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SCREENING OF SOIL STREPTOMYCES FOR THE SYNTHESIS OF SILVER NANOPARTICLES AND ITS ANTIMICROBIAL PROPERTY AGAINST SELECTIVE PATHOGENS

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ABSTRACT

The recent exploration of nanoparticles in the various field of applications have paved way to generate nanoforms using eco friendly approach, the most important research interest in the field of nanotechnology. In the present study, extracellular components mediated synthesis of silver nanoparticles (AgNPs) using different soil Streptomyces spp. was investigated. The synthesized AgNPs as characterized using UV- Vis spectroscopy, field emission scanning electron microscopy (FESEM), energy dispersive X-ray analysis (EDS) and X-ray diffraction (XRD). The synthesized nanoparticles were found to be spherical in shape with size range from 5 to 20 nm. The crystalline nature of AGNPs was confirmed with XRD analysis and the elemental composition was also determined using EDX analysis. The potent isolate which mediated the synthesis was identified conventionally and also by using molecular 16S rDNA sequence analysis and found to be Streptomyces strain SJ6. The antimicrobial potency of the biologically synthesized AgNPs as tested against bacterial pathogens and maximum activity was found against Bacillus subtilis.

Keywords: *AgNPs, Biosynthesis, Streptomyces sp., Antibacterial activity.*

INTRODUCTION

Recently, there is huge interest in the field of nanoparticles due to their potential applications in different fields such as medicine, electronic, agriculture, optical etc., [1, 2].The intense research of nanoparticles in the field of biomedicine has become one of the leading thrust areas in the current scenario. However the synthesizing of nanoparticles with less toxicity, highly specific, effective and cheap were considered uphill task for the current researchers [3-6].

Recently, the biologically mediated synthesis of AgNPs using microbial systems such as bacteria, fungi and algae has become a huge interest [7, 8]. However the use of actinomycetes sp for the synthesis of nanoparticles is still not much exploited. Most of the cases, actinomycetes were best utilized for the production of various antibiotics and secondary metabolites in the biomedicine field [9]. This important property of actinomycetes may also aid in the bio inspired synthesis of AgNPs with richer antimicrobial property due to the presence of capping agents resulting from the exgtracellular components. Among the different actinomycetes, *Streptomyces* sp were found to play a major role in the antibiotic production and other secondary metabolites.

On this basis, in the present study we demonstrate the bio inspired synthesis of AgNPs using extracellular components of *Streptomyces* sp. The synthesized AgNPs was characterized using UV-Vis spectroscopy, FESEM, EDS and XRD. Further the antimicrobial property of biologically synthesized AgNPs was tested against selective human pathogens.

MATERIALS AND METHODS

Chemicals and reagents:

All the chemicals used in the present study were of analytical grade chemicals purchased from Merck Inc. (Mumbai, India); Qualigen, Mumbai, India and the microbiological media were purchased from Himedia Pvt Ltd, Mumbai, India.

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Sample collection and isolation of *Streptomyces:*

Soil samples were collected from various locations in Chennai using sterile plastic bags and pretreated with calcium carbonate, incubated at 28°C for 7 days. The pre-treated soil sample was serially diluted and plated on starch casein agar (composition g/L: soluble starch: 10 g, K_2HPO_4: 2 g, KNO_3: 2 g, casein: 0.3 g, $MgSO_4.7H_2O$: 0.05 g, $CaCO_3$: 0.02 g, $FeSO_4.7H_2O$: 0.01 g, agar: 15 g, pH: 7.0±0.1) amended with cyclohexamide (25µg/mL) to minimize fungal contamination. The plates were incubated for 7-14 days at 28°C and monitored for *Streptomyces* colonies. The representative colonies were selected, subcultured in starch casein agar slant and utilized for further studies [10].

Screening of *Streptomyces* for AgNPs synthesis:

The isolated *Streptomyces* sp. was grown individually in starch casein broth with continuous shaking at 30 °C for 9 days at 120 rpm. At the end of incubation the biomass was separated by centrifugation at 20 °C for 20 min at 5,000 rpm and the mycelia mass were washed thrice with sterile distilled water under aseptic conditions. Then the biomass was brought into contact with 100 ml sterile distilled water and kept for agitation at 150 rpm for 24 h at room temperature. After incubation, the cell free filtrate was collected by filtering though Whatman No. 1 filter paper and then it was treated with $AgNO_3$ to reach final concentration of 1 mM. The reaction was carried out in dark for 24 h at room temperature and observed for the formation of AgNPs [11].

Characterization of biologically synthesized Nanoparticles:

The change in color was visualized and the biologically synthesized AgNPs was further characterized using UV-Vis spectroscopy. The best isolate which mediated the synthesis of stable AgNPs was selected and utilized for further study. The biologically synthesized AgNPs were then characterized using X-ray powder diffraction, field emission scanning electron microscopy (FESEM) and energy dispersive spectroscopy (EDS). The synthesized AgNPs were lyophilized, powdered and used for X-ray diffraction analysis at a high operating voltage and the intensities were recorded from 30 to 80 2θ angles [12].

Antibacterial activity of biologically synthesized AgNPs:

For the antibacterial assay, the following bacterial pathogens *E. coli* ATCC 8739, *P. aeruginosa* MTCC 741 (Gram negative) and *S. aureus* ATCC 29736, and *B. subtilis* ATCC 6633 (Gram-positive) were procured from microbial-type culture collection (MTCC), IMTECH, Chandigarh, India 160 036. All the

pathogens were grown and sub cultured in Mueller Hinton agar medium (MHA) at 37 °C of 24 h incubation.

The antibacterial property of biologically synthesized AgNPs was tested against the four different pathogens using standard method described by Bauer et al. [13]. Briefly, the Mueller Hinton agar plates were prepared and the overnight test cultures were evenly swabbed on the medium, allowed standing for 10 mins. Four different wells were punched using sterile cork borer and marked as A, B, C and D. Thirty µL of cell free supernatant, standard antibiotic solution (streptomycin 1mg/mL), biologically synthesized AgNPs and sterile distilled water were added to the well marked as A, B, C and D respectively. Then plates were incubated at 37 °C for 24 h and the diameters of the zone of inhibition were calculated and expressed in millimeters.

Identification of potent AgNPs producing *Streptomyces* sp SJ6:
Cultural characteristics:

The potential isolate which mediated the synthesis of stable AgNPs was identified using PIBwin (probabilistic identification of bacteria) software based on the various cultural, morphological and biochemical characteristics. Different morphological and physiological characteristics feature of test strain such as morphology of the spore, antibiotic sensitivity, tolerance level; nutritional requirements and pigmentation were studied [14, 15]. The colony morphology, the mycelium pattern was observed based on the media recommended by Shirling and Gottlieb of International Streptomyces Project (ISP) after two weeks of incubation at 28°C [16-18].

Molecular identification of *Streptomyces* sp SJ6:

The identification of *Streptomyces* sp SJ6 was performed using 16S rDNA partial sequencing method. Briefly, the genomic DNA was isolated and the 16S rDNA gene of the potent *Streptomyces* strain was amplified using forward primer, StrepF; 5′-ACGTGTGCAGCCCAAGACA-3′ and reverse primer StrepR; 5′-ACAAGCCCTGGAAACGGGGT-3′ [19]. The reaction was carried out using PCR mixture composed of 10 to 50 ng of DNA; 5 p moles of each primer; 200 µM of dNTPs; Taq polymerase of 2.5 units to final volume of 50 µl of polymerase buffer containing 1.5 mM $MgCl_2$. The amplification reaction was conducted for 30 cycles with 1 min at 94 °C, annealing at 53 °C for 1 min, and finally extension at 72 °C for 2 min. The amplified PCR product was then purified using QIA quick PCR purification kit (Qiagen, USA) and then sequenced using Dye terminator cycle sequencing kit by ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA [20, 21]. The obtained sequence data was analyzed for their degree of DNA similarity using the BLAST program (www.ncbi.nlm.nih.gov/blst) and the molecular phylogenetic tree of the amplified sequence was constructed using MEGA6 software [22]. Further, the sequence of *Streptomyces* SJ6 strain was deposited in the Genbank database under the accession no. KU981019.

RESULTS AND DISCUSSION

The present study was aimed to synthesize biocompatible AgNPs by biological method using cell free supernatant of *Streptomyces* sp. isolated form soil samples. In the present study soil samples were collected from 3 different locations, Arumbakkam, Koyambedu market and Vadapalani in Chennai, Tamil Nadu, India. A total of 27 strains were isolated in which the maximum number of isolates were found from sample collected form Arumbakkam of 13 isolates, followed by Koyambedu market with 10 isolates.

Silver were used since from the ancient time and currently their antimicrobial property has paved way to enormous applications in various fields such as drug delivery, medical diagnostics, cosmetics, personal care products and also in sensors, optics, painting due to their unique properties in nanosized forms [23, 24]. One of the most popular areas where AgNPs gained most of their importance is due to their antimicrobial property against wide variety of pathogens [25]. Most of the studies also reveal that the effect of antimicrobial property is majorly depended on the particles size and it increases with decrease in the size of particles [26].

The 27 isolates were subjected for the screening of AgNPs using $AgNO_3$ at concentration of 1 mM in dark condition for 24 h. Among the different isolates tested, change in color of the extract from colorless to brown color were observed in 3 different cell free supernatant treated with $AgNO_3$ thus indicating the formation of AgNPs. The synthesized nanoparticles were characterized for the UV – Vis spectrum analysis using UV-Vis Spectrophotometer (Systronic 119) showing maximum absorption spectra between 410 to 420 nm. The AgNPs synthesized using three different *Streptomyces* sp were further checked for their stability studies, such as precipitation of AgNPs and maximum UV –Vis spectra. Among the three strains tested, one strain synthesized AgNPs with good stability without any precipitation for about 2 months with maximum spectra of 420 nm and it was chosen as potential isolate and designated as *Streptomyces* SJ6 (Fig. 1) which was utilized for further study.

The change in the color from colorless to brown is due to excitation phenomenon of surface Plasmon resonance of synthesized AgNPs (Fig. 2a and b) which is well reported by several researchers [27-29]. It was also noted that the intensity of AgNPs synthesized using *Streptomyces* sp SJ6 increased after 24 h and it was maintained further incubation period. However in case of other different extracts treated with $AgNO_3$ results in no color change confirming the inability to reduce $AgNO_3$ to AgNPs. The maximum spectrum of 420 nm is due to observance of surface plasmon resonance resulting in the formation of AgNPs where the position of spectra absorbance depends on several factors such as nanoparticles size, medium etc. [30, 31].

The EDX spectra confirms the presence of elemental silver in the biologically synthesized AgNPs and the XRD spectra also confirms the crystalline nature of AgNPs with diffraction peaks of at 37.12°, 43.15° and 63.81° corresponding to the lattice planes of 111, 200 and 220 of the FCC cubic silver pattern (figures not shown). The obtained XRD pattern is well matched with JCPDS file no 04-0783. Similar studies were also reported with Philip and Unni who have synthesized AgNPs using *Ocimum sanctum* extract showing an XRD patterns of five speaks corresponding to as (111), (200), (220), (311) and(222) planes of fcc silver nanforms [32]. Shaligram et al and Bhainsa and D'souza also reported similar XRD pattern during their investigation of AgNPs characterization [33, 29]. The FESEM studies reveal the shape of biologically synthesized AgNPs of spherical in shape with size ranging from 5 to 20 nm with uniform dispersity (Fig. 3).

The antibacterial activity of biologically synthesized AgNPs was investigated against four different pathogens namely, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*. Interestingly, all the pathogens showed zone of inhibition when tested against the *Streptomyces* sp SJ6 mediated AgNPs. Among the tested pathogens, maximum activity was found when AgNPs treated against *Bacillus subtilis* (27 mm), followed by *P. aeruginosa* (25 mm) and E. coli (23 mm). The lowest activity was found when AgNPs treated against *S. aureus* with an inhibition zone of 21 mm. The observation clearly indicates that the biologically synthesized AgNPs showed potential antibacterial activity against all the tested pathogens (Fig. 4).

The findings are in the agreement with the earlier studies where antimicrobial activity of AgNPs was tested against *B. subtilis* and *C. albicans* by Sadhasivam and co-workers [34]. They have synthesized AgNPs using extracellular components of *Streptomyces hygroscopicus* with an average size of 20-30 nm of spherical in size. Though there are no clear reports on the mechanism of AgNPs of the bacterial pathogens, several studies suggested that the penetration of AgNPs into the bacteria resulting in damage of cell membrane leading to release of cellular components [35]. Similarly, Kim et al. and Li et al. investigated the antimicrobial effect of AgNPs on different pathogens suggested that the release of Ag ions from the nanoforms may play major role in the bactericidal property [36, 37].

The morphological and biochemical properties of the potential isolate were characterized based on the International *Streptomyces* Project (ISP) and Bergey's manual of systematic bacteriology was tabulated in table 1 and 2. The SEM image show the Rectus-Flexibilis (RF) form *of* spore bearing hyphae and grey color spore mass with smooth surface. The substrate mycelium was found to be yellow in colour without any diffusible pigment and melanin formation. The isolate was found to be Gram positive, non motile, catalase positive, oxidase positive, with an NaCl tolerance of 0-4% and absence of nitrate reduction. The isolate was able to hydrolyze chitin and gelatin and found to be resistant against Colistin, Penicillin, Ampicillin, Chloromphenical, Neomycin and Novobiocin (table 3). In ISP 2, the substrate mycelium were found to be brown in colour and in case of ISP 4 and 5, there was a moderate growth with white colour substrate mycelium formation. Similar studies were performed by various researchers who have also identified the *Streptomyces* sp strains using physiological, morphological and biochemical parameters [30-40]. The potential strain was further characterized for partial sequencing by 16S rRNA method, and sequence was identified as *Streptomyces* sp. SJ6 which was deposited under the GenBank accession number of KU981019.

Table No. 1: Growth characateistics of *Streptomyces* sp SJ6 on different ISP medium

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
ISP- 2 Yeast extract-malt extract agar	Abundant	White	Brown	-
ISP-3 Oatmeal agar	Moderate	White to yellow	Dull yellow	-
ISP- 4 Inorganic salts starch agar	Moderate	White to greenish	White	-
ISP- 5 Glycerol asparagine agar	Moderate	White to creamish	White	-

Table No. 2: Physiological and biochemical characteristics of the isolate *Streptomyces* sp SJ6

Name of the test/ Criteria	Characteristic features of *Streptomyces* sp SJ6
Gram's staining	Gram positive
Motility	-
Spore chain	RF
Spore surface	Smooth
Spore mass	Gray
Aerial mycelium	Gray – white
Substrate mycelium	Yellow
Diffusible pigment	-
Melanin production	-
Oxidase	+
Catalase	+
Nitrate reduction	-
NaCl Tolerance	0 - 4%
Growth in lysozyme	+
Gelatin hydrolysis	+
Cellulose hydrolysis	-
Pectin hydrolysis	-
Chitin hydrolysis	+

+ (Positive) ; ***- (Negative)***

Table No. 3: Antibiotic sensitivity analysis of *Streptomyces* sp SJ6

Antibiotic	Sensitivity
Colistin	-
Penicillin	-
Ampicillin	-
Chloromphenical	-
Neomycin	-
Novobiocin	-
Streptomycin	+
Methicillin	+
Ciprofloxcin	+
Amikacin	+

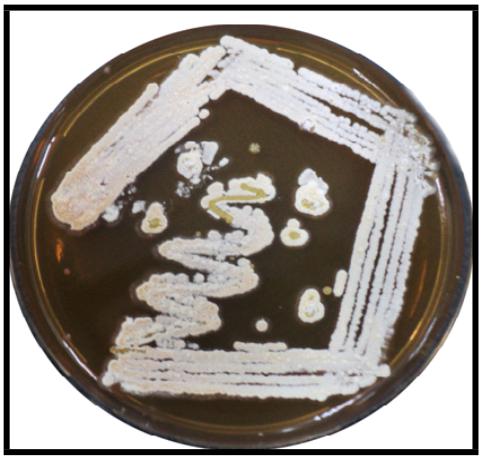

Fig. 1: Growth of *Streptomyces* sp SJ6 on Starch casein agar medium

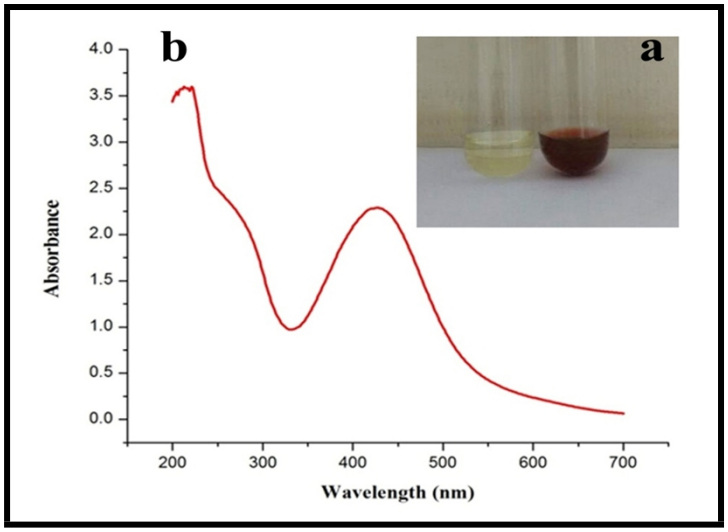

Fig 2: Synthesis of AgNPs (a) visual color change from colorless to brown color (b) UV-visible spectra of biologically synthesized AgNPs showing maximum peak at 420 nm

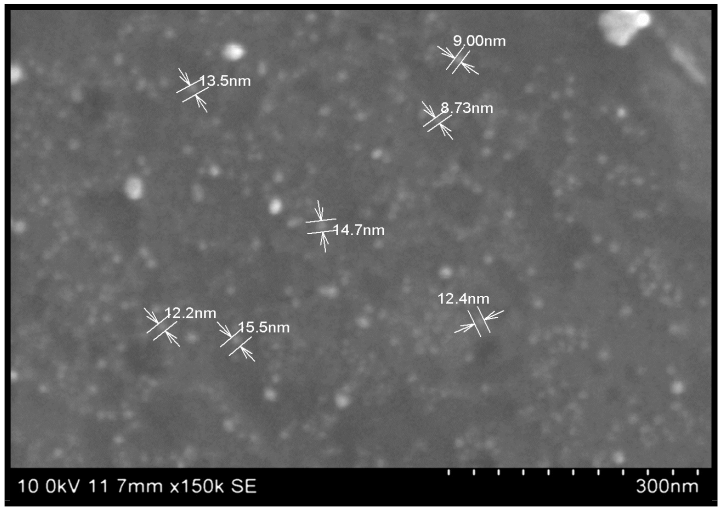

Fig 3: FESEM image of biologically synthesized AgNPs

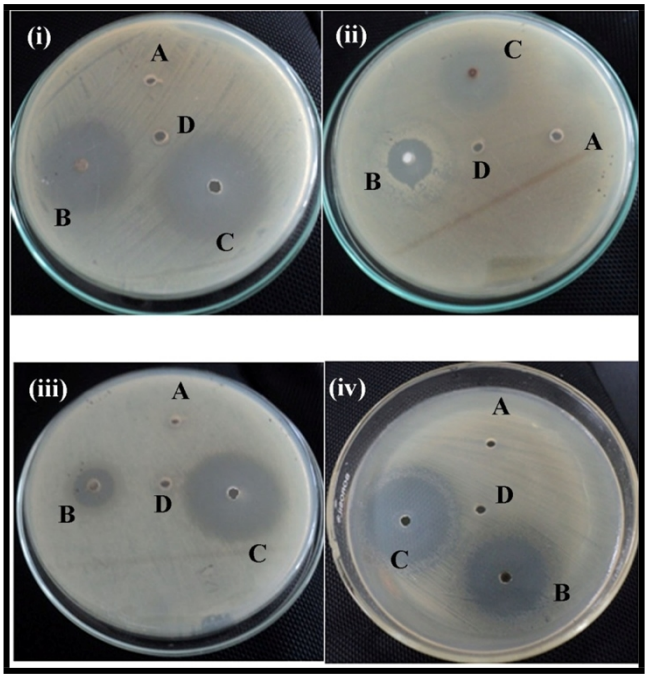

Fig 4: Antibacterial activity of biologically synthesized AgNPs against human pathogens
(i) ***B. subtilis;*** **(ii)** ***S. aureus;*** **(iii)** ***E. coli;*** **(iv)** ***P. aeruginosa;*** [A - cell free supernatant, B - standard antibiotic; C - biologically synthesized AgNPs and D - sterile distilled water]

CONCLUSION

To conclude, the utilization of soil actinomycetes for the bioreduction of $AgNO_3$ to silver nanoforms was attempted. The potential soil isolate *Streptomyces* sp SJ6 supported the synthesis of AgNPs with size ranging from 5 to 20 nm with good stability. The nanoparticles synthesized were well characterized and their effect as an antimicrobial agent was tested against selective pathogens. The bio inspired mediated synthesized AgNPs showed good activity towards all the tested pathogens which clearly indicates the antibacterial effectiveness. Thus these nanoforms synthesized using *Streptomyces* sp SJ6 could be applied as an antimicrobial agent however extensive studies, including toxicity, molecular and genomic investigation is required to understand the clear picture of the role and mode of action of synthesized AgNPs.

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