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Research Article

Synthesis of Silver Nano Particles from Marine Bacteria *Pseudomonas aerogenosa*

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ABSTRACT

Metal-tolerant microorganisms have been exploited in recent years to synthesize nanoparticles due to their potential to offer better size control through peptide binding and compartmentalization. Biological synthesis of silver nanoparticles using microorganisms has received profound interest because of their potential to synthesize nanoparticles of various size, shape and morphology. In the Present study, synthesis of silver nanoparticles by a bacterial strain PSK09 isolated from marine cost is reported. Molecular identification of the isolate showed it as a strain of *Pseudomonas aeruginosa*. On treating the bacteria with 1 mM AgNO3, it was found to have the ability to form silver nanoparticles at room temperature within 24 h. SNPs were characterized by UV-visible spectrophotometry, X-ray diffraction (XRD), and scanning electron microscopy (SEM). UV-visible absorption scan of a 48 h culture exposed to 5mM silver nitrate revealed a broad peak at 450nm indicative of the surface plasmon resonance of SNPs. This was confirmed by the visual observation and UV–Vis absorption at 450 nm. Therefore, the current study is a demonstration of an efficient synthesis of stable silver nanoparticle by a bacillus strain.

INTRODUCTION

Marine microbial biotechnology has opened up unexpected new ways for finding new organism for trapping their potential resources. Silver nanoparticles (AgNPs), are the noble metal nanoparticles that has being studied extensively due its various biological properties Chu et al. (1988). Nowadays, synthesis and characterization of nanoparticle studies playing a major role in nanotechnology and it is an important area of research Mritunjai et al .(2008). Silver is a nontoxic, safe inorganic antibacterial agent used for centuries and it has the capability of killing different type of diseases causing microorganisms Jeong (2005). Silver has been known to be a potent antibacterial, antifungal and antiviral agent, but in recent years, the use of silver as a biocide in solution, suspension, and especially in nano-particulate form has experienced a dramatic revival. Due to the properties of silver at the nano level, nanosilver is currently used in an increasing number of consumer and medical products. The remarkably strong antimicrobial activity is a major reason for the recent increase in the development of products that contain nanosilver. The main challenge in nanomaterials synthesis is the control of their physical properties such as obtaining uniform particle size distribution, identical shape, morphology, chemical composition and crystal structure.

There are an extensive number of synthesis methods of silver nanoparticles that are readily available. Nanomaterials are nanoparticles that have special physicochemical properties as a result of their small size Buzea *et al.* (2007). It is well known that silver possess good antimicrobial activities. The use of

silver is well documented in scientific literatures and it is known to have been used by the Persians, by ancient Phoenicians, Greeks, Romans and Egyptians for the treatment related to bacterial infections (21-22). Alexander (2009) Nanoparticles have found uses in many applications in different fields, such as catalysis, photonics, and electronics. Nanoparticles usually ranging in dimension from 1-100 nanometers (nm) have properties unique from their bulk equivalent. With the decrease in the dimensions of the materials to the atomic level, their properties change. The nanoparticles possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired applications Feynman (1991) Hence the present study is aimed to synthesis and characterize silver nanoparticles obtained by use of a marine bacterial strain and its various biological activities.

MATERIALS AND METHODS

Chemicals

Silver nitrate Merck (Germany) , Zobell media was procured from Hi media (India

Sample collection

The marine sediment sample was collected from Nellore Coast, Andhra Pradesh India at 10 m length and 5 m depth in sterilized glass bottle. The collected marine water sample was stored in ice box and then transported to the laboratory within 1 hour.

Isolation of bacteria:

10µl of sea water sample was spreaded over the surface of the marine agar (Zobell Marine agar) with composition of Peptone 5.0 g, Yeast Extract. 1.0 g, Ferric Citrate 0.1 g, Sodium Chloride 19.45 g, Magnesium Chloride 8.8 g, Sodium Sulfate 3.24 g, Calcium Chloride1.8g, Potassium Chloride 0.55 g, Sodium Bicarbonate 0.16g, Potassium Bromide.0.08 g, Strontium Chloride 34.0 mg, Boric Acid 2.0 mg, Sodium Silicate 4.0 mg, Sodium Fluoride 2.4 mg, Ammonium Nitrate 1.6 mg, Disodium Phosphate 8.0 mg, Agar 15.0. The individual black Colony was taken and maintained as stock culture at 30 ºC in marine agar test tubes slants. The selected strain was streaked on the Petri plate and continued for the further process (Fig -1)

Fatty acid methyl esters:

The bacterial strain were cultured onto Trypticase Soya Broth Agar (TSBA) media at 30°C for 24 hours. The fatty acids were extracted and methylated to form Fatty Acid Methyl Esters (FAME). These FAME’s were analyzed using Gas Chromatography (Agilent 6850 Series II) with the help of MIDI Sherlock software for FAME. The Aerobic library was used for comparing the results.

16S rRNA Gene Sequence

The purified bacterial isolates were cultured in saline nutrient broth (7% NaCl). After centrifugation at 4500 rpm for 10 min, at 4^0C, and twice washing with distilled water, the pellets were selected for DNA extraction an PCR amplification. Bacterial DNA was extracted by heat extraction method. The 16S rRNA gene was amplified by PCR, using the universal prokaryotic primers 5^I-ACGGGCGGTGTGTAC- 3^I and 5^I-CAGCCGCGGTAATAC-3^I which amplify a *800-bp region of the 16S rRNA gene. PCR was performed in a final volume of 50 µl containing PCR amplification buffer, Taq DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 lM) and template DNA (4 ng). Amplification conditions were initial denaturation at 94^0C for 5 min, 10 cycles at; 94^0C for 30 s, 50^0C for 30 s and 72^0C for 2 min. 20 cycles at; 92^0C for 30 s, 50^0C for 30 s, and 72^0C for 2.5 min with a final extension of 72^0C for 5 min. Taq polymerase was added to the reaction after initial denaturation. The lower denaturation temperature (92^0C) during the 20 cycle step was used to avoid loss of enzyme activity Fiore., (2000).The samples were electrophoresed in a 1% (w/v) agarose gel, using TBE buffer containing ethidium bromide (1 µg/ml). A single *800 bp DNA fragment was cut and extracted from the gel, using a Core Bio Gel Extraction Kit. The sequence similarity searches were done using the BLAST program that is available from the National Centre for Biotechnology Information (NCBI)

Biosynthesis of Ag-NPs

Bacterial strain was grown in Zobell marine broth. The final pH was adjusted to 7.0. The flask were incubated at 200 rpm at 28^0C. After 24 of incubation, the biomass was separated by centrifugation The supernatant and pellet was challenged with equal amount of with various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mM) of silver nitrate solution (prepared in deionized water) and incubated in dark condition at 28^0C. Simultaneously, a positive control of silver nitrate solution and deionized water and a negative control containing only silver nitrate solution were maintained under same conditions.

UV–visible spectral analysis:

The synthesized silver nanoparticle solution was observed in UV–visible spectra, the change in colour of this solution were recorded in ELICO SL-159 Spectrophotometer in the range of 350–470 nm.

Scanning electron microscope

After freeze drying of the purified silver particles, the size and shape were analyzed by scanning electron microscopy (JOEL-Model 6390).

Fourier-transform infrared (FT-IR) chemical analysis:

Fourier-Transform Infra-Red spectroscopy measurements, the biotransformed products present in extracellular filtrate were freeze-dried and diluted with potassium bromide in the ratio of 1:100. The FT-IR spectrum of samples was recorded on a FT-IR instrument (Digital Excalibur 3000 series, Japan) with diffuse reflectance mode (DRS-800) attachment. All measurements were carried out in the range of 400–4,000 cm-1 at a resolution of 4 cm-1 (Saifuddin et al. , 2009).

X-ray diffraction analysis:

The samples were embedded with the silver nanoparticles was freeze-dried, powdered and used for XRD analysis. The spectra were recorded in Philips_ automatic X-ray Diffractometer with Philips PW 1830 X-ray generator. The diffracted intensities were recorded from 30 to 90 2h angles

Antibacterial activity:

Muller Hinton Agar was prepared according to the manufacturer's instructions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure and was used to determine the antibacterial activity of Ag-NPs from pigmented black bacteria. Sterile molten cool (45°C) agar was poured aseptically into sterile petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate micro organisms by streaking evenly on to the surface of the medium with a sterile spreader and wells (8 mm diameter) were cut out from the agar plates using a sterile stainless steel bore and filled with 0.1ml of the each synthesized silver nanoparticles solution in respective wells. Tetracycline and double distilled water were used as positive and negative control respectively. Then the plates were incubated at 37°C for 24 hrs in the next day the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were read by the presence or absence of zone of inhibition.

Anti fungal activity:

To determine the antifungal activity of Ag-NPs from black pigmented bacteria. Sterile molten cool (45°C) agar was poured aseptically into sterile petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate micro organisms by spreading evenly on to the surface of the medium with a sterile spreader and wells (8 mm diameter) were cut out from the agar plates using a sterile stainless steel bore and filled with 0.1ml of the each synthesized silver nanoparticles solution in respective wells. Nystatin and distilled water were used as positive and negative control respectively. Then the plates were incubated at 37°C for 4 days, the zones of inhibition were measured.

RESULTS AND DISCUSSION

Marine water samples collected from Nellore marine area were used to isolate potential microbial strains using Zobell marine agar plates. After incubation at 30°C several colonies developed, and individual morphologically different bacterial colonies were isolated and purified subsequently All the isolated strains were screened further for their ability to produce silver nanoparticles synthesis. A total of 30 different bacterial strains were isolated, purified and preserved. Among the isolated strains, the strain which produced a brown pigment with good antibacterial activity against both gram positive and gram negative bacteria was selected for further studies and designated as PSK09. (Fig 1).

Figure 1: The pure culture of marine isolate PSK09

Molecular identification:

The bacterium was identified as *Pseudomonas aeruginosa*. Using 16S rRNA analysis. The bacterial DNA was isolated (Fig 2) and the 16S rRNA sequence was amplified and sequenced. The 16S rRNA sequence of the bacterium obtained was compared with the non-redundant BLAST database to obtain the sequences that displayed maximum similarity. All the sequences reported by BLAST revealed that the bacterial species showed a very high percentage of similarity (99%) with the sequences of *Pseudomonas aeruginosa* KC121042.1. with a reasonably high score and E-value being zero. The sequences showing the

maximum similarity were used for alignment using CLUSTAL W2 to derive the phylogenetic relationship. There exists a clear evolutionary relation between all the 16S rRNA sequences as this represents a highly conserved sequence.

Fig 2: Showing the 16s rRNA band of *Pseudomonas aeruginosa*

Synthesis of silver nano particle :

For the conformation of synthesis of nanoparticles in the medium was characterized by the changes in color of the reaction mixture from light yellow to light brown after 24h of incubation. Addition of Ag+ ions to the supernatant and pellet culture, samples showed the results as color formation to brown,(Fig 3) the color intensity increased with period of incubation due to the reduction in Ag0 .Control (without silver nitrate) showed no color formation in the culture when incubated for the same period and condition. In the supernatant culture no color changes seen on incubation period and the pellet culture containing Ag+ ions shown the change of color to brown as shown in figure. Synthesis of silver nanoparticles also depends on incubation period of the culture stated in previous studies Vaishali *et al*.(2012).

Fig:3 Showing Positive and control of silver nanoparticles synthesized from marine bacteria extracts

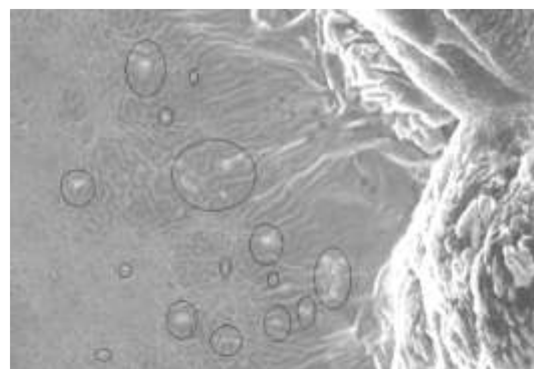

Fig:4 Showing SEM micrographs of silver nanoparticles synthesized from marine bacteria extracts

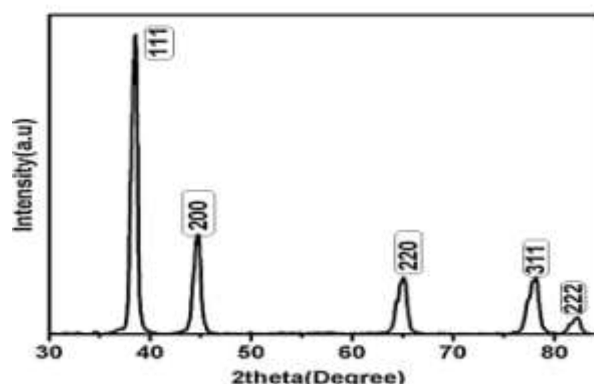

Figure 5: XRD analysis of silver nanoparticle

UV spectrophotometer:

Reduction of silver ions into silver nanoparticles using bacterial cultures was evidenced by the visual change of colour from yellow to reddish brown due to excitation of surface plasmon vibrations in silver nanoparticles. The synthesized silver nanoparticles was then characterized by UV spectrophotometer**.** The UV-visible spectra recorded at different time intervals showed increased absorbance with increasing time of incubation. The absorbance spectra of reaction mixture containing aqueous solution of 1mM silver nitrate and the pellet of *Pseudomonas aeruginosa* after incubation. The band corresponding to the surface plasmon resonance at 410 to 430 nm. The strong peak at 420 nm.

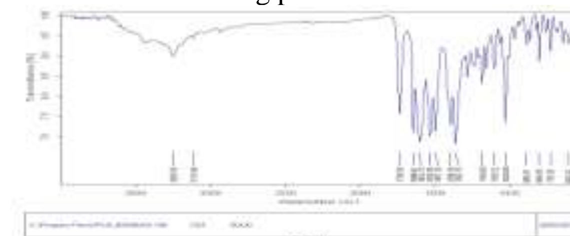

Fig:6:FT-IR spectrum recorded with synthesized silver nanoparticles

Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) analysis image (Fig.4) provided further insight into the shape and size of the nanoparticles. It is evident from the figure that the biosynthesized silver nanoparticles are in small and spherical in shape. Silver nanoparticles in the range of 35–46 nm by *Pseudomonas stutzeri* Klaus et al. (1999) 20–50 nm particles by *Lactobacillus* sp. Nair (2002).

FTIR analysis:

The characterization of the nanoparticles and the resulting resulting silver nanoparticle Was analyzed by FTIR.FTIR absorption spectra of the silver nano particle solution was shown in figure(Fig 5). The absorbance bands analysis in bioreduction and absorbed in the regions are 1735.85.61, 1602.70, 1533.93, 1396.34, 1363.15.

X-ray diffraction analysis:

XRD analysis of lyophilized cell pellets is powdered and used for XRD analysis. The spectra were recorded. All patterns indicate the occurrence of five diffraction peaks which are consistent with the (1 1 1), (2 0 0), (2 2 0), (3 3 1) and (2 2 2) diffraction of face centre cubic silver.(Fig 6)

Antibacterial activity:

To check the Silver and its derivatives are widely used in medicine for a long time in the treatment of bacterial infections. Thus it is now must to investigate the antibacterial activity of synthesized nanoparticles. The antibacterial activity of AgNPs was studied against the pathogenic bacterial strains using agar well diffusion method against standard antibiotic streptomycin. The highest activity was 35mm diameter of zone inhibition observed against *Micrococcus luteus* followed by 28.0 mm diameter of zone inhibition against *Proteus mirabilis*, *Bacillus cereus* and *Salmonella paratyphi* the zone of inhibitions produced by the synthesized silver nanoparticles was observed to compare with the standard streptomycin.(Fig7.)

Antifungal activity:

The antifungal activities of silver nanoparticles of *Pseudomonas aeruginosa* were determined against five pathogenic fungi (Table 2). The highest activity was 6.0 mm diameter of zone inhibition observed against *Aspergillus fumigatus* followed by 3.0 mm diameter of zone inhibition against *Aspergillus niger* The silvernanopaticles have been reported for their antifungal properties Vaishali Arjun (2012) which supports our present findings.

DISCUSSION

Reduction of silver ion into silver particles during exposure to the pellet could be followed by color change. Silver nanoparticles exhibit dark yellowish-brown color in aqueous solution due to the surface plasmonresonance phenomenon. The synthesis of nanoparticles is in lime light of modern nanotechnology. The development of biologically motivated experimental processes for the synthesis of nanoparticle is evolved into an important branch of modern nanotechnology. The present study emphasizes the use of marine bacteria for the synthesis of silver nanoparticles with potent biological effect In this study, the application of silver nanoparticle as an antimicrobial and anti fungal activity. Microorganism plays a very important function in sustaining soil health, ecosystem functions and production. Many Nanoparticles have already been reported to have anti-microbial properties and therefore exactly affect microorganisms. The enormous interest in the biosynthesis of nanoparticles is due to their odd optical, chemical, photochemical, electrical devices and magnetic properties. Utilizing promise biological systems from higher angiospermic plants or microbes, biosynthesis of nanoparticles is currently under wide exploration Extracts from microorganisms may reduced as decreasing and capping agents in AgNPs synthesis. The decrease of silver ions by combinations of bio-molecules found in these extracts such as enzymes, proteins, amino acids, polysaccharides and vitamins is environmentally benign, yet chemically complex. But, the mechanism which is broadly acknowledged for the synthesis of silver nanoparticles is the occurrence of enzyme ‘‘Nitrate reductase’ (Anil Kumar et al., 2007) (Kalimuthu et al., 2008). Time dependent reliant analysis of surface plasmon resonance demonstrated an increase in peak worth with a function of time mostly at 410 nm in both\ conditions, and little change in positive (silver nitrate in fresh medium) and negative (medium encompassing biomass without silver nitrate) controls. These facts and figures farther sustained that the marine isolate, *Pseudomonas aeruginosa* , has promise to reduce the silver ions to nanoparticles, and biosynthesis of metal

nanoparticles is associated with the metabolic nature of the microbial strain employed. Khosravi-Darani (2010) furthermore observed similar UV spectra for answers containing reduced silver ions with high absorption intensity. With previous data agreement with the results of silver nanoparticle Production output by stationary phase *Bacillus licheniformis*. Kalimuthu et al (2008), while Natarajan et al.(2010) described production of silver nanoparticles by mid-log phase heritage of *Escherichia coli* . FTIR is a powerful device for identifying types of chemical bonds in a molecule by making an infrared absorption spectrum that is like a molecular "fingerprint" Senapat (2005). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum.

The size ranges of silver nanoparticles produced by the CS 11 (42–94 nm) fall closer to the size of silver nanoparticles produced by other bacteria (Gurunathan et al. 2009).

CONCLUSION

In conclusion, silver nanoparticles are having wide applications in various fields like antimicrobials, paints preservatives, and cosmetics. So improving of synthesis for nanoparticles production is the major object in the field of nanotechnology. The characterization of silver ion exposed to microbial strain and the reduction of silver nitrate to silver nanoparticles was confirmed by UV visible spectrophotometer. The toxicity study of The usage of marine bacteria is the good approach to the production of Eco-friendly and costs effectual silver nanoparticles. Silver nanoparticles on human pathogens opens a door for a new range of antibacterial activity.

In this paper we report the marine bacterium *Pseudomonas aeruginosa* for synthesis of silver nano particles using the culture filtrate. The synthesized nanoparticles have been characterized by Uv-vis, SEM, XRD and FTIR measurements. The nanoparticles proved excellent antimicrobial activity. Hence, the biological approche appears to be cost efficient alternative to conventional physical and chemical methods of silver nanoparticles synthesis and would be suitable for developing a biological process for commercial large scale-production.

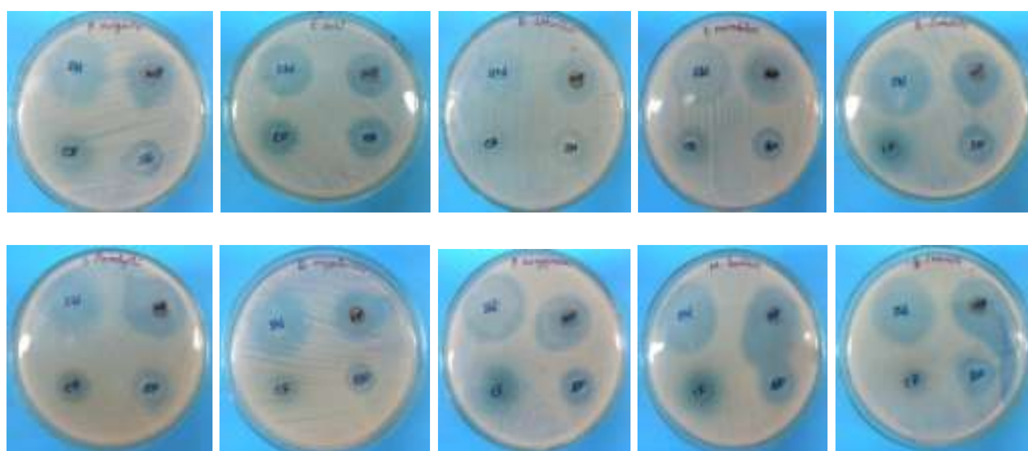

Figure:7. Antibacterial activity of silver nanoparticles (Ag-NPs) against bacteria

Table 1: Table showing the antibacterial activity of silver nanoparticle synthesized from bacterial pellet Zone of inhibition (ZOI in mm).

Strain Name	Silver nano particles	Culture filtrate	Silver nitrate	Streptomycin
Bacilllus sphericus	22 mm	8 mm	18 mm	25 mm
Micrococcus luteus	35 mm	22 mm	21 mm	33 mm
Pseudomonas aeruginosa	20 mm	18 mm	15 mm	30 mm
Bacillus cereus	28 mm	10 mm	20 mm	30 mm
Proteus vulgaris	22 mm	18 mm	18 mm	32 mm
Escherichia coli	22 mm	18 mm	20 mm	25 mm
Bacillus subtilis	25 mm	20 mm	20 mm	34 mm
Salmonella paratyphi	28 mm	18 mm	20 mm	30 mm
Bacillus megaterium	20 mm	10 mm	11 mm	30 mm
Proteus mirabilis	28 mm	15 mm	15 mm	30 mm

Table:2 Table showing the antifungal activity of silver nanoparticle synthesized from bacterial pellet Zone of inhibition (ZOI in mm).

Test Organisms	Silvernanoparticles	Standard
Aspergillus fumigatus	06	12
Aspergillus flavus	03	18
Aspergillus niger	0	19
Candida	03	20

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