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Extracellular biosynthesis of silver nanoparticles using *Bacillus* sp. GP-23 and evaluation of their antifungal activity towards *Fusarium oxysporum*

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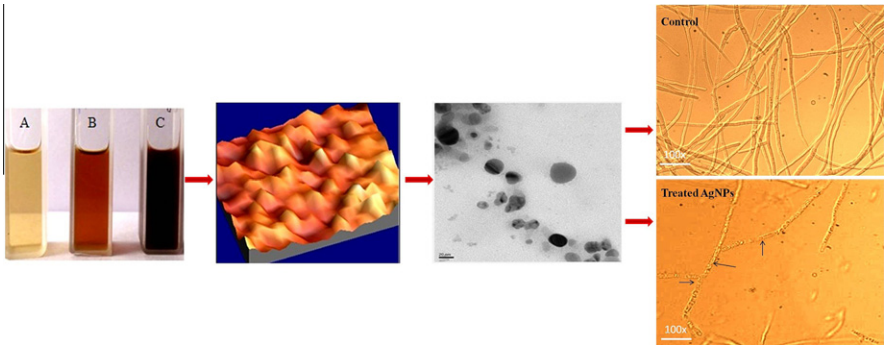
HIGHLIGHTS

- The work emphasizes on extracellular synthesis of AgNPs from *Bacillus* sp. GP-23.
- The synthesized particles were stable after five months period at 37 °C.
- A spherical shaped AgNPs was recorded by HRTEM.
- The resultant AgNPs shows excellent antifungal activity.

GRAPHICAL ABSTRACT



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ABSTRACT

In last few decades nanoparticles have attracted and emerged as a field in biomedical research due to their incredible applications. The current research was focused on extracellular synthesis of silver nanoparticles (AgNPs) using cell free culture supernatant of strain GP-23. It was found that the strain GP-23 belonged to *Bacillus* species by 16S rRNA sequence analysis. Biosynthesis of AgNPs was achieved by addition of culture supernatant with aqueous silver nitrate solution, after 24 h it turned to brown color solution with a peak at 420 nm corresponding to the Plasmon absorbance of AgNPs by UV–Vis Spectroscopy. The nanoparticles were characterized by FTIR, XRD, HRTEM, EDX and AFM. The synthesized nanoparticles were found to be spherical in shape with size in the range of 7–21 nm. It was stable in aqueous solution for five months period of storage at room temperature under dark condition. The biosynthesized AgNPs exhibited strong antifungal activity against plant pathogenic fungus, *Fusarium oxysporum* at the concentration of 8 μg ml^{-1}. The results suggest that the synthesized AgNPs act as an effective antifungal agent/fungicide.

Introduction

In the recent years, noble nanoparticles are considered important in the field of biology, medicine and electronics owing to their unique particle size and shape dependent physical, chemical and biological properties. The nanoparticles have been synthesized by using toxic chemicals and high energy physical procedures. To overcome this problem, biological materials have been used for the synthesis of various metal and oxide nanoparticles. Among the metal nanoparticles, silver nanoparticles (AgNPs) has received much attention in various fields, such as antimicrobial activity [1], therapeutics [2], bio-molecular detection [3], silver nanocoated medical devices [4] and optical receptor [5]. Hence, biogenic approach, in particular the usage of natural organisms has offered a reliable, simple, nontoxic and environmental friendly method [6–8].

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Marine soil is an extensively explored ecological niche for sources of microorganisms that are involved in various interactions. Metal-microbe interactions have important roles with fascinating applications such as bioremediation, biomineralization, bioleaching and microbial corrosion. However, recently that microorganisms have been explored as potential biofactory for synthesis of metallic nanoparticles such as cadmium, gold and silver, [9,10]. Among the microbes, use of bacteria, like in this study, is rapidly gaining importance due to its growing success, ease of handling and genetic modification [11]. Klaus et al. demonstrated that the *Pseudomonas stutzeri* AG259, isolated from a silver mine, produced silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria [12]. In recent study various bacterial strains such as *Bacillus amyloliquefaciens, Acinetobacter calcoaceticus, Escherichia coli*(S30 and S78) and *Bacillus megaterium*(S52) could effectively induce the synthesis of silver nanoparticles [13,14]. Biosynthetic methods can be categorized into intracellular and extracellular synthesis according to the place where nanoparticles are formed [15,16]. Of which, the extracellular synthesis of nanoparticles is still continually emerging in order to understand the mechanisms of synthesis, easy downstream processing and rapid scale-up processing. For these reasons, a bacterial system could prove to be a potential source for the extracellular synthesis of metal nanoparticles instead of physical and chemical procedures.

The present study, we report a newly isolated *Bacillus* sp. GP-23 from marine soil that possess extracellular synthesis of spherical shaped AgNPs and evaluate their effect on suppression of *Fusarium oxysporum*, which is one of the most important Fusarium wilt disease of tomato, tobacco, legumes, cucurbits, sweet potatoes and banana. Furthermore, the synthesized nanoparticles were characterized by UV–Vis spectrophotometer, FTIR, XRD, HRTEM and AFM.

Materials and methods

Chemicals and fungal pathogen

All the chemicals and media components were purchased from Sigma Aldrich (St. Louis, USA). The plant pathogenic fungus *F. oxysporum* was obtained from culture collection of Microbial technology laboratory, SRM University, Chennai. Double sterilized Milli-Q water was used throughout the experiments.

Isolation and identification of the bacteria

Soil samples were collected from different sites of the marine soils at Kovalam, Chennai, Tamil Nadu. The collected sample was serially diluted and plated on Luria–Bertani (LB) agar medium and the plates were incubated at 37 °C for 24 h. After the incubation period the bacterial colonies were observed and it was further sub-cultured on the same medium to obtain the pure colonies. Totally 27 strains were isolated and used for nanoparticle synthesis. Based on the rapid reduction of silver metal ions, strainGP-23 was selected and identified by 16S rRNA sequencing analysis.

Extracellular synthesis of AgNPs

In order to screen an efficient strain for the synthesis of AgNPs, all the isolated bacterial strains were freshly inoculated in an Erlenmeyer flask containing LB broth. The flasks were incubated at 37 °C for 24 h. After incubation period, the culture supernatant was obtained by centrifugation at 6,000 rpm for 10 min. Then 100 ml of cell free supernatant was transferred into 250 ml Erlenmeyer flask and the final volume concentration was adjusted to 1 mM $AgNO_3$. The cell free supernatant without addition of $AgNO_3$ was maintained as a control. Consequently, the bio reduction reaction was monitored by visual color change and UV–Vis absorbance of the reaction mixture. Based on the rapid reduction of $AgNO_3$ into AgNPs a proficient bacterial strain was selected and used for further characterization.

Characterization of silver nanoparticles

The biologically synthesized silver nanoparticles using the cell free supernatant were characterized by UV–Vis spectroscopy (Perkin Elmer preclsley, Lambda 25) instrument scanning in the range of 200–700 nm, at a resolution of 1 nm. Cell free supernatant without addition of silver nitrate was used as a control throughout the experiment. The FTIR analysis was performed with cell free culture reduced silver nanoparticles. The synthesized AgNPs samples were mixed with KBr to make a pellet and the spectrum was recorded at a resolution of 4 cm^{-1} at the range of 500–4000 cm^{-1}. The biologically synthesized silver nanoparticles were freeze dried on lyophilzer and the powdered sample was used for X-ray diffraction (XRD) analysis. The XRD analysis was performed by X'PertPro A Analytical X-ray diffractometer instrument using Cu Kα radiation (k = 1.54056 Å) in the range of 20–80° at 40 keV.

The morphological analysis of the particle was done with High resolution transmission electron microscopy (JEOL 2100) instrument accelerating voltage of 80 keV equipped with EDX. A drop of aqueous AgNPs sample was loaded on the carbon coated copper grid which was allowed to dry for an hour. The micrographic images of silver nanoparticles were observed and recorded in different range of magnifications. The morphology and size of the synthesized silver nanoparticles were further characterized by the atomic force microscopy (AFM) images. The microscopic images were recorded with silicon cantilever with force constant 0.22–0.77 N/m, tip height 10–12 nm in the contact mode.

Antifungal activity of silver nanoparticles

The antifungal activity of synthesized particles was assayed by well diffusion method against *F. oxysporum*. The 4 days grown fresh fungal culture was inoculated into the centre of the potato dextrose agar (PDA) plate. The wells 6 mm diameter was made on PDA plate using gel puncture. The well in the right side well was loaded with 8 μg ml^{-1} of AgNPs solution and the left side was loaded with same volume of cell free supernatant used as a control. Then the plate was incubated for 4 days at 30 °C and the zone of inhibition was observed around the well. Moreover, the antifungal effect of AgNPs against *F. oxysporum* was observed by a light microscope (Nikon, Tokyo, Japan). In order to determine the deformation of hyphae, fungal suspension was treated with 8 μg ml^{-1} concentration of AgNPs and the culture without treating with AgNPs (using cell free supernatant) was used as a control.

Results and discussion

Biosynthesis of silver nanoparticles

In our pilot scale screening, 27 marine bacteria were isolated and evaluated for their extracellular silver nanoparticles synthesis. Among the isolates, a bacterial strain that exhibited the rapid production of AgNPs, was designated to be strain GP-23, and was identified as a member of *Bacillus* species by 16S rRNA analysis and referred as *Bacillus* sp. GP-23 (GenBank Accession No: JX156301). Subsequently, biogenic synthesis of AgNPs was performed by using 100 ml of cell free supernatant of *Bacillus* sp. GP-23 treated with the final concentration was adjusted to 1 mM aqueous $AgNO_3$. The reaction mixture was kept in dark room to avoid photolytic