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FACILE SYNTHESIS OF SILVER NANOPARTICLES USING *STREPTOMYCES* SP.VSMGT1014 AND THEIR ANTIMICROBIAL EFFICIENCY

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Microbial synthesis of nanoparticles has a prospective to develop simple, cost-effective and eco-friendly method for production of technologically important materials. The present investigation demonstrates the role of rice rhizosphere *Streptomyces* sp. mediated silver nanoparticles in bio control of various plant fungal pathogens and nosocomial infection causing bacteria by *in vitro* antagonistic activity and agar well diffusion method respectively. Isolated actinomycetes were screened for antagonism against plant fungal pathogens and the potent strain was further screened against human pathogenic bacteria. The potential strain, VSMGT1014 was identified by microscopic, physiological, biochemical characteristics and used for synthesis of silver nanoparticles. The nanoparticles were characterised by UV-Vis Absorption spectroscopy, AFM, FTIR and X-ray diffraction. The AgNPs were determined to be spherical through the observation under AFM. X-ray diffraction patterns displayed typical peaks from 24°-85° at position 2 Θ for crystalline silver. It was found that the synthesised nanoparticles showed MIC range of 10-50µg/mL against selected plant fungal pathogens. Moreover these nanoparticles showed significant level of inhibition as MIC range from 10- 25 µg/mL against clinically important pathogenic bacteria. Through this investigation, we concluding with *Streptomyces* sp. VSMGT1014 mediated silver nanoparticles would be a potential nanomedicine against various pathogenic organisms.

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1. Introduction

Nanotechnology deals with the synthesis, characterization, exploration and application of nanosized (1-100 nm) materials for the development of science. The synthesis of silver nanoparticles extensively studied by using chemical and physical methods, but the development of reliable technology to produce them is an important aspect of nanotechnology. Biological synthesis provides a wide range of environmentally acceptable methodology, low cost production and minimum time. However for the past few years, various rapid chemical methods have been replaced by green synthesis because of avoiding toxicity of the process and high quality. The properties of many conventional materials change, when created into nanoparticles. Nanoparticles

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have a greater surface area per weight than larger particles which causes them to be more reactive to certain other molecules.

Nanoparticles are used, or being evaluated for their use in many fields. Nanoparticles, because of their small size, have distinct properties compared to the bulk form of the same material, thus offering many new developments in the fields of biosensors, biomedicine, and bio nanotechnology. Current research in inorganic nanomaterials with good antimicrobial properties has opened a new era in pharmaceutical and medical industries. Silver nanoparticles showed very good bactericidal activity against gram positive as well as gram negative bacteria including multidrug resistant strains [1] and also it was found to be in biological studies [2- 3]. Microbial source to produce the silver nanoparticles shows the great interest towards the precipitation of nanoparticles due to its metabolic activity. Microorganisms have been explored as potential bio factories for synthesis of metal nanoparticles and nanocomposites [4]. The metal microbe interaction has made the material scientist aware of the immense potential of the microorganisms as ecofriendly nanofactories [5-7]. Extracellular synthesis of nanoparticles using cell filtrate could be beneficial over intracellular synthesis and bacteria being extremely good candidates for extracellular process and also ecofriendly. Nano-encapsulated agrochemicals are designed in such a way that they possess all necessary properties such as effective concentration, time controlled release in response to certain stimuli, enhanced targeted activity and less ecotoxicity with safe and easy mode of delivery [8- 12].

Actinobacteria are gram-positive bacteria, which comprise a group of branching unicellular microorganisms. They are often regarded as the prokaryotic equivalent of fungi and have been found to produce many commercially vital bioactive compounds such as antitumor agents in addition to enzymes of industrial interest. A number of these isolates were capable of suppressing the fungal pathogens such as *Rhizoctonia solani* both *in vitro* [13] and in plants indicating the potential of the actinobacteria to be used as biocontrol agents. Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Modern agriculture apart from improving the overall production and productivity has also caused destruction to the environment. The use of chemical fertilizers has been necessitated due to cultivation of high yielding varieties. Biological methods offer an excellent alternate strategy for effective control of various diseases and plant growth promotional activity. Microorganisms as biocontrol agents have high potential to control plant pathogens and no effect on the environment or other non target organisms. One of the potential applications in which silver can be utilized is in management of plant diseases. The present investigation focuses on *Streptomyces* sp. mediated silver nanoparticles (AgNPs) involved in bio controlling plant fungal pathogens and nosocomial infection causing human pathogens.

2. Experimental details

2.1 Rhizosphere soil collection

Soil samples were collected from rhizosphere region of rice plants in the agriculture fields in Madurai district (9° 58’ N, 78° 10’ E) of Tamil Nadu, India. The collected samples were transported to laboratory for isolation.

2.2 Isolation of actinomycetes

10 g of rice rhizosphere soil samples were serially diluted and plated (0.1mL) on International *Streptomyces* Project-2(ISP-2) [14] medium supplemented with cyclohexamide and nystatin (50 μg/mL) in order to minimize bacterial and fungal contamination. The plates were incubated for one week at 30°C. Colonies of actinomycetes on the agar plates were picked on the basis of their morphological characteristics such as coloration of aerial mycelium (on the surface of agar), substrate mycelium (underside of agar) diffusible pigment and purified on ISP- 2 agar.

2.3 Characterization of actinomycetes

The potent actinomycetes were characterized by morphological and biochemical methods. The microscopic characterization was done by cover slip culture method [15]. The mycelium

structure and arrangements of conidiospores on the mycelium were observed through Atomic force microscope. Different physiological tests suggested in Bergey's Manual of Determinative Bacteriology [16] were carried out to identify the isolate.

2.4 Screening of actinomycetes

All 40 actinomycete strains were screened by dual plate technique [17- 18] against *Rhizoctonia solani*, the sheath blight pathogen of rice. A mycelial plug of 5.0 mm diameter from a 3 day old fungus was cut using sterile cork borer and transferred to the actinomycete pregrown PDA plate besides fungal control. The radial fungal growth in the direction of the antagonist in both the control and the dual culture plates was measured at 2 days after inoculation of *R. solani*. These strains were again screened for their broad spectrum of antagonistic activity against *Macrophomina phaseolina, Fusarium udum and Alternaria alternata.*

2.5 Biosynthesis and characterisation of AgNPs

50 mL of the culture filtrate was mixed with 50 mL of 1 mM silver nitrate ($AgNO_3$) solution and incubated at 28˚C in orbital shaker at 150 rpm under dark condition along with control (Silver nitrate solution alone) for 72- 96 hrs. Also cell free supernatant was challenged with 1 mM silver nitrate solution. For UV – Vis spectral analysis (Shimadzu 1800), 1 mL sample were withdrawn at 24 h intervals and the absorbance was measured at 420 nm. FT-IR analyses were performed by using a Perkin-Elmer spectrometer, equipped with a Globar source and a DTGS (deuterated tryglycine sulphate) detector. The spectrum were scanned from 400 to 4000 cm^{-1} range and plotted as intensity versus wave number. The powdered samples of silver nanoparticles (AgNP's) were investigated with X-Ray diffraction (XRD) method. X-ray diffraction analysis was performed by using a Philips X'Pert Pro X-Ray diffractometer with the copper anode (40 KV and 30 mA) and scanning from 24°-85° at position 2 Θ. For atomic force microscopic studies of silver nanoparticles were done by placing a drop of the colloidal solution of silver nanoparticles on a cover slip and allowed to dry overnight at room temperature.

2.6 Antibacterial activity

The antibacterial activity of *Streptomyces* sp. VSMGT1014 was determined by using perpendicular streak method. Single streak of the actinomycetes was made on the surface of the nutrient agar. The test organisms used were *E.coli, Streptococcus viridans, Streptococcus* sp., *Staphylococcus aureus, Pseudomonas* sp, *Klebsiella* sp., *Bacillus* sp., *Salmonella* sp., *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, obtained from Department of Microbial Technology, Madurai Kamaraj University, Madurai, Tamil Nadu. After observing good growth of the actinomycetes on the petri plates, the test organisms were streaked at right angles to the original streak of actinomycetes and plates were incubated at 30°C for 24- 48 hours. After incubation, length of streak growth was measured. Decrease in length of growth than the inoculate streak indicate inhibition of growth.

2.7 Antifungal activity

The antifungal activity of the synthesised silver nanoparticles was done by agar well diffusion method against plant fungal pathogens. Briefly, the various concentrations of the AgNPs (5- 60 µg/mL) were prepared and introduced in PDA wells. Carbendazim at the same concentrations as above were kept as positive control. A pregrown agar plug of fungi was kept at centre for the test and PDA plate with pathogen alone kept as control. After incubation period the minimal inhibitory concentration were observed for all fungi.

2.8 Minimal inhibitory concentration (MIC)

The AgNP's were tested for antibacterial activity by agar well-diffusion method [19] against human pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia and Pseudomonas aeruginosa*. The pure cultures of bacteria (both Gram positive and Gram Negative) were grown in Nutrient broth for 24hours. AgNP's was dissolved in 10% DMSO to get different concentrations (5- 50 µg/mL). Each well received 50 µL solution of each compound. Similar concentrations of commercially available tetracycline used as positive control.

The plates were incubated for 24 hours at 37°C. After incubation, the different levels of zone of inhibition (ZOI) of bacteria were measured using a meter ruler and the MIC [20] for each organism was recorded compared to positive control.

3. Results

3.1 Isolation and screening of actinomycetes

A total of 40 actinomycetes was isolated from rice rhizosphere soils and screened for antifungal activity against *R.solani*. Among 40, 14 isolates exhibited antagonistic activity and produced inhibition zones ranging from 12 mm to 30mm (Table 1). Among 14, the isolate designated VSMGT1014 had shown highest antifungal activity with broad spectral antagonistic activity against phytopathogens such as *A. alternata, F. udum* and *M. phaseolina*. The selected strain VSMGT1014 was identified as *Streptomyces* sp. based on the physiological, morphological, biochemical tests [13] and atomic force microscopic studies (Fig. 1). The antagonistic isolates had given varied zone of inhibition levels against human pathogenic bacteria.

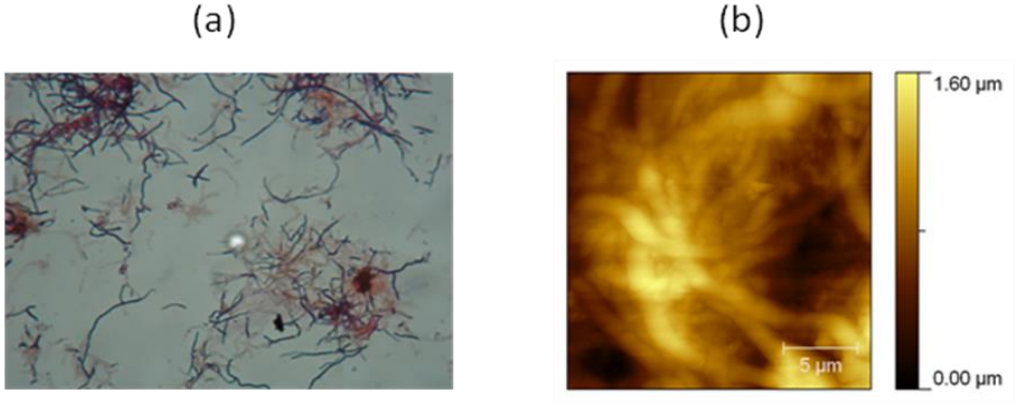

Fig.1 Streptomyces sp. VSMGT14 a) Light microscope, b) Atomic force microscope

Table 1 Antagonistic activity actinomycetes against plant fungal pathogens

Plant pathogens	No. of Antagonistic isolates	Zone of inhibition (cm)
Rhizoctonia solani	14	0.5- 3.0
Macrophomina phaseolina	16	0.4- 2.5
Fusarium udum	19	0.2-1.2
Alternaria alternata	20	0.4- 2.4

3.2 Synthesis of Silver nanoparticles

The solution- turned brown after incubation of the cell free filtrate with silver nitrate solution for both organisms, indicating the synthesis of silver nanoparticles. Thus *Streptomyces* sp. VSMGT1014 incubated in silver nitrate solution yielded silver nanoparticles. These cells were characterised for confirmation of silver nanoparticles.

3.3 Characterization of silver nanoparticles

The UV spectrum of the *Streptomyces* sp.VSMGT1014 mediated AgNPs showed λ max at 420 nm. The absorbance (Fig. 2) at 420 nm increased with incubation time. The highest fraction of AgNP's where formed at 72 hrs of synthesis. There is a loss of Ag reduction observed after 72 hrs, may be related to a loss of nitrate reductase activity [21]. FTIR spectrum of *Streptomyces*

*sp.*VSMGT1014 AgNP's exhibited absorption at 2923.04 cm^{-1} which indicate alkane C-H stretching, and the absorption at 1644.34 cm^{-1} indicating a double bond of amide. The FTIR spectrum (Fig. 3) exhibited absorption at 3442.81 cm^{-1}, which indicate alcoholic hydroxyl group stretching. X-ray diffraction patterns displayed typical peaks from 24°-85° at position 2 Ɵ for crystalline silver (Fig. 4).The AFM images (Fig. 5) of silver nanoparticles were more uniform in size, mostly spherical in shape but aggregated to a greater extent. The AFM results indicate that the process of formation of silver nanoparticles takes place on the surface of the cells.

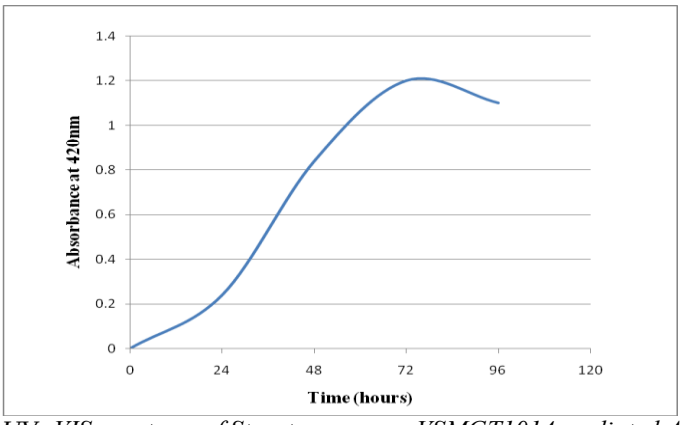

Fig. 2 UV- VIS spectrum of Streptomyces sp. VSMGT1014 mediated AgNP's

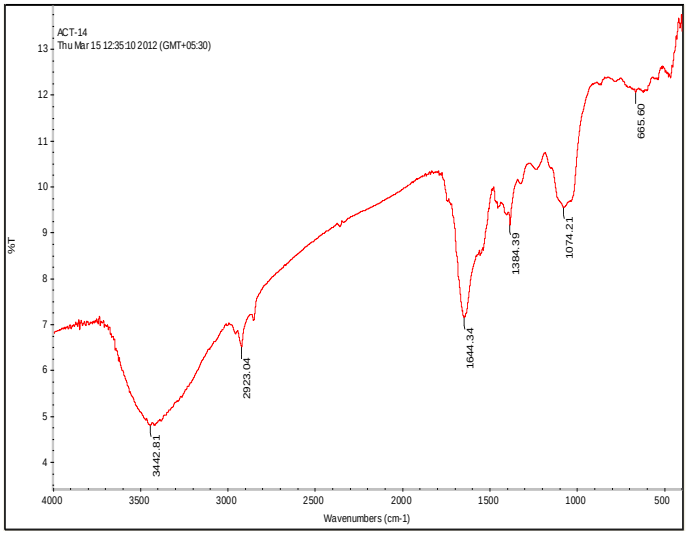

Fig. 3 FTIR spectrum of Streptomyces sp. VSMGT1014 mediated AgNP's

3.4 Antibacterial activity

It was found that the *Streptomyces* sp. VSMGT1014 mediated AgNPs given maximum zone of inhibition (Fig. 6) against *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa.* It has no inhibitory action with other tested human pathogens. The selected isolate might produce more than one antibacterial metabolites that made them effective inhibitor to both Gram positive and Gram negative bacteria. The lowest concentration of silver nanoparticles at which no growth of microorganisms was observed upon visual observation after incubating at 37°C for 18 h is considered the minimal inhibitory concentration (MIC) value.

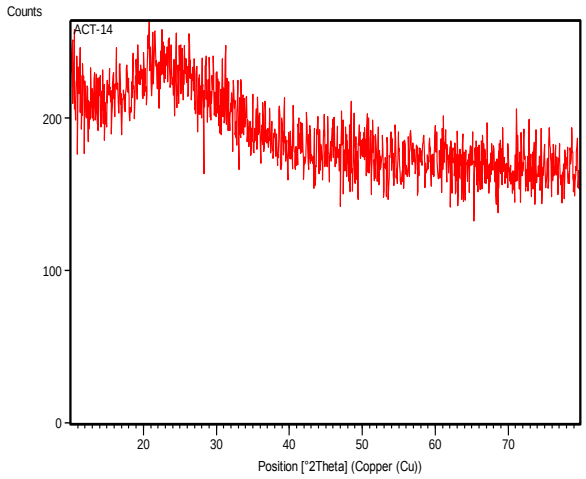

Fig. 4 X-ray diffraction pattern of synthesised silver nanoparticles

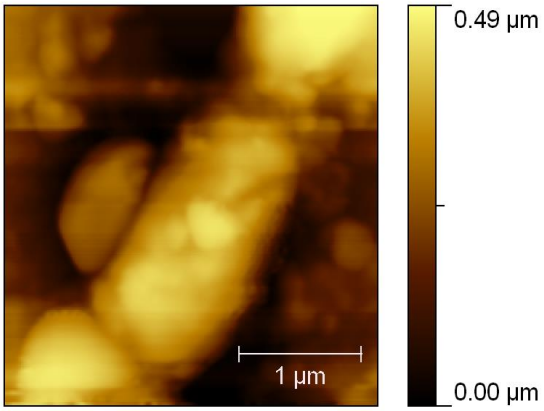

Fig. 5 Atomic Force micrographs of Streptomyces sp. VSMGT1014 mediated AgNPs

The MIC values of silver nanoparticles against human pathogenic bacteria were determined and tabulated (Table 3). The results were compared with commercial antibiotic tetracycline as positive control and universal solvent water as negative control.

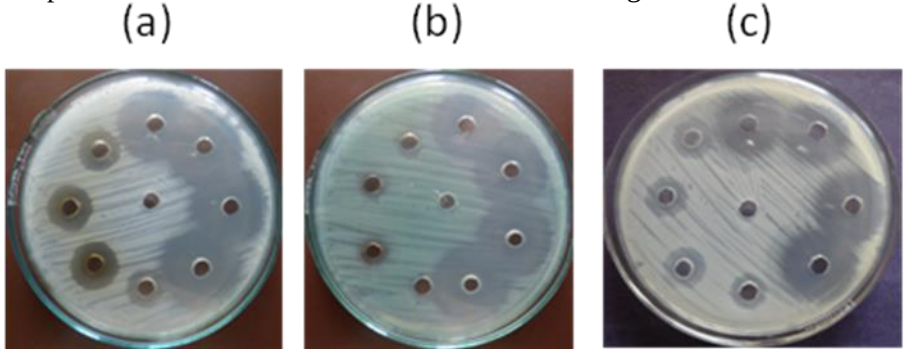

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Fig. 6 Antibacterial activity of Streptomyces sp. VSMGT1014 mediated AgNPs against (a) Pseudomonas aeruginosa, (b) Klebsiella pneumoniae and (c) Staphylococcus aureus

3.5 Antifungal activity

The MIC values of synthesised silver nanoparticles against plant fungal pathogens were obtained and tabulated (Table 3). The results were compared with commercial fungicide carbendazim as positive control and universal solvent, water as negative control.

Table 2 MIC value of silver nanoparticles against human pathogenic bacteria

Human bacterial pathogens	**MIC of Ag NP (µg/ml)**
Klebsiella pneumoniae	25
Staphylococcus aureus	20
Escherichia coli	10
Pseudomonas aeruginosa	15
Plant Fungal pathogens	**MIC of Ag NP (µg/ml)**
Rhizoctonia solani	10
Macrophomina phaseolina	30
Fusarium udum	50
Alternaria alternata	40

4. Discussion

Biological application of nanoparticles is very much interesting and increasingly recognized for their utility especially in bio-nanomedicine [22] and in controlling infectious diseases [23]. The brown color is the result of excitation of surface plasmon vibration in the metal nanoparticles and is typical of the silver nanoparticles [24]. The role of NADH (Nicotinamide adenine dinucleotide) dependent nitrate reductase from fungi in the biosynthesis of silver nanoparticles was recently reported [25]. However, different NADH-dependent reductase may be produced also by *Streptomyces* sp. [26- 27] explained the successful use of silver nanoparticles Ag NPs in diverse medical streams as antifungal and antibacterial agents has led to their applications in controlling phytopathogens.

The use of a supernatant formed from the autolysed biomass permitted the formation of nanoparticles in the absence of any mycelial materials, resulting in a clear solution and avoiding complications due to nanoparticles / cell interactions. It has been suggested that DNA loses its replication ability once the bacteria are treated with silver ions [28].

It is noteworthy that the metabolites or other enzymes produced by the strain which causing the reduction of $AgNO_3$ to Ag metal nanoparticles is intracellular. This was confirmed by the observation that when the cell free supernatant from the microbial cultures was mixed with silver nitrate solution and incubated at 28°C for 96 h, there was no color change or silver nanoparticles. However, when the biomass was washed, autolysed and the cell-free filtrate was mixed with $AgNO_3$, the solution changed to brown indicating the formation of silver nanoparticles, confirming that the formation depends on intracellular components. Silver displays multiple modes of inhibitory action to microorganisms which can be used for controlling various plant pathogens in a relatively safer way compared to synthetic fungicides [29]. Limited research has been provided some evidence of the applicability of silver for controlling plant diseases.

Our results contrast with the results by Sadhasivam [30], who used a culture filtrate to produce Ag nanoparticles. The formation of Ag nanoparticles was confirmed by UV–VIS spectroscopy, where the maximum absorbance occurred at 420 nm, which is the wavelength reported by others for silver nanoparticles [31]. *Streptomyces* continued to show increasing production and relatively higher concentrations. The results from the UV–Vis spectrum were verified by X-ray diffraction (XRD). However, the nanoparticles were not in direct contact even within the aggregates, indicating good stabilization of the nanoparticles. The results for antifungal action by the synthesised silver nanoparticles shows the strain have significant role in disease

suppression *invitro.* With the results of antibacterial activity, it is evident that these silver nanoparticles have not only antifungal action but also antibacterial efficiency. Hence the successful use of silver nanoparticles in diverse agricultural streams as antifungal and medical streams as antibacterial agents [26- 27] as led to their applications in controlling phytopathogens.

5. Conclusion

Application of nanotechnology in the field of nanomedicine and agriculture are very much needy globally. Nanoscience leads towards the development of low cost nanotech applications for enhanced disease resistance. This investigation guarantees that AgNPs play a dual role as an effective nanomedicine and as an agrochemical in the form of silver nanoparticles for the control of nosocomial infection causing pathogens and phytofungal pathogens. Hence *Streptomyces sp.*VSMGT1014 mediated silver nanoparticles warranties to be a potential candidate in drug delivery system in both plants and humans for effective disease management.

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