Production and Characterization of Protein Encapsulated Silver Nanoparticles by Marine Isolate *Streptomyces parvulus* SSNP11

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Abstract Production of protein encapsulated silver nanoparticles (AgNPs) assisted by marine actinomycetes strain has been investigated. The selective isolate was identified as *Streptomyces parvulus* SSNP11 based on chemotaxonomic and 16S rRNA analysis. Maximum AgNPs production was observed within 24 h incubation time. The produced AgNPs are spherical in shape with mono-dispersive and crystalline in nature. The particle size distribution ranges from 1.66 to 11.68 nm with a mean size of 2.1 nm. The biosynthesized AgNPs revealed stretching vibrations of primary and secondary amines along with C–H and C–N, suggesting that metabolically produced proteins are involved in size regulation of reduced AgNPs. These particles possess an average negative zeta potential value of 81.5 mV with an electrophoretic mobility of 0.000628 cm²/Vs. The biosynthesized nanoparticles revealed antimicrobial property against gram negative as well as gram positive bacterial strains.

Keywords Silver nanoparticles · *Streptomyces parvulus* SSNP11 · Zeta potential · FT-IR spectroscopy

Introduction

The synthesis of nanoparticles has been considered as the priority area in the nanotechnology sector due to their

ability to engineer the properties of materials based on size. This is mainly due to acquisition of optical, chemical, photochemical and electrical properties to the materials that may significantly different from its counterpart materials because of materials dimensions reduction [1, 2]. Though, a number of approaches such as chemical reduction using reductants like NaBH₄, N₂H₄, NH₂OH, ethanol, ethylene glycol and *N,N*-dimethylformamide, electrochemical, sonochemical deposition, photochemical, laser irradiation, etc. have been developed, however in these synthesis procedures there is an unavoidable need for the use of stabilizing agent for improving the stability and homogeneity of nanoparticles. These stabilizing agents invariably operate via one of the four principles: electrostatic, steric, electrosteric stabilizations and stabilization by a ligand or a solvent [3]. These methods have some significant disadvantages regarding nanoparticle formation, monodispersity of the particles and thermodynamic stability. In addition, use of these stabilizing agents deserves capital investment and hazardous for the environment. In this context, green nanotechnology created world wide interest to develop biocompatible and environment friendly manufacturing process, as well as their use as catalysts in synthesis reactions to minimize or eliminate the use of toxic materials, to reduce the pollution, improve the efficiency of solar/fuel cells/other biotechnological purposes [4].

Cell metabolic processes are considered as novel, simple and viable alternative to chemical synthetic procedures in modern biotechnology and continued to be exploited for production of various industrially useful products in eco-friendly manner [5, 6]. In general all most all microbial strains have potential to absorb and accumulate metals and help in reduction of environmental pollution as well as recovery of metals from environment to avoid the toxicity. However, only a few microbial strains have ability to

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selectively reduce certain metal ions by electron shuttling using enzymes. For example, hydroquinones released by microorganisms are capable of reducing ions to nanoparticles [7]. This potential of microbes to reduce metals has led to new dimension for production of biogenic nanoparticles [4]. Several microbial strains belonging to bacteria, fungal and actinomycetes strains have been isolated and characterized for their unique metabolic mechanisms for synthesizing reproducible particles with well-defined size and structure. Furthermore, biogenic nanoparticles often exhibit water soluble as well as biocompatible properties which are essential for biotechnological application especially in therapeutic uses.

Although extensive studies have been carried out on microbial synthesis of nanoparticles, it was noticed that the physico-chemical properties of the produced nanoparticles differ with the metabolic nature of microbial strains. For example, production of various sizes ranging from 15 to 500 nm size nanoparticles using *Lactobacillus* sp. has been reported [8, 9]. Similarly, *Fusarium* sp. reported to produced 2–50 nm [10], 2–20 nm [11], 20–40 nm [12] and 8–14 nm [13] particles. Marine strains belonging to actinomycetes is less explored compared to terrestrial and a very few reports are available on biosynthesis of nanoparticles using marine microbial flora [4]. Keeping this in view, in the present study effort has been made to isolate marine actinomycetes species and evaluate its biogenic properties for production of silver nanoparticles (AgNPs).

Materials and Methods

Isolation, Screening and Identification of Microorganism

Marine soil sediment samples were collected from the coast of Bay of Bengal near Visakhapatnam, Andhra Pradesh. The strains were isolated according to the method described by Prakasham et al. [4].

The strain was identified based on their cell wall composition (chemotaxonomic properties) according to Cummins and Harris [14]. Molecular characterization based on ribotyping of 16S rRNA was performed at Microbial Type Culture Collection Center, IMTECH, Chandigarh, India.

Phylogenetic Analysis

Nucleotide sequences were compared with those maintained in the GenBank Database through NCBI Blast (<http://www.ncbi.nlm.nih.gov>). Alignment of nucleotide sequences was done using a cluster method of the DNASTAR software program (DNASTAR Inc., Madison, WI,

USA). All analyses were performed on a bootstrapped dataset containing 1,000 replicates. In order to determine the genetic relationship between these strains, a phylogenetic tree was generated based on the percentage difference between the sequences.

Synthesis of AgNPs

Synthesis of AgNPs was performed based on the method described by Prakasham et al. [4].

Characterization of AgNPs by Using Transmission Electron Microscopy (TEM)

The TEM analysis of extracellular biosynthesized AgNPs was performed by drop-coating biosynthesized AgNPs solution on carbon-coated copper TEM grids (40 × 40 μm mesh size). Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. TEM measurements were performed using Tecnai-12, FEI, Netherlands, electron microscope operated at an accelerating voltage at 120 kV. Selected area electron diffraction (SAED) analysis of the particles was performed.

Particle Size Analysis and Zeta Potential

The particle size distribution and zeta potential of biosynthesized AgNPs were evaluated using dynamic laser light scattering measurements conducted with a nanoparticle size analyzer (HORIBA Nanoparticle Size Analyzer SZ-100, JAPAN). Data obtained were analyzed using instrument software.

FT-IR Spectroscopy Analysis

For Fourier transform infra-red (FT-IR) spectroscopy measurements, the bio-transformed products present in extracellular filtrate were freeze dried and diluted with potassium bromide in the ratio of 1:100. FT-IR spectrum of samples was recorded on FT-IR instrument with diffuse reflectance mode (DRS-800) attachment. All measurements were carried in the range of 400–4,000 cm⁻¹ at a resolution of 4 cm⁻¹.

Anti-Bacterial Activity

Antibacterial activity of biosynthesized AgNPs were evaluated by testing them against strains of both gram positive and gram negative bacteria (*Pseudomonas putida*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Salmonella typhi*) by well diffusion method as described by Prakasham et al. [4].

Synergistic Activity

Disk diffusion method is followed to assay the synergistic effect of synthesized AgNPs with commonly used antibiotics, was adopted to test the bactericidal efficacy of these nanoparticles alone and in combination with antibiotics. The standard antibiotic disks were purchased from HiMedia (Mumbai, India) (Penicillin (10 units/disk), Ampicillin (10 µg/disk), Cefalexin (30 µg/disk) and Streptomycin (10 µg/disk)). To determine the synergistic effects, each standard antibiotic disk was impregnated with 10 µl of freshly prepared AgNPs and was placed onto the nutrient agar medium inoculated with test organisms. Standard antibiotic disks were used as positive control and along with this AgNP control also placed (10 µl impregnated to empty disks). These plates were incubated at 37 °C for 16–24 h. After incubation, the zones of inhibition for the control and treated plates were measured.

Results and Discussion

Isolation and Identification of Actinomycetes Strains

Several actinomycetes strains were isolated preliminarily using Actinomycetes specific growth medium from soil samples collected from the different sea belts of Visakhapatnam, Andhra Pradesh, India. One of the strains designated as SSNP11 was selected for further studies based on biosynthesis of nanoparticles. Hence, this strain was analyzed further for chemotaxonomic properties to identify based on the cell wall composition. Cell wall compositional analysis revealed presence of glycine and diamino

pimelic acid and mannose and ribose, indicating that the designated SSNP11 strain belongs to Type-I class actinomycetes which contains major genus *Streptomyces*.

Identification of *Streptomyces* strain at species level was performed based on ribotyping. The 16S rRNA gene sequencing analysis of the isolate yielded 1,263 base pairs and NCBI BLAST search analysis showed that the sequence was 96 % similar to the sequence of *Streptomyces parvulus* 1044 strain (Fig. 1). A neighbor joining tree using Maximum-parsimony method was constructed based on 16S rRNA sequences denoting that the isolate occupies a distinct Phylogenetic position within the radiation including representatives of the *Streptomyces* family. Hence, this isolate is designated as *S. parvulus* SSNP11.

Production and Characterization of AgNPs

Silver nanoparticles biosynthesis potential of isolated marine actinomycetes strain, *S. parvulus* SSNP 11, has been further evaluated using positive (without silver nitrate supplementation) and negative (without biomass/cell free fermentation broth) control experimentation by monitoring silver nanoparticle production against time line continuously for the period of 24 h by measuring absorbance in the range of 280–600 nm. The primary confirmation of synthesis of AgNPs in the medium was identified based on the change in color. This color change from yellowish white to dark brown is due to surface Plasmon resonance of deposited AgNPs and considered as indication for biogenesis of nanoparticles [15]. In fact, visual monitoring of positive and negative control revealed little variation in color change even after 24 h of incubation while silver nitrate treated cell free fermentation broth turned dark

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences from strain *Streptomyces parvulus* SSNP11 and related organisms

Fig. 2 Transmission electron microscopy (TEM) analysis of Silver nanoparticles (**a** TEM micrograph; **b** SAED pattern)

Fig. 3 Particle size distribution histogram of silver nanoparticles

Fig. 4 Zeta potential analysis of silver nanoparticles

brown color. This suggested that isolated marine *S. parvulus* SSNP11 has potential to reduce the Ag^+ to Ag^0 ions under normal atmosphere and room temperature effectively

in extracellularly. Though the basic information on the reduction process of silver ions by microbial strains and their biochemical process is not clear, it is believed that protein molecules and enzyme such as nitrate reductase are reported to act as regulators of silver nanoparticle biosynthesis in *Bacillus licheniformis* [16], *Klebsiella pneumoniae* [17], *Rhodobacter capsulatus* [18] and *Fusarium oxysporum* [19].

Transmission Electron Microscopy (TEM)

The morphology and shape of AgNPs produced by *S. parvulus* SSNP11 were determined using TEM. TEM micro-graphs recorded from drop-coated films of the AgNPs synthesized after the reaction with silver nitrate solution for 24 h showed that the sample is composed of a large quantity of AgNPs (Fig. 2a). The produced AgNPs were predominantly spherical in shape and uniformly distributed without significant agglomeration. Careful observation of TEM micrographs further revealed that some AgNPs appeared as clusters which may be due presence of

different nanoparticle in the same angle of focused beam. This may be further confirmed by the appearance of a longitudinal surface plasmon resonance band in the UV–Vis spectra recorded from the AgNPs solution (Fig. 2a). The Fig. 2b shows the SAED pattern recorded from the AgNPs shown in the Fig. 2a. The diffraction spots in the SAED pattern indicated the reflections of different planar angles of *fcc* silver. The sharp spots in the SAED pattern indicated that the produced AgNPs are crystalline in nature.

Particle size Analysis and Zeta Potential

Figure 3 represents the histogram of particle size distribution of AgNPs produced by isolated marine actinomycetes strain, *S. parvulus* SSNP11. Analysis of the distribution pattern revealed that the biosynthesized particles size ranges from 1.66 to 11.68 nm with a maximum average size of $2.11 \text{ nm} \pm 1.1$. Thus, the isolated strain is unique in nature to produce very small size AgNPs compared to literature reports [2, 4]. However, Ahmad et al. [20] reported production of 5–200 nm size AgNPs, while 449–491 nm ranges nanoparticles production observed by Sanghi and Verma [21]. Further analyses of size distribution revealed that >85 % of AgNPs produced by *S. parvulus* SSNP11 are in the range of 1.66–3.05 nm and that a very small number of biosynthesized AgNPs are in the range of 3.45–11.68 nm as shown in Fig. 3. This could be further evidenced from the frequency distribution curve

where it is observed that 86 % of the particles are in a range of 1.66–3.05 nm.

Analysis of dispersive property of biosynthesized silver particles revealed the polydispersity index ratio of 0.963, indicating that particles obtained are mostly mono-dispersive or uniform distribution of shape. Such a high polydispersity index further confirm earlier observation of TEM studies where most of the particles viewed as spherical in shape and well distributed as single particles. The observed Z-average value for biosynthesized AgNPs was 185.9 nm while zeta potential value noticed to be -81.0 mV with an electrophoretic mobility of $-0.000628 \text{ cm}^2/\text{Vs}$, suggesting that these particles are highly stable as shown (Fig. 4).

FT-IR Spectroscopy Analysis

FTIR measurements of the powder sample (freeze dried) were carried out to identify the possible interactions between silver and bioactive coating agent, which may be responsible for synthesis and stabilization (capping agent) of AgNPs. FT-IR spectral data revealed two vibration bands at $1,645.5$ ($\text{C}=\text{O}$ stretching) and $1,402.81 \text{ cm}^{-1}$ (CN stretching, NH bending) that corresponds to bending vibrations of the amide I and amide II bands. While the observed stretching vibrations at $3,392.19$ and $2,928.08 \text{ cm}^{-1}$ respectively indicating NH stretching suggesting the proteinaceous material presence on surface of nanoparticles. The amide linkages between amino-acid residues in proteins give rise to well-

Fig. 5 FTIR spectrum of silver nanoparticles synthesized from *Streptomyces parvulus* SSNP11

Table 1 Antibacterial activity of synthesized silver nanoparticles

Sl.no	Microorganism	Zone of growth inhibition diameter (mm)	
		Standard (streptomycin)	Silver nanoparticles (AgNP)
1	<i>Bacillus subtilis</i>	30	30
2	<i>Salmonella typhi</i>	28	26
3	<i>Pseudomonas putida</i>	00	21
4	<i>Klebsiellapneumoniae</i>	29	26

known peaks in the infra-red region of the electromagnetic spectrum. Three bands observed at 1,110.78, 1,075.06 and 1,035.85 cm^{-1} can be assigned to the C–N stretching vibrations of aliphatic amines as shown in Fig. 5. It is well known that protein–nanoparticle interaction can occur either through free amino groups or cysteine residues in proteins and through the electrostatic attraction of negatively charged carboxylate groups in the enzymes. These observations suggested metabolically produced proteins play an important role in the stabilization of AgNPs especially when synthesized by biological methods.

Anti-Bacterial Activity

Silver has been used for its well-known antimicrobial properties since ancient times. But the production of AgNPs have made possible that the use of silver as a powerful bactericide recently. In order to evaluate bactericidal role of synthesized bionanoparticles, anti-bacterial activity of AgNPs was investigated against various pathogenic bacteria of both gram positive and gram negative (*P. putida*, *S. typhi*, *B. subtilis* and *K. pneumoniae*). Table 1 indicated the antibacterial activity of AgNPs produced extracellularly by the filtrate of the *S. parvulus* SSNP11 against control (extracellular filtrate without silver nitrate supplementation). Control did not show any zone of inhibition indicating the produced AgNPs exhibited the antibacterial activity against these tested microbial strains as shown in Fig. 6. Comparable antibacterial activity was noticed with produced AgNPs and streptomycin against three tested strains, *B. subtilis*, *S. typhi* and *K. pneumoniae*. Further, it is interesting to note that *P. putida* did not show any inhibitory zone for standard antibiotic, streptomycin whereas the produced AgNPs revealed an inhibitory zone of 21 mm. This data further supported that the biosynthesized nanoparticles have different anti-bacterial mechanism

Fig. 6 Antibacterial activity of Silver nanoparticles produced from *Streptomyces parvulus* SSNP11

Table 2 Synergistic activity of synthesized silver nanoparticles

Microorganism	Zone of growth inhibition diameter in (mm) for different antibiotics							
	Penicillin		Cephalexin		Ampicillin		Streptomycin	
	Without AgNP	With AgNP	Without AgNP	With AgNP	Without AgNP	With AgNP	Without AgNP	With AgNP
<i>Bacillus Subtilis</i>	–	17	10	13	–	15	8	25
<i>Klebsiella pneumoniae</i>	–	14	9	13	–	17	15	20
<i>Pseudomonas putida</i>	–	10	–	11	12	19	23	27
<i>Salmonella typhi</i>	–	–	20	23	12	19	21	23

to that of streptomycin, which is known to inhibit protein synthesis in prokaryotic organisms. This data have been reported by Shirley et al. [22]. The proposed mechanism of anti-bacterial action of AgNPs is generation of reactive oxygen species that directly act on the DNA of the microorganism and inhibits their growth.

Antibiotic Synergistic Activity

The combination of AgNP with different antibiotics was investigated against gram-positive (*S. typhi* and *B. subtilis*) and gram-negative bacteria (*P. putida* and *K. pneumoniae*) using the well diffusion method. The diameter of inhibition zones (mm) of different antibiotics with and without AgNP against test strains is shown (Table 2). The antibacterial activity of Ampicillin, Streptomycin, Cefalexin and Penicillin increased in the presence of AgNP against test strains. From the table it is evidenced that penicillin at (10 units/disk) shows resistance to all the organisms used but when the same antibiotic disks were impregnated with AgNP the activity is enhanced against all the test strains except for *S. typhi*. When it comes to ampicillin which is improved form of penicillin shows lower enhanced activity compared to penicillin. When it comes to newer antibiotics like Cefalexin (a new generation cephalosporin) the enhanced activity is much lowered. For aminoglycoside's (Streptomycin) the enhanced activity is tripled for *B. subtilis* and little enhanced for rest of the test strains. However, enhanced antimicrobial activities of commonly used antibiotics were observed in combination with the AgNPs. Therefore, it can be concluded that AgNPs alone or their formulations in combination with commonly used antibiotics can be used as effective bactericidal agents.

Conclusion

In recent years nanotechnology has been emerging as a rapidly growing field with wide application spectra in science and technology in health and daily life, especially in better drug delivery, chemical deposition for environmental

pollution cleanup, medical imaging, as well as military purposes. Biocompatible nanoparticles production plays significant role in hydrophilic based technological application especially in therapeutic sector over chemical methods of synthesis. In the present investigation, biocompatible nanoparticles were produced using cellular metabolic mechanism of marine isolate *S. parvulus* SSNP11 and evaluated their therapeutic application potential. The nanoparticles production is time-dependent and associated with biocatalytic based reduction process. The biocompatible nanoparticles were characterized for structural properties, surface chemistry and surface electrical potential in addition to biological activities especially antibacterial properties. The produced nanoparticles revealed high stability, polydisperse, crystalline and spherical nature with very small size (1.6–3.5 nm with median size of 2.11 nm). The particles revealed -81.0 mV zeta potential and -0.000628 cm²/Vs electrophoretic mobility. FT-IR characterization revealed the presence of N–H, C–H and C–C vibration stretching's on the surface of nanoparticles indicating a protein is involved as desizing and stabilization agent for produced AgNPs. The bio-generated AgNPs denoted antibacterial properties against gram negative and gram positive bacterial species. Antibiotic synergistic studies confirm the enhanced activity of conventional antibiotics when they were combined with AgNPs. These biocompatible nanoparticles could be used as ligand molecules to drug delivery which could be employed as double sword in controlling microbial infections and targeted drug application since silver, this precious metal can be used as an effective antimicrobial agent and as a disinfectant, as it exerts relatively free of adverse effects.

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