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Extracellular Synthesis of Silver Nanoparticles Using *Pseudomonas aeruginosa* KUPSB12 and Its Antibacterial Activity

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Abstract

The use of microorganisms like bacteria in the synthesis of nanoparticles emerges as an eco-friendly approach and an alternative to the chemical method. In the present investigation, we report the biosynthesis of silver nanoparticles (AgNPs) using the phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12. Silver nanoparticles were synthesized through the reduction of aqueous Ag^+ ion using the bacterial culture supernatants at room temperature. Synthesis of AgNPs was initially observed by color change from greenish yellow to brown which was confirmed by UV-visible spectroscopy. The silver nanoparticles were further characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopic (SEM) analyses. The synthesized nanoparticles were found to be spherical in shape with a size in the range of 50-85 nm. The synthesized AgNPs were found to have antibacterial activity against six tested pathogenic bacteria (*Escherichia coli* MTCC 443, *Vibrio cholera*e MTCC 3904, *Shigella flexneri* MTCC 1457, *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 3160 and *Micrococcus luteus* MTCC 1538). Thus, the biosynthesis of silver nanoparticles using *Pseudomonas aeruginosa* culture supernatant deserves to be a good candidate as an antibacterial agent.

Keywords: *Pseudomonas aeruginosa*, Silver nanoparticles, Antibacterial activity, Phosphate solubilizing bacterium (PSB).

1. Introduction

Nanotechnology involving synthesis and applications of nanoscale materials is an emerging field of nanoscience with significant applications in biology, medicine and electronics owing to their unique particle size and shape dependent physical, chemical and biological properties (Albrecht *et al*., 2006; Mahasneh, 2013). To date, nanoparticles are mostly prepared from metals, i.e. silver (Sinha and Paul, 2014), gold (Arunachalam *et al*., 2014), copper (Lee *et al*., 2013), zinc (Darroudi *et al*., 2013), iron (Nadagouda *et al*., 2010), palladium (Khazaei *et al*., 2013) and titanium (Rajakumar *et al*., 2012). Among the metal nanoparticles, silver nanoparticles (AgNPs) have received much attention in various fields, such as antimicrobial activity (Agarwal *et al*., 2014), therapeutics (Mukherjee *et al*., 2014), water treatment (Con and Loan, 2011), bio-molecular detection (Tomšič *et al*., 2009), silver nanocoated medical devices (Furno *et al*., 2004) and optical receptor (McFarland and Van Duyne, 2003).

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. Hence, the biogenic approach, the usage of natural organisms or materials in particular, has offered a reliable, simple, nontoxic and eco-friendly method (Gopinath *et al*., 2013). The microbial synthesis of nanoparticles has significant advantages over other processes since it takes place at relatively ambient temperature and pressure (Gade *et al*., 2008; Mukherjee *et al*., 2008; Wei *et al*., 2012). In such a situation, screening of unexplored microorganisms for AgNPs synthesizing property is very important, as the size and shape of nanoparticles can also be controlled in microbial synthesis (Narayanan and Sakthivel, 2010).

Microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly (Kowshik *et al*., 2003, Korbekandi *et al*., 2013). Extracellular biosynthesis is cheap and it requires a simpler downstream processing than the intracellular biosynthesis which requires additional steps such as ultrasound treatment or reactions with suitable detergents to release the synthesized nanoparticles (Kalimuthu *et al*., 2008). This favors large-scale production of silver nanoparticles to explore its potential applications. Because of this, many studies focused on extracellular methods for the synthesis of metal nanoparticles (Duran *et al*., 2005). *Escherichia coli* (Gurunathan *et al*., 2009), *Staphylococcus aureus* (Nanda and Saravanan, 2009), *Bacillus megaterium* (Saravanan *et al*., 2011), *Bacillus cereus* (Sunkar and

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Nachiyar, 2012), *Salmonella typhimurium* (Ghorbani, 2013), *Serratia nematodiphila* (Malarkodi *et al.*, 2013), *Pseudomonas fluorescens* (Silambarasan and Jayanthi, 2013) etc., proved its property to form extracellular nanoparticles very effectively. Biofabrication of silver nanoparticles (AgNPs) has offered a consistent, nontoxic and eco-friendly approach for the management of plant diseases owing to their strong antimicrobial properties (Navrotsky, 2000; Hu *et al.*, 2006; Moonjung *et al.*, 2010). Phosphate solubilizing bacteria are found to be agriculturally important. As a result, the development and application of biosynthesized nanoparticles has opened new avenues in agricultural research oriented to developing eco-friendly and effective means of controlling plant diseases. Though several works regarding the synthesis of nanoparticles of a large number of bacteria have been made, no comprehensive work is their relating to the nanoparticles synthesis using phosphate solubilizing bacteria. Furthermore, considering the significance of agriculturally important microbes, their utilization to synthesize AgNPs with potent antimicrobial properties can certainly provide an alternate means for plant protection.

Therefore, the present investigation deals with the phosphate solubilizing bacteria *Pseudomonas aeruginosa* KUPSB12 mediated extracellular synthesis and characterization of silver nanoparticles and their biomedical application.

2. Materials and methods

2.1. Chemicals and Tested Bacteria

All the chemicals were purchased from Merck, India. All the chemicals used were of an analytical grade. The tested bacterial strains for the antibacterial activity were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Figure 1. Map of the sampling site.

2.2. Strain used for Silver Nanoparticles Synthesis

Pseudomonas aeruginosa KUPSB12, a phosphate solubilizing bacterial strain isolated from a jute mill effluent exposed area of river Ganga at Bansberia (22°58’17”N and 88°24’03”E), West Bengal, India has been used for the synthesis of silver nanoparticles (Figure 1). Previously, the bacterium was isolated and screened on Pikovskaya’s agar medium by pour plate technique (Pikovskaya, 1948). After 48 h of incubation, discreet colonies showing halo zones were picked up with an inoculating needle and reinoculated in Pikovskaya’s broth for further plating and isolation by streaking on Pikovskaya’s agar. The methods were followed three times to procure a pure colony of phosphate solubilizing bacteria. Physiological, morphological and biochemical tests of the selected bacterial strain were carried out for their identification as per the procedures outlined in Bergey’s Manual of Determinative Bacteriology (Holt *et al.*, 1994) (Figure 2). The bacterium was also characterized based on 16S rRNA technique and the sequence has been submitted to the Genbank with the accession number KJ131180 (Thompson *et al*., 1997).

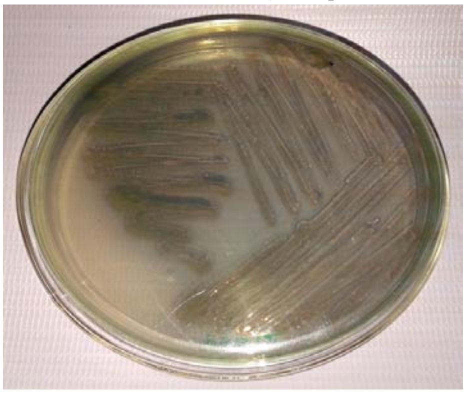

Figure 2. Pure culture of *Pseudomonas aeruginosa* KUPSB12 used for synthesis of AgNPs

2.3. Extracellular Synthesis of Bacterial Silver Nanoparticles

The *P. aeruginosa* KUPSB12 strain was freshly inoculated in an Erlenmeyer flask containing 100 ml nutrient broth. The flasks were incubated in orbital shaker at 37^{o}C and agitated at 200 rpm for 24 h. After incubation, the cell filtrates were obtained by centrifugation at 10,000 rpm for 10 min and followed by decantation. The final concentration of 1 mM $AgNO_3$ was added in to 100 ml of cell filtrate in 250 ml Erlenmeyer flask. The flasks were incubated in a dark room condition up to 48 h. The control was maintained without addition of $AgNO_3$ with the experimental flask containing cell filtrate. The brown colored solution of silver nanoparticles was stored under ambient condition for further characterization and applications.

2.4. Characterization of Silver Nanoparticles

The bioreduction of the Ag^+ ions in the solution was monitored by changes in color. The absorption spectrum of this solution was recorded using a UV–visible spectrometer (Shimadzu UV-2450) from 300 nm to 800 nm at regular intervals. Further characterization of AgNPs involved Fourier Transform Infrared Spectroscopy (FTIR) by scanning the spectrum in the range 400–4000 cm^{-1} at resolution of 4 cm^{-1}. To reveal the shape and the size, AgNPs Scanning Electron Microscopic (SEM) analysis was applied using Hitachi S-4500 SEM machine.

2.5. Antibacterial Activity

Antibacterial activity was performed with synthesized silver nanoparticles by Well diffusion method against three Gram negative (*Escherichia coli* MTCC 443, *Vibrio cholerae* MTCC 3904 and *Shigella flexneri* MTCC 1457) and three Gram positive (*Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 3160 and *Micrococcus luteus* MTCC 1538) bacteria. The bacterial cultures were brought into broth culture for antibacterial assay. Approximately 7 mm diameter of well was made on Mueller Hinton agar plate with the help of sterilized cork borer. The cultures were uniformly spread on solid culture media with the help of sterilized glass spreader. 25 µl of synthesized AgNPs were poured into the well, and then the plates were incubated for 37^{O} C for 24 h and the zones of inhibition were measured.

2.6. Statistical Analyses

All experiments were carried out in triplicate, and the results were expressed as the mean. Means and standard deviations (SD) were analyzed by using the SPSS 13.0 software package.

3. Results and Discussion

A study on extra-cellular biosynthesis of AgNPs by the culture supernatant of *Pseudomonas aeruginosa* KUPSB12 was carried out in this work. Physiological, morphological and biochemical characteristics of isolate KUPSB12 were outlined in Table 1. On the basis of above characteristics as well as 16S rRNA study, the isolate was identified as *Pseudomonas aeruginosa.*

Visual observation of the culture supernatant incubated with $AgNO_3$ showed a color change from greenish yellow to brown (Figure 3). The appearance of a brown color in $AgNO_3$-treated culture supernatant due to reduction of silver ions suggested the formation of AgNPs (Priyadarshini *et al*., 2013; Ranjitham *et al*., 2013). This supports the fact that change in color as observed in the experiment can be considered as an indication of AgNPs formation.

The confirmation of the particle synthesis and stability of the AgNPs in colloidal solution was monitored by UV–vis spectral analysis for which aliquots of the reaction mixture (after completion of the reaction) were withdrawn and used for UV–vis spectroscopy measurements. In the UV–vis absorption spectrum, a strong, broad peak, located at about 442 nm, was observed for nanoparticles synthesized using the culture supernatant (Figure 4). This peak indicated a surface plasmon resonance (SPR), which has already been well documented for various metal nanoparticles with sizes ranging from 2 nm to 100 nm (Henglein, 1993; Ravindra and Rajasab, 2014). As evident from previous reports, the presence of single SPR peak indicates spherical shape of AgNPs which was further confirmed by scanning electron microscopy (Kanchana *et al.*, 2011).

Table 1. Morphological, physiological and biochemical characteristics of *Pseudomonas aeruginosa* KUPSB12

Characters/tests	*Pseudomonas aeruginosa* KUPSB12
Cell shape	Rod
Gram reaction	-
Motility	+
Growth at 5% NaCl	+
Catalase	+
Oxidase	+
IMViC test	
Indole production	-
Methyl red	-
Voges-Proskauer	-
Citrate	+
Urease	-
H_2S production	-
NO_3^- reduction	-
Gelatine liquefaction	+
Starch hydrolysis	-
Hugh-Leiffson (O/F) reaction	O/F
Utilization of carbon source	
Glucose	+
Fructose	+
Sucrose	+
Raffinose	-
Cellobiose	-
Xylose	+
Mannitol	-
Sorbitol	-
Dulcitol	-

+ indicates presence or positive; - indicates absence or negative; O= Oxidation; F=Fermentation

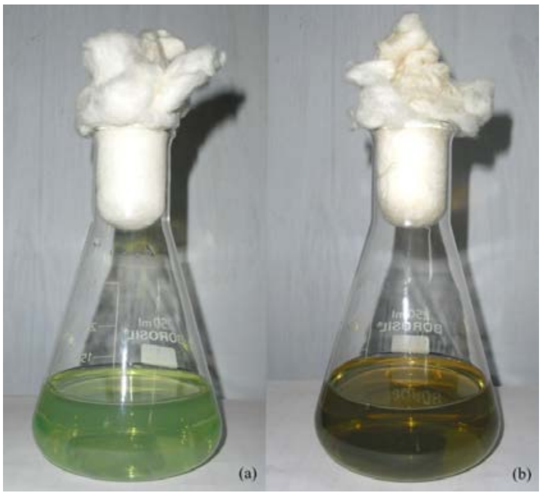

Figure 3. (a) Cell filtrate of *Pseudomonas aeruginosa* KUPSB12 without silver nitrate (control), (b) cell free extract with $AgNO_3$ after 24 h incubation**.**

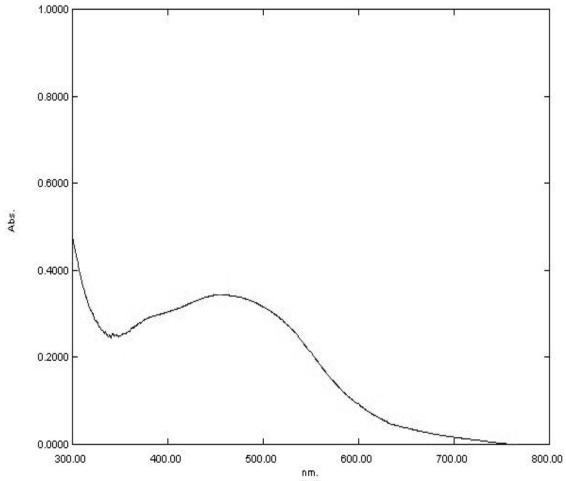

Figure 4. UV-visible spectra of synthesized silver nanoparticles

To explore the reduction process of $AgNO_3$ by the culture supernatant of *P. aeruginosa*, FTIR measurements were carried out to identify possible interactions between silver salts and protein molecules, which could account for the reduction of Ag^+ ions and stabilization of AgNPs (Figure 5). The amide linkages between amino acids residues in proteins give rise to the well known signatures in the infrared region of the electromagnetic spectrum. The bands seen at 3449.08 cm^{-1} and 2633.72 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines respectively. The band observed at 1863.63 cm^{-1} is characteristic of –C=O carbonyl groups and –C=C stretching. The band seen at 1487.23 cm^{-1} is due to amine group. The overall FTIR pattern confirms the presence of proteins in synthesized nanoparticles. The free amine and carbonyl groups present in the bacterial protein could possibly perform the function for the formation and stabilization of silver nanoparticles (Babu and Gunasekaran, 2009; Balaji *et al*., 2009). Thus, the higher stability of the synthesized AgNPs could be attributed to the complex nature of the *Pseudomonas aeruginosa* KUPSB12 strain culture supernatant (Malhotra *et al.*, 2013; Mishra *et al.*, 2014).

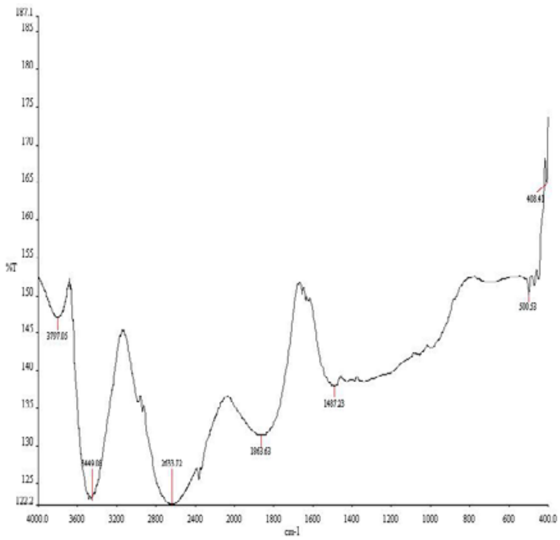

Figure 5. FTIR spectra of synthesized silver nanoparticles

Scanning electron microscopy (SEM) was used to determine the size and shape of the synthesized nanoparticles. SEM images revealed the average size of particles as 50-85 nm. SEM images show that they are relatively uniform in diameter and have a spherical shape (Figure 6). The size ranges of silver nanoparticles produced by the *P. aeruginosa* KUPSB12 fall closer to the size of silver nanoparticles produced by other bacteria (Shahverdi *et al*., 2007; Das *et al.*, 2014).

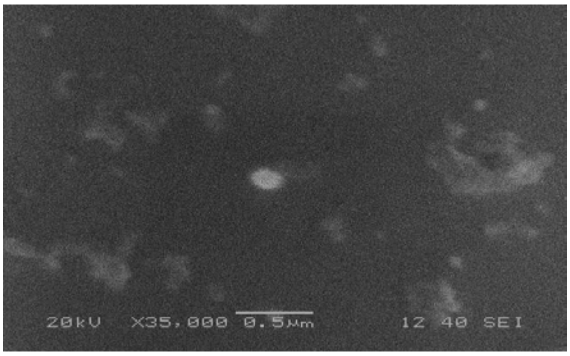

Figure 6. SEM image of synthesized silver nanoparticles

The antibacterial activities of synthesized silver nanoparticles were tested against six pathogenic bacteria as shown in Table 2. The silver nanoparticles exhibited antibacterial activity against both Gram positive and Gram-negative bacteria. The highest inhibition zone of 19.0 mm diameter was formed against *Escherichia coli* and the lowest of 13.6 mm was produced against *Staphylococcus aureus* by the synthesized nanoparticles. In general, Ag ions from nanoparticles are believed to become attached to the negatively charged bacterial cell wall and lyse it, leading to protein denaturation and finally cell death (Lin *et al.*, 1998). Priyadarshini *et al.* (2013) reported that the Gram negative bacterium *E. coli* showed a greater antibacterial activity compared to that of the Gram positive bacteria *Bacillus cereus* and *Streptococcus pyogenes* which was probably due to their thick cell walls.

Table 2. Antibacterial activity of synthesized silver nanoparticles against tested pathogenic bacteria (mean ± SD)

Tested bacteria	Zone of inhibition (mm in diameter)
Escherichia coli	19.0 ± 0.24
Vibrio cholerae	15.3 ± 0.28
Shigella flexneri	16.0 ± 0.34
Bacillus subtilis	17.6 ± 0.21
Staphylococcus aureus	13.6 ± 0.36
Micrococcus luteus	18.6 ± 0.18

The exact mechanism behind the extracellular synthesis of nanoparticles using microbes is not clearly established. But it is believed that enzymes like nitrate reductase secreted by microbes help in the bioreduction of metal ions to metal nanoparticles (Duran *et al*., 2005). Such a mechanism was found to be operative in *Bacillus licheniformis* where nitrate reductase secreted by the bacteria was found to be responsible for the reduction of Ag^+ to nanoparticles (Kalimuthu *et al*., 2008). Nangia *et al.* (2009) also suggested that the biosynthesis of nanoparticles and their stabilization via charge capping in *Stenotrophomonas maltophilia* involved NADPH-dependent reductase enzyme through electron shuttle enzymatic metal reduction process.

4. Conclusions

In conclusion, we have reported the simple biological way for synthesizing the silver nanoparticles using the culture supernatant of *P. aeruginosa* KUPSB12. The present investigation indicates the extracellular synthesis of highly stable silver nanoparticles. The results of FTIR suggested that the protein might have played an important role in the stabilization of silver nanoparticles. Synthesized silver nanoparticles showed a potent antibacterial activity against six pathogenic bacterial strains. These study results demonstrated that the phosphate solubilizing bacteria *P. aeruginosa* KUPSB12 is a cheap and environment-friendly bio-resource for the synthesis of silver nanoparticles with antibacterial activity. Considering the significance of phosphate solubilizing bacteria an agriculturally important microbes, their utilization to synthesize AgNPs with potent antibacterial properties can certainly provide an alternate means for plant protection. Further studies are required on fundamental understanding of the mechanism of nanoparticles synthesis at cellular and molecular levels.

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