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Research Paper

Antibacterial properties of silver nanoparticles synthesized by marine *Ochrobactrum* sp.

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Abstract

Metal nanoparticle synthesis is an interesting area in nanotechnology due to their remarkable optical, magnetic, electrical, catalytic and biomedical properties, but there needs to develop clean, non-toxic and environmental friendly methods for the synthesis and assembly of nanoparticles. Biological agents in the form of microbes have emerged up as efficient candidates for nanoparticle synthesis due to their extreme versatility to synthesize diverse nanoparticles with varying size and shape. In the present study, an eco favorable method for the biosynthesis of silver nanoparticles using marine bacterial isolate has been attempted. Very interestingly, molecular identification proved it as a strain of *Ochrobactrum anhtropi*. In addition, the isolate was found to have the potential to form silver nanoparticles intracellularly at room temperature within 24 h. The biosynthesized silver nanoparticles were characterized by UV-Vis spectroscopy, transmission electron microscope (TEM) and scanning electron microscope (SEM). The UV-visible spectrum of the aqueous medium containing silver nanoparticles showed a peak at 450 nm corresponding to the plasmon absorbance of silver nanoparticles. The SEM and TEM micrographs revealed that the synthesized silver nanoparticles were spherical in shape with a size range from 38 nm - 85 nm. The silver nanoparticles synthesized by the isolate were also used to explore its antibacterial potential against pathogens like *Salmonella typhi, Salmonella paratyphi, Vibrio cholerae* and *Staphylococcus aureus.*

Key words: biosynthesis, silver nanoparticles, *Ochrobactrum anhtropi*, purification, antibacterial activity.

Introduction

Nanoparticles exhibit unique properties because of their size, distribution and morphology compared with larger particles of the bulk material. Thus, development of environmental friendly methods for the synthesis and assembly of nanoparticles is of considerable importance to explore their potential applications (Babu and Gunasekaran, 2009). Among the various methods of nanoparticle synthesis, biological methods are much promising because of its effectiveness, flexibility and environment friendly approach. Considering the diverse biological applications of silver nanoparticles(AgNPs), it is necessary to explore various biological systems for AgNPs production. Many microbes including bacteria, yeast and fungi have been found to be capable of synthesizing silver nanoparticles. Among these microbes, bacterial systems are excellent option because they are easy to handle and can be manipulated genetically without much difficulty (Vaidhyanathan *et al.*, 2010).

In microorganism mediated nanoparticle synthesis, the reduced metal in its elemental form can get accumulated either intracellularly or extracellularly (Ahmad *et al.*, 2007; Kalimuthu *et al.*, 2008; Saifuddin *et al.*, 2009; Jain *et al.*, 2011, Janardhanan *et al.*, 2013). Studies on the biosynthesis of silver nanoparticles using the bacterium *Pseudomonas stutzeri* AG 259 isolated from silver mine demonstrated the intracellular synthesis of silver nano-

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particles with distinct size and morphology and was the first report on biosynthesis of AgNPs (Haefeli *et al.*, 1984; Klaus *et al.*, 1999). Later many bacterial species were studied for its ability to form nanoparticles. *Bacillus licheniformis* was shown to form AgNPs both intracellularly and extracellularly assisted by the enzyme nitrate reductase (Kalimuthu *et al.*, 2008). Culture supernatants of *Enterobacteriaceae* (*Klebsiella pneumoniae, Escherichia coli* and *Enterobacter cloacae*) were found to form silver nanoparticles rapidly with a size range of 28.2 to 122 nm by reducing Ag+ to Ag0(Shahverdi *et al.*, 2007). There are also several reports on the biosynthesis of AgNPs using bacteria like *Bacillus subtilis* (Kannan *et al.*, 2011), *Bacillus megaterium* (Saravanan *et al.*, 2011) and *Acinetobacter sp.* (Zaki *et al.*, 2011), *Bordetella sp*.(Thomas *et al.*, 2012a), *Pseudomonas aeruginosa* (Ramalingam *et al.*, 2013).

Biologically produced metal nanoparticles, especially silver in a nanometric scale (less than 100 nm) have received considerable application as antimicrobials, therapeutics and in biomolecular detection and catalysis (Christopher *et al.*, 2011; Elechiguerra *et al.*, 2005; Sadhasivam *et al.*, 2010; Shrivastava *et al.*, 2009; Wei *et al.*, 2008). This is due to their well-developed surface which provides maximum contact with the environment (Krutyakov *et al.*, 2008). Antimicrobial activity of silver nanoparicles against medically important pathogenic microorganisms such as *Bacillus subtilis*, *Enterococcus faecalis, Escherichia coli, Salmonella typhimurium* and *Candida albicans* were reported (Sadhasivam *et al.*, 2010). Extracellular silver nanoparticles synthesized using *Bacillus flexus* were proved to have antibacterial effect on clinically isolated multidrug resistant (MDR) *E. coli, B. subtilis, S. pyogenes* and *P. aeruginosa* (Priyadarshini *et al.*, 2013). Also silver nanoparticles generated extracellularly by *Bacillis megaterium*(NCIM 2326) were found to be effective against multi drug resistant clinical pathogens like *S.pneumoniae* and *S.typhi* (Saravanan *et al.*, 2011). Furthermore, methicillin-resistant *Staphylococcus aureus,* methicillin-resistant *Staphylococcus epidermidis* and *Streptococcus pyogenes* were found to be susceptible to silver nanoparticles with size ranges from 160-180 nm (Nanda and Saravanan, 2009).

Thus the studies on antimicrobial activity of AgNPs generated by diverse microorganisms are of considerable clinical significance.

Although silver nanoparticle formation by various microorganisms is well known, the potential of marine microbes for this property is least explored. In the present study, a bacterial isolate from marine water collected from Calicut beach was used for the synthesis of silver nanoparticles. Very interestingly, the molecular identification proved the isolate (CB2) as species of *Ocrobactrum anthropi* and biosynthesis of AgNPs using this marine isolate is least investigated. Also the biosynthesized AgNPs showed promising antibacterial activity against medically important pathogenic bacteria (*Salmonella typhi*, *Salmonella paratyphi*, *Vibrio cholerae* and *Staphylococcus aureus*).Thus this study highlights the importance of exploring diverse microbial communities for nanoparticle synthesizing properties and their potential as nanomedicine to manage pathogenic microorganisms.

Materials and Methods

Isolation of metal resistant bacteria

Seawater samples were collected from Calicut beach, Kerala, India. Samples were serially diluted in sterile 0.8% NaCl and were plated onto Marine agar (ZoBell Marine agar media - Peptone 5 g, Yeast extract 1 g, $FePO_4.4H_2O$ 0.01 g, Agar 15 g, Aged sea water 750 mL, and distilled water 250 mL pH 7.5) and incubated at room temperature for 48 h. The colonies obtained were further inoculated on marine agar supplemented with 1 - 20 mM concentrations of filter sterilized $AgNO_3$ and incubated at room temperature for 48 h for the screening of Ag resistant bacterial strains. After the incubation period, the resistant colonies formed were sub cultured and from this, a colony named as CB2 was randomly selected for nanoparticle synthesizing studies.

Molecular identification

Genomic DNA isolation of the selected isolate was conducted as per the method described by Ausubel *et al.* (1995). The isolates were cultured overnight in Luria-Bertani broth and the cells were collected by centrifugation. The cells were then resuspended in 567 µL of TE buffer followed by lysis using 30 µL of 10% SDS and 3 µL of 20 mg/mL protienase K. The mixture was then incubated for 1 h at 37 °C. After which the lysate was mixed thoroughly with 100 µL of 5 M sodium chloride and 700 µL chloroform: isoamyl alcohol (24:1) and this was centrifuged at 7,500 x *g* for 10 min. The aqueous layer was then transferred to a fresh tube and equal volume of isopropanol was added. This was then inverted several times and centrifuged again at 7,500 x *g* for 10 min. The pellet was washed in 70% ethanol (v/v) and air dried at room temperature. The dried DNA pellet was resuspended in 100 µL TE buffer and visualized on a 0.8% agarose gel (w/v). The isolated genomic DNA was used as template for PCR amplification of 16S rDNA using the primers 27F (5'-AgA gTTTgA TCM Tgg CTC-3') and 1525R (5'-AAg gAggTg WTC CAR CC-3') specific to 16S rDNA(Chun and Goodfellow., 1995). The PCR was carried out in a total volume of 50 µL containing 50 ng of genomic DNA, 20 pmoles of each primer, 1.25 units of *Taq* DNA polymerase, 200 µM of each dNTPs and 1X PCR buffer as components. The PCR was performed for 35 cycles in a Mycycler (Bio-Rad, USA) with the initial denaturation for 3 min at 94 °C, cyclic dena-

turation for 30 s at 94 °C, annealing for 30 s at 58 °C and extension for 2 min at 72 °C with a final extension of 7 min at 72 °C. After the PCR, the reaction product was analyzed by agarose gel electrophoresis. The product was then gel purified and was further subjected to sequencing PCR using the Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). This was then subjected to sequencing using ABI 310 Genetic Analyser.

The sequence data was checked for similarity analysis with BLAST program (Zhang *et al.*, 2000).

The phylogenetic analysis of the 16S rDNA sequence of the isolates obtained in the study was also conducted with MEGA 5 using neighbor-joining method with 1,000 bootstrap replicates (Tamura *et al.*, 2011).

Synthesis of silver nanoparticles

For nanoparticles synthesis studies, the bacterial isolate was freshly inoculated in an Erlenmeyer flask containing 100 mL marine broth. The flask was incubated in a rotating shaker at room temperature and agitated at 200 rpm for 48 h. After incubation, the biomass and supernatant were obtained by centrifugation at 12000 rpm for 10 min and were used separately for the synthesis of silver nanoparticles. For the extracellular production of silver nanoparticles, supernatants were mixed with filter sterilized $AgNO_3$ solution with a final concentration of 1 mM. For intracellular production; 2 g of bacterial wet biomasses were resuspended in 100 mL aqueous solution of 1 mM $AgNO_3$ in a 250 mL Erlenmeyer flask. Then the mixtures were kept on rotating shaker set at 200 rpm for a period of 72 h at room temperature in light. The heat killed biomass and heat inactivated supernatant incubated with silver nitrate and silver nitrate solution alone were also maintained as control. The bioreduction of Ag+ ions was monitored by changes in color. Also the optical characteristics of synthesized silver nanoparticles were measured using UV-visible spectrophotometer (Hitachi U5100) at 200-800 nm range with control as reference.

Purification of silver nanoparticles from biomass

For purification, the bacterial pellets were collected by centrifugation at 8,000 rpm for 10 min, resuspended in 0.9% NaCl solution, and centrifuged again at 8,000 rpm for 10 min. The washing process was repeated three times to ensure removal of any undesirable materials. The pellets were then transferred to a test tube and were disrupted by ultrasound sonicator. This was then resuspended and centrifuged (12,000 rpm) in a solution containing 0.5 M NaCl and 0.5 M sucrose and was finally resuspended in 35 mL of complete salt solution [NaCl (17.5 g/L), KCl (0.74 g/L), $MgSO_4$-$7H_2O$ (12.3 g/L), Tris HCl (0.15 g/L), and pH 7.5]. For the lysis of remaining cells, this was then treated with 20 mg of egg white lysozyme and incubated at 22 °C for 18 h. Then the nanoparticles were separated from the lysed mixture by wash with the complete salt solution and for further characterization and application, the cleaned nanoparticles were resuspended in deionized water and stored under ambient condition. This process of purification was conducted by methods described earlier (Oremland *et al.*, 2004).

Characterization of silver nanoparticles

Purified silver nanoparticles produced from biomass were air-dried and analyzed using SEM. SEM analysis of dried samples was performed by mounting nanoparticles on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined under JEOL 6390 SEM JSM at 10 kV. The morphological characteristics of silver nanoparticles were analyzed using TEM. Samples for TEM analysis were prepared on carbon-coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 min, the extra solution was removed using a blotting paper, and the grid was allowed to dry prior to measurement. TEM measurements were recorded using a JEOL-JEM-1011 instrument at 80 kV.

Analysis of antibacterial activity of AgNPs

The antimicrobial activity of AgNPs synthesized by CB2 was tested against *Salmonella typhi, Salmonella paratyphi, Vibrio cholerae* and *Staphylococcus aureus* by standard well diffusion method in Muller Hinton Agar (MHA) plates (Saravanan *et al.*, 2011). Pure cultures of bacterial pathogens were grown in Muller Hinton broth at 37 °C for 18-24 h. The MHA plates were inoculated by swabbing these bacterial pathogens to create a confluent lawn of bacterial growth. Wells were made on the Muller-Hinton agar plates using a gel puncture and 40 µL of the biosynthesized AgNPs solution from CB2, 40 µL of AgNPs synthesized by *Bacillus sp.* (positive control) and 1 mM $AgNO_3$ were added to separate wells. After incubation, the diameter of zone of inhibition was measured. The assays were performed in triplicates.

Results and Discussion

Water samples from Calicut beach were used as source material for the isolation of microorganisms with the potential to form silver nanoparticles. After screening for the resistance towards silver, the isolate named as CB2 showed higher resistance to silver nitrate and was selected for further investigation. The sequence data of the strain was subjected to BLAST analysis and the result of CB2 showed 100% identity to recently reported 16S rDNA sequence of *Ochrobactrum anthropi* strain (JQ435714). The 16S rDNA sequence of the isolates used in the study was also used for phylogenetic analysis and the result showed clustering of the 16S rDNA sequence of CB2 with the sequence of *Ochrobactrum anthropi* (Figure 1). So the isolate can be considered as strain of *Ochrobactrum anthropi* and

silver nanoparticle synthesis property of this species is least explored.

The synthesis of AgNPs by the isolate CB2 was monitored by visible observation of changes in color of biomass and supernatant in the presence of 1 mM $AgNO_3$ based on methods used for screening microbial isolates for silver nanoparticle synthesis (Kalimuthu *et al.*, 2008). The observations indicated that intracellular components of the strain reduced Ag+ ions as observed by change in color of the samples from pale yellow to brown within 24 h of incubation in the presence of light (supplementary data). This color change is due to the excitation of surface plasmon resonance of silver nanoparticles (Ahmad *et al.*, 2003, Kalimuthu *et al.*, 2008). It was also noted that the intensity of the brown color increased up to 24 h and was maintained throughout the 72 h period of observation. However, no extracellular reduction of Ag+ ions was observed with CB2 in the presence of light or in the dark. This observation indicates that the reduction of the Ag+ ions takes place intracellularly under visible-light irradiation. Visible light irradiation is known to have significant increasing effect on the biosynthetic rate of silver nanoparticles formation (Mokhtari *et al.*, 2009; Wei *et al.*, 2012, Thomas *et al.*, 2012 b). In addition, experimental controls like heat killed biomass and supernatant of CB2 incubated in presence of silver nitrate and silver nitrate solution alone showed no color changes. Thus, this visual observation of color changes in samples containing bacterial biomass can be taken as an indication of intracellular synthesis of silver nanoparticles. Similar results were previously reported for the biomass of *B. cereus* and *Bacillus sp.* due to the reduction of aqueous silver ions to silver nanoparticles (Babu and Gunasekaran, 2009; Das *et al.*, 2013).

The intracellular production of AgNPs in the reaction flasks were monitored by UV-Vis spectral analysis, which is a valuable tool for the preliminary characterization of

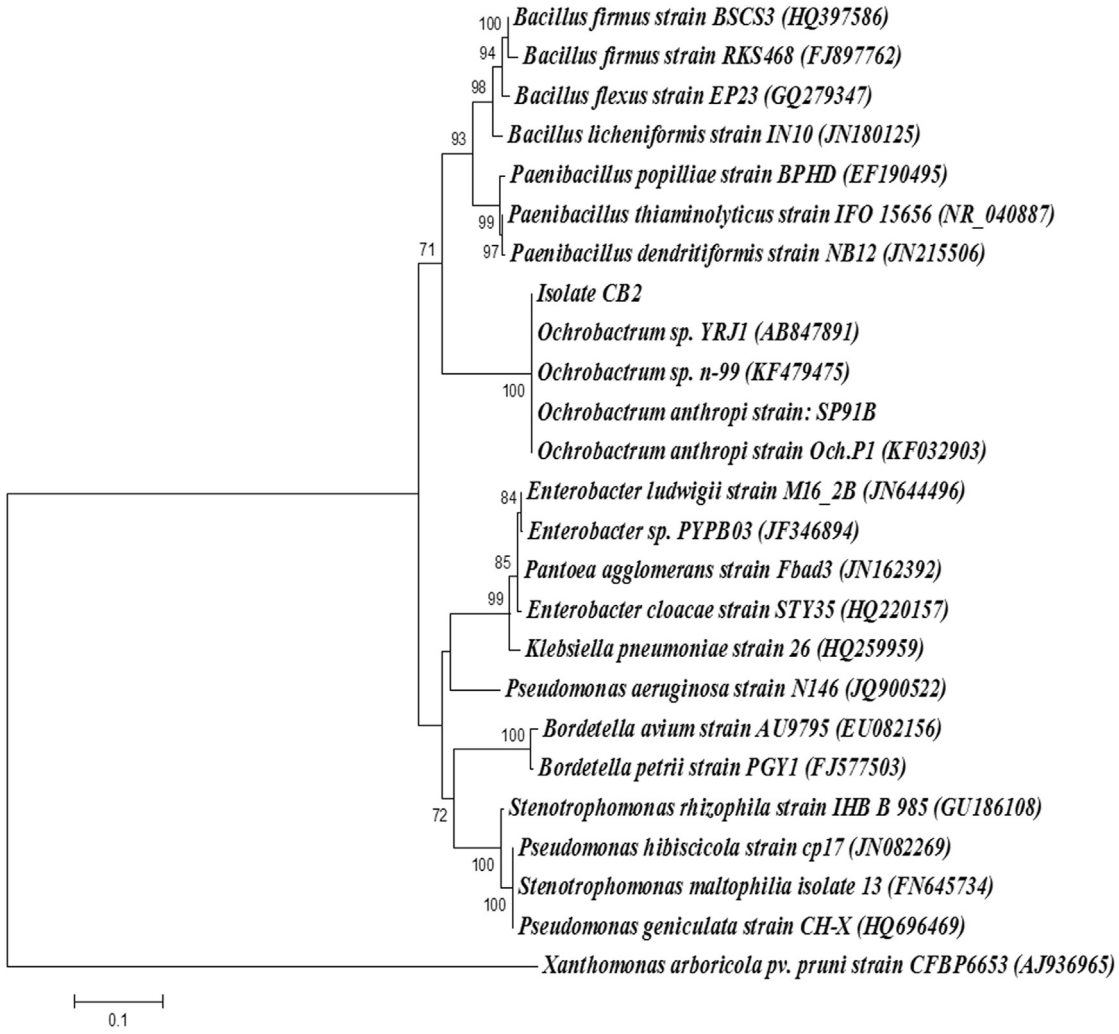

Figure 1 - The phylogenetic analysis of the 16S rDNA sequence of the marine bacterial isolate CB2 obtained in the study along with other selected sequences from database. The analysis was conducted by constructing a rooted tree [outgroup used was *Xantomonas arboricola pv. pruni* strain CFBP 6653 (AJ93965)] using neighbor - joining method in MEGA4.

nanoparticles (Sastry *et al.*, 1998). UV- visible spectra of silver nanoparticle for the test samples was obtained within 24 h where a strong absorption band at 450 nm was observed for CB2 indicating the presence of AgNPs (Figure 2).The absorption spectra of silver nanoparticles exhibited an intense absorption peak range due to its surface plasmon excitation, which describes the collective excitation of conduction electron in metal. Presence of such peak assigned to a surface plasmon resonance of silver nanoparticles was previously reported in the case of *Bacillus licheniformis* and *Neurospora crassa* (Kalimuthu *et al.*, 2008; Longoria *et al.*, 2011).

For further confirmation of the presence of AgNPs, the samples were subjected to purification process and purified nanoparticles were resuspended in deionized water and were used for SEM and TEM analysis. The SEM micrograph of purified nanoparticle*s* showed the presence of silver nanoparticles at a magnification of 20,000 x and is shown in Figure 3 (a). Transmission electron microscopic analysis provided further insight into size and morphology of synthesized nanoparicles (Figure 3 (b)). The AgNPs were more or less spherical in shape in the TEM images with sizes ranges from 35 - 85 nm. The size ranges of silver nanoparticles synthesized by the marine isolate used in the study fall closer to the size of silver nanoparticles produced by other bacteria.

The antibacterial activity of biosynthesized silver nanoparticles was investigated against both gram positive (*Staphylococcus aureus)* and gram negative (*Salmonella typhi, Salmonella paratyphi* and *Vibrio cholerae*) bacteria and AgNPs synthesized by CB2 (*Ochrobactrum anthropi*) showed excellent antibacterial activity against all tested bacterial strains at the volume of 40 µL/well. In order to compare the activity of CB2, AgNPs synthesized by another *Bacillus* strain standardized in our laboratory (unpublished data) was used. For *Salmonella typhi*, AgNPs synthesized by *Bacillus sp.* showed zone of inhibition of 13 mm and that from CB2 showed 14 mm zone of inhibition, for *Salmonella paratyphi* it was 17 mm for *Bacillus sp.* and 15 mm for CB2, for *Vibrio cholerae* it was 17 mm for *Bacillus sp.* and 16 mm for CB2. In the case of *Staphylococcus aureus* AgNPs from both *Bacillus sp.* and CB2 showed 15 mm zone of inhibition (Table 1). There is no zone of inhibition around the well filled with 1 mM $AgNO_3$ and this indicates the antimicrobial activity is due to biosynthesized silver nanoparticles. These findings support the previous reports of antibacterial activity of silver nanoparticles against *Salmonella typhi* (Saravanan *et al.*, 2011; Sadhasivam *et al.*, 2010), *Staphylococcus aureus* (Nanda

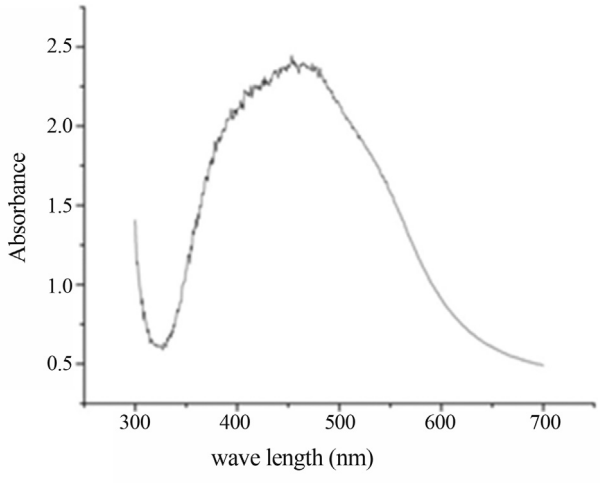

Figure 2 - The UV-Vis absorption spectrum of silver nanoparticles synthesized by Ochrobactrum sp. The absorption spectrum of silver nanoparticles exhibited a strong broad peak at 450 nm.

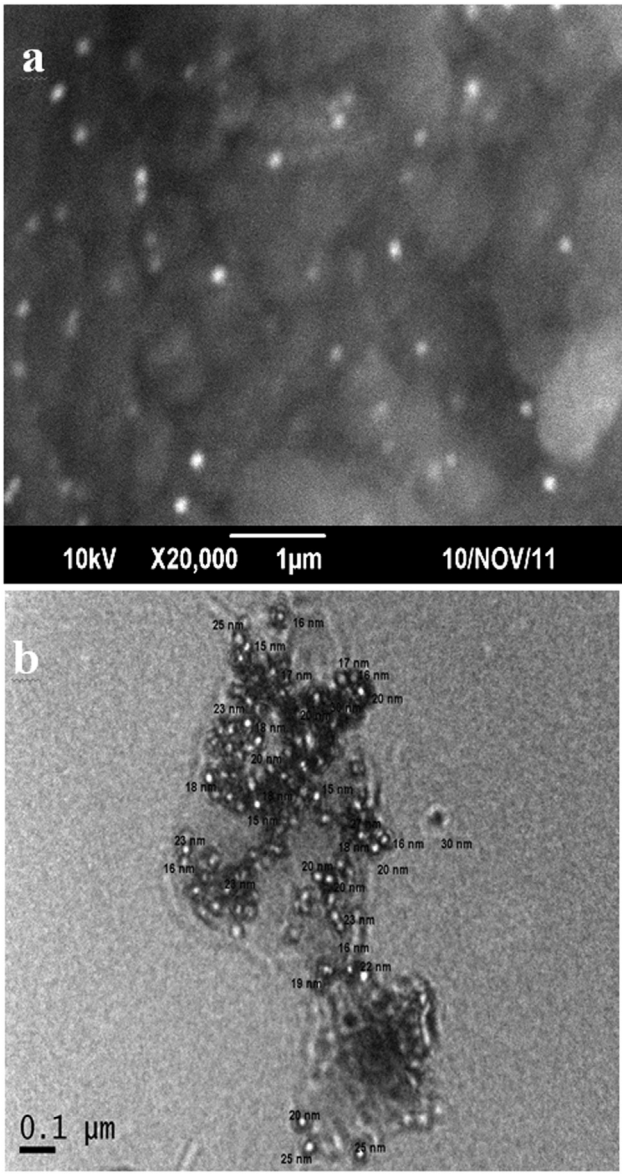

Figure 3 - Scanning Electron Microscopy (a) and Transmission electron microscopy (b) image of silver nanoparticles synthesized by *Ochrobactrum* sp.

Table 1 - Diameter of zone of inhibition by biosynthesized AgNPs against pathogenic bacteria.

Bacteria	Zone of inhibition (mm)		
	AgNPs synthesized by *Ochrobactrum anthropi*	AgNPs synthesized by *Bacillus* sp.	Control ($AgNO_3$)
S. aureus	15	15	No zone
S. typhi	14	13	No zone
S. paratyphi	15	17	Nozone
V. cholerae	16	17	No zone

and Saravanan, 2009) and *Vibrio cholerae* (Renugadevi and Aswini, 2012). The bactericidal activity of silver nanoparticles is due to their small size and large surface area to volume ratio (Baker *et al.*, 2005).

Conclusion

The nanoparticle synthesizing ability can vary among various microbial isolates. Also changes in physico-chemical properties of the nanoparticles generated through diverse sources can directly affect its applications. Therefore, isolation of novel bacterial isolates from unexplored sources is a promising approach for the identification of bacteria with the potential to form nanoparticles. The marine bacterial isolate used in the study was identified as *Ochrobactrum anthropi* strain. This isolate was found to have the ability to form silver nanoparticles with activity against pathogens, which indicates its potential applications. This also make it sure the biological production of metallic nanoparticles as promising alternative to the known and established physical and chemical methods.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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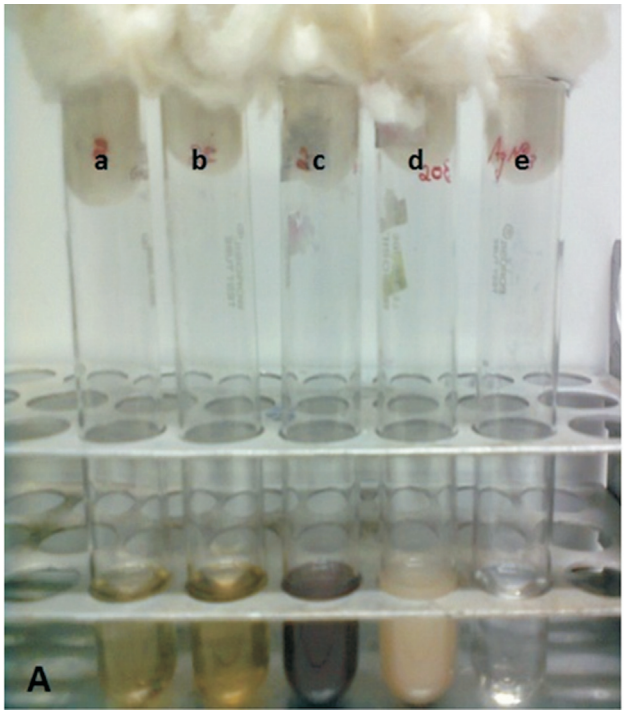
Supplementary Material

Figure S1 - Visual observation of the biosynthesis of silver nanoparticles by CB2 isolate selected for the study along with control after 24 h of incubation. (a) supernatant with $AgNO_3$ solution (no color change). (b) heat inactivated supernatant with $AgNO_3$ solution (no color change). (c) bacterial biomass with $AgNO_3$ solution (color change from pale yellow to brown). (d) heat inactivated biomass with $AgNO_3$ solution (no color change). (e) $AgNO_3$ solution alone (no color change).



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