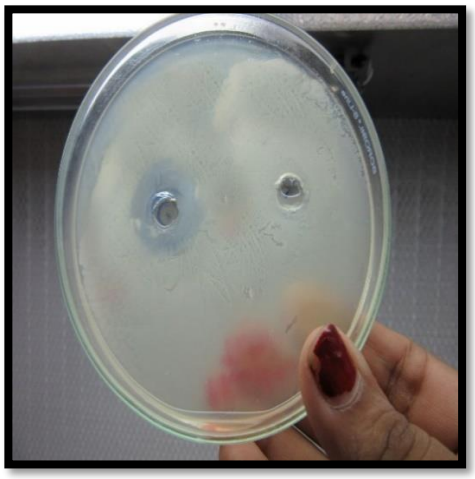


Fig.6 FT-IR spectroscopy of Ag-NPs synthesised.

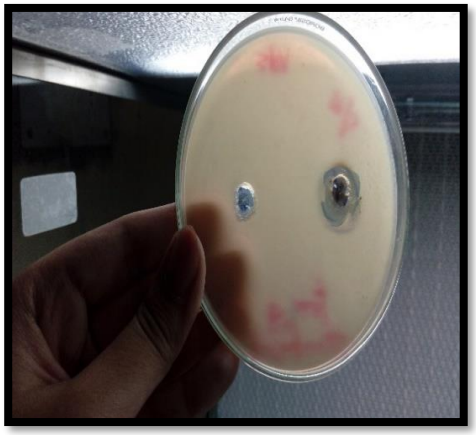
Anti-bacterial activity of AgNPs: Well distinct Zone of Inhibition could be detected around all the Gram positive and Gram negative bacterial strains tested (Fig. 7), and the effectiveness was more prominent against Gram positive ones (Table 1).



Staphylococcus aureus



Bacillus subtilis



Escherichia coli

Klebsiella variicola

Pseudomonas aeruginosa

Fig.6. Antibacterial activity of silver nano silver particles against target bacterial strains

Table 1. Diameter of Zone of Inhibition for AgNP treated bacteria using Well Diffusion method

Bacteria	Halo diameter (cm)	Well diameter (cm)	Anti-microbial activity (halo: well diameter)
Bacillus subtilis	1.3	0.6	2.16
Klebsiella variicola	0.9	0.6	1.50
Staphylococcus aureus	2.5	0.6	4.16
Pseudomonas aeruginosa	1.1	0.6	1.83
E.coli	1.05	0.6	1.75

It could be clearly shown that due to the presence of anisotropy in the native structure of synthesised AgNPs, sharp edges were causing a charge transfer reaction at the AgNP- bacterial interface [**10**]. Such charge transfer interaction led to the penetration of the AgNPs inside the bacterial species resulting into the production of free radicals like O-, O^{2-} and $O2^{2-}$ [12] Such free radicals upon conjunction with the bacterial DNA caused damage and that led to inhibition in the basic transcription and translational machinery causing production of adequate protein and metabolite required for the bacterial growth and development, which in turn caused their ultimate destruction.

CONCLUSION

Although the production of nano silver particle is an expensive procedure, employment of natural resources, particularly *Rhizopus oryzae* was proved to be a much cost effective one. Moreover, the production of nanosilver particles utilising fungal reductase was longer lasting one. The antibacterial efficacy of the nano silver particles could aid in designing different therapeutic silver-based agents which could pave the way in exploring the new nano-therapeutic arena.

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