Materials Science-Poland, 31(2), 2013, pp. 173-179
http://www.materialsscience.pwr.wroc.pl/
DOI: 10.2478/s13536-012-0085-1

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Biosynthesis of silver nanoparticles by a *Bacillus sp.* of marine origin

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This study was aimed to explore the nanoparticle synthesizing properties of a silver resistant *Bacillus sp*. isolated from a marine water sample. The 16SrDNA sequence analysis of the isolate proved it as a *Bacillus* strain. Very interestingly, the isolate was found to have the ability to form intracellular silver nanoparticles at room temperature within 24 hours. This was confirmed by the UV-Vis absorption analysis which showed a peak at 430 nm corresponding to the plasmon absorbance of silver nanoparticles. Further characterization of the nanoparticles was carried out by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis. The presence of silver nanoparticles with the size less than 100 nm was confirmed. These particles were found to be extremely stable as confirmed by the TEM analysis after three months of purification. So, the current study is the demonstration of an efficient synthesis of stable silver nanoparticles by a marine *Bacillus* strain.

Keywords: *silver nanoparticle; biosynthesis; purification; marine Bacillus sp.*

1. Introduction

Nanoparticles are particles with a size range of 1 – 100 nm. Nanomaterials such as metal nanoparticles, metal oxides and semiconductor materials are well known for their unique and amazing properties [3]. Biological methods, especially those using microorganisms are considered as efficient methods for the synthesis of nanoparticles [13]. The emerging interest on microbial nanoparticle synthesis is caused by the fact that these synthesis processes take place at ambient temperature and pressure and are also environment friendly [6, 14] Due to their innate biosynthetic potential, microorganisms can act as biofactories for nanoparticles synthesis. The biosynthesized nanoparticles are of high importance in material research with potential applications in drug delivery, cancer treatment, gene therapy, DNA analysis, biosensors, enhancing reaction rates, magnetic resonance imaging (MRI) and as antimicrobial agents [22]. In the case of silver nanoparticles, they have various applications in optics, electronics and biomedicine [4, 5]. The diverse utility of silver nanoparticles is due to the difference in their sizes and shapes which can be better controlled by biological methods of synthesis. Thus, the physical and chemical properties of nanoparticles produced through various biological methods can have diverse functions and applications. Therefore, the exploration of novel microbial habitats for the identification of microorganisms with potential nanoparticle synthesizing properties is very attractive.

Many microorganisms aggregate inorganic material and form nanoparticles (NPs) intracellularly or extracellularly [1, 2, 14]. The studies on reduction of Ag^{+} ions to silver nanoparticles by the bacterium *Pseudomonas stutzeri* AG259, isolated from silver mine, gave the basic explanation of microbial potential

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to form nanoparticles [8, 15, 23]. Since then, various groups of microorganisms have been studied to understand the nanoparticle formation and properties. Studies on culture supernatants of *Pseudomonas proteolytica, Pseudomonas meridiana, Arthrobacter kerguelensis, Bacillus indicus etc.* proved their ability to form extracellular silver nanoparticles very effectively [18]. When the biomass of *Brevibacterium casei* was treated with aqueous solutions of chloroaurate ions and Ag^+ ions, it formed both Au nanoparticles (AuNP) and Ag nanoparticles (AgNP) with a size range of 10 – 50 nm, intracellularly [12]. In the case of fungi like *Verticillium*, *Fusarium oxysporum* and *Aspergillus flavus* it was shown that the AgNPs were synthesized in the form of a film, dispersed in the solution or accumulated on the cell surface [10, 18, 21].

Considering the great deal of microbial metabolic features, much diversity can be expected in the mechanisms of nanoparticle formation. So, investigating the nanoparticle synthesizing properties of the microorganisms from the least explored habitats, such as marine environment, can have promising applications. Thus, in the current study, a bacterial isolate obtained from marine water sample was studied for the synthesis of silver nanoparticles. The isolated strain was identified as a strain of *Bacillus sp.* by molecular methods. The microbial reduction of metal ions was routinely monitored by visual inspection as well as by measuring the UV-visible spectra of the resulting solution. Further characterization of the Ag nanoparticles formed was carried out by transmission electron microscopy and scanning electron microscopy analysis. This confirmed the biosynthesis of stable silver nanoparticles with a size range of 35 – 85 nm.

2. Experimental

2.1. Isolation of metal resistant bacteria

Seawater samples collected from Calicut beach were used as a source of bacteria. The samples were serially diluted in sterile 0.8 % NaCl and were plated onto marine agar medium containing 5 g peptone, 1 g yeast extract, 0.01 g $FePO_4 \cdot 4H_2O$, 15 g agar, 750 ml aged sea water, and 250 ml distilled water at pH 7.5. The plates were incubated at room temperature for 48 h. The colonies obtained were further sub cultured on marine agar supplemented with 1 to 20 mM concentration of filter sterilized $AgNO_3$. This was further incubated at room temperature for 48 h for the screening of Ag resistant bacterial strains. After the incubation period, the plates were observed for bacterial growth and the isolated colonies were sub cultured and prepared in the form of pure culture. Predominant bacterial colony with the highest resistance to $AgNO_3$ was selected for further studies.

2.2. Molecular identification

Molecular identification was carried out by 16S rDNA sequence based method. For this purpose, total genomic DNA was isolated from the selected metal resistant strain. Quality of the DNA was checked by agarose gel electrophoresis and quantified using spectrophotometer. Isolated genomic DNA was used as a template for polymerase chain reaction (PCR). Forward and reverse primers used for the PCR amplification of 16S rDNA were 27F (5'-AgA gTTTgA TCM Tgg CTC-3') and 1525R (5'-AAg gAggTg WTC CAR CC-3'), respectively. PCR was conducted in a 50 µL reaction volume 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. The reaction was performed for 35 cycles in a Mycycler^{TM} (Bio-Rad, USA) with the initial denaturation at 94 °C for 3 min, cyclic denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 2 min with a final extension of 7 min at 72 °C. The PCR product was analysed by agarose gel electrophoresis. The product was then gel purified and used as a template for sequencing PCR. Sequencing PCR was carried out using the Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequencing product was purified and the sequence

run was carried out in the DNA sequencer ABI 310 Genetic Analyser. The 16SrDNA sequence data thus obtained were aligned using BioEdit programme and subjected to BLAST analysis. The sequence was submitted to NCBI under the accession number JX949179. The phylogenetic analysis of the 16SrDNA sequence of the isolates obtained in the study was also conducted using neighbor-joining method in MEGA5 [20].

2.3. Synthesis of silver nanoparticles

For biosynthesis studies, the bacterial isolate was inoculated in to 250 ml Erlenmeyer flask containing 100 ml sterile marine broth. The cultured flask was incubated in a rotating shaker set at 200 rpm for 48 h at room temperature. After incubation, the culture was centrifuged at 12,000 rpm for 10 min and both the biomass and supernatant were collected and used separately for the synthesis of silver nanoparticles. For extracellular production of silver nanoparticles, supernatant was mixed with filter sterilized $AgNO_3$ solution at a concentration of 1 mM. For intracellular production 2 g of bacterial wet biomass was resuspended in 100 ml of 1 mM aqueous solution of $AgNO_3$ in a 250 ml Erlenmeyer flask. Then the mixtures were kept on a rotating shaker (200 rpm) at room temperature for a period of 72 h under visible light illumination. The biosynthesis of silver nanoparticles was supervised visually. As controls, the heat killed biomass and supernatant incubated with silver nitrate and silver nitrate solution alone were maintained. The optical characteristics of synthesized silver nanoparticles were measured using UV-visible spectrophotometer. The absorption spectra of the biosynthesized AgNPs were taken on UV-visible double beam spectrophotometer (Hitachi U5100) at a range of 300 nm to 700 nm with control as reference.

2.4. Purification of silver nanoparticles from biomass

For purification, the cell suspension containing silver nanoparticles was centrifuged at 8,000 rpm for 10 min. The pellet was collected and 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 the cells were disrupted by ultrasound sonicator. The product 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 a complete salt solution [NaCl (17.5 g/L), KCl (0.74 g/L), $MgSO_4 \cdot 7H_2O$ (12.3 g/L), Tris HCl (0.15 g/L), and pH 7.5]. Lyse the remaining cells with 35 ml of the complete salt solution containing 20 mg of egg white lysozyme by incubation at 22 °C for 18 h. The lysed mixture was rinsed away from the nanoparticles by washing and then it was centrifuged at 12,000 rpm with the complete salt solution and deionized water [16]. Finally the cleaned nanoparticles were resuspended in deionized water for further characterization.

2.5. Characterization of silver nanoparticle

Purified silver nanoparticles produced from the biomass were air-dried and analyzed using SEM and TEM. SEM analysis of the dried samples was performed by mounting the nanoparticles on specimen stubs with double adhesive tape and coating with platinum in a sputter coater and examining with JEOL 6390 SEM JSM at 10 KV. Size, morphology and stability of the silver nanoparticles were characterized by 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, then the excess of 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. Images of the silver nanoparticles were recorded at different magnifications.

3. Results and discussion

Among the various colonies formed on marine agar plates supplemented with 1-20 mM of $AgNO_3$, the predominant colony forming bacteria CB1 resistant to 20 mM $AgNO_3$ was selected

for exploring its potential to form nanoparticles. The selected isolate was further subjected to identification. Molecular identification of the bacterial strain was done by 16SrDNA sequencing based method. The sequence data was subjected to BLAST analysis and the result showed its maximum identity of 99 % to various *Bacillus sp.,* mainly *Bacillus firmus*. The 16SrDNA sequence of the isolates obtained in the study was also used for phylogenetic analysis; the result showed clustering of the 16SrDNA sequence of CB1 with the sequence of *Bacillus firmus* (result not shown). So, the isolate can be considered as a strain of *Bacillus sp.* which can be represented as *Bacillus sp.* CB1.

The formation of silver nanoparticles was primarily investigated through the visual observation of color change of biomass and supernatant in the presence of 1 mM $AgNO_3$. Positive result was observed only with bacterial biomass where the color change from pale yellow to brown occurred within 24 h of incubation in the presence of light (Fig. 1). Notably, the intensity of the brown color increased dramatically for the first 24 h and was maintained throughout the 72 hour period of observation. However, no colour change was observed in the tubes containing culture supernatant with 1 mM $AgNO_3$ solution neither in the presence of light nor in the dark. In addition, experimental controls like heat killed biomass and culture supernatant incubated in the presence of silver nitrate and silver nitrate solution alone also did not show any color change. Therefore, the color change observed in the samples containing bacterial biomass can be taken as indication of silver nanoparticle synthesis. The brown color is the result of excitation of surface plasmon vibration in the metal nanoparticles and is a typical feature of the silver nanoparticles. Similar change in color from pale yellow to brown was previously reported for the biomass of *B. licheniformis* due to the reduction of aqueous silver ions to silver nanoparticles [11].

The color change observed was further confirmed by UV-Vis spectral analysis as a part of primary characterization. UV-Vis spectroscopy has proven to be a very useful technique for the analysis of nanoparticles [17]. The UV-visible absorption spectra of silver nanoparticles could exhibit an intense absorption peak due to its surface plasmon excitation, which describes the collective excitation of conduction electrons in a metal [3]. In the UV-Vis absorption spectrum of biomass of the sample used, a strong, broad peak, located at about 430 nm, was observed indicating the presence of silver nanoparticles (Fig. 2). The presence of such peak, assigned to a surface plasmon, is well documented for metal nanoparticles [11]. Since the colour change was observed in the biomass of *Bacillus sp.* CB1 used in this study, it can be noted that the reduction of $AgNO_3$ to Ag metal nanoparticles by the bacterial isolate can be intracellular. This was further confirmed by the observation that after mixing the cell free supernatant from the bacterial culture with silver nitrate solution and incubating at room temperature for 72 h, there was no color change or silver nanoparticles production. Intracellular formation of silver nanoparticles by other *Bacillus sp*. was also reported previously [3].

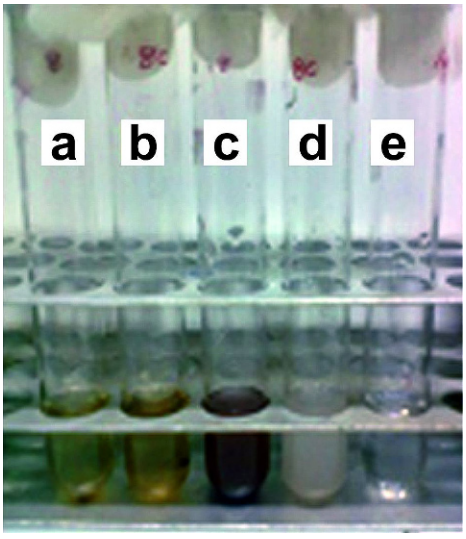

Fig. 1. Visual observation of the biosynthesis of silver nanoparticles by the *Bacillus sp.* CB1 after 24 h (a) supernatant with $AgNO_3$ solution, (b) heat killed supernatant with $AgNO_3$ solution, (c) bacterial biomass with $AgNO_3$ solution, (d) heat killed biomass with $AgNO_3$ solution, (e) $AgNO_3$ solution alone

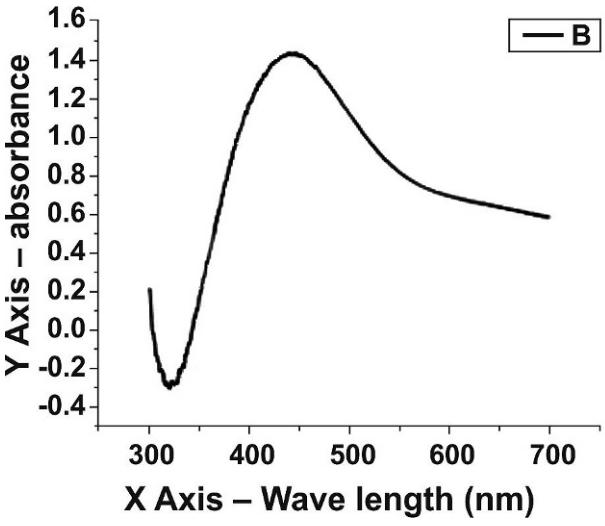

Fig. 2. The UV-Vis absorption spectrum of silver nanoparticles synthesized by biomass of *Bacillus sp*. CB1. The absorption spectrum of silver nanoparticles exhibits a strong broad peak at 430 nm.

For the analysis of nanoparticles formed by bacterial biomass and for the confirmation of the observation with UV-Vis spectrum data, the samples were subjected to purification process. The nanoparticles purified from the biomass were finally resuspended in deionized water and used for SEM and TEM analysis. The SEM micrograph of purified silver nanoparticles is shown in Fig. 3 c. It shows the presence of silver nanoparticles at a magnification of 20,000x. In this micrograph, silver nanoparticles in the size less than 100 nm can be observed. Similar observations were previously reported for the silver nanoparticles formed by other bacterial isolates [9, 17].

Transmission electron microscopic analysis was utilized to confirm and prove the previously detected morphology of silver nanoparticles using SEM. It also provided insight into the size and stability of the silver nanoparticles. The analysis revealed that the silver nanoparticles produced were generally spherical in shape and ranged in size from 35 to 85 nm (Fig. 3a). The morphology and size of the particles was also analyzed at higher magnification (Fig. 3b). The size of nanoparticles formed by various groups of microorganisms can vary. In the case of *Escherichia coli*, it was shown that the size of silver nanoparticles formed can range from 42.2 to 89.6 nm [7]. In the current study, the TEM analysis also proved that the nanoparticles formed were not in direct contact and this can be taken as an indication of good stabilization of the nanoparticles. The silver nanoparticles synthesized in this study were extremely stable because the TEM analysis was carried out after three months of their purification. The TEM analysis clearly confirmed that even after the long period of storage, there was no sign of aggregation of the silver nanoparticles. The particles might have been stabilized in the solution by the capping agent which may be the proteins present in the biomass. The role of proteins in the stabilization of silver nanoparticles, as reported in the case of *Bacillus cereus,* supports this observation [3]. All these properties confirm the promising potential of the silver nanoparticles formed by the *Bacillus sp.* CB1 identified in this study.

Microbial interaction with metals is the basis of its potential to form nanoparticles. In the current study a metal resistant bacterial strain was isolated from unexplored marine water samples. The selected isolate was found to have resistance to 20 mM silver nitrate. Interestingly, the molecular identification showed that the isolate belongs to a strain of *Bacillus.* So, the isolate was further subjected to exploring its nanoparticle synthesizing property. The biogenic method of nanoparticle synthesis used in the study is free from any toxic chemicals or solvents and is environmentally friendly. Also the detailed analysis of the synthesized nanoparticle by TEM analysis proved the size range of the formed particles and their stability which highlights the potential of the marine bacterial isolate identified in the study.

4. Conclusions

Microbial biogenesis of nanoparticles is an area of great scientific interest because of its promising opportunities. Silver nanoparticles have drawn much attention because of their extensive application to new technologies in chemistry, electronics, medicine and biotechnology. Microbially unexplored water samples from

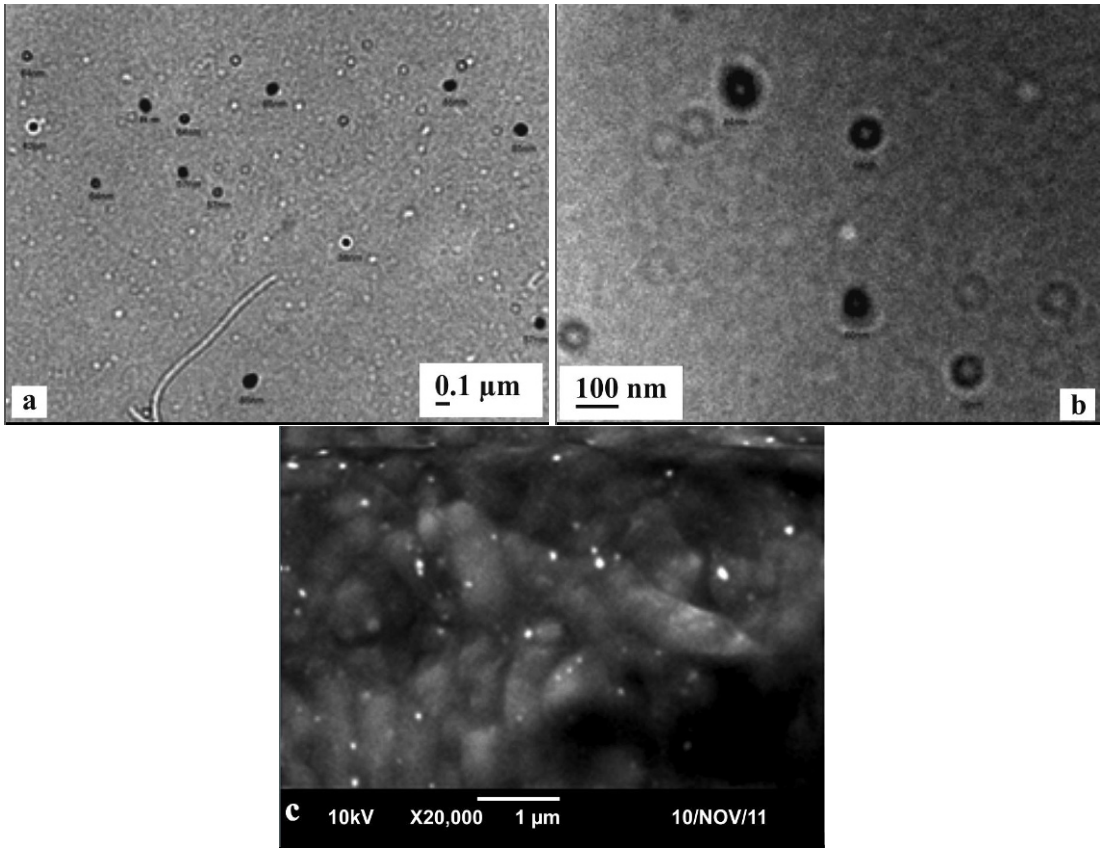

Fig. 3. (a) TEM image of the silver nanoparticles synthesized by *Bacillus sp.* CB1; (b) TEM image of silver nanoparticles at higher magnification; (c) SEM image of the silver nanoparticles synthesized by the *Bacillus sp.* CB1.

Calicut beach used in this study formed an ideal source for the selection of microorganisms with the potential to form nanoparticles. The results show that marine *Bacillus sp*. could be used effectively for the production of silver nanoparticles from silver nitrate.

Acknowledgements

The authors gratefully acknowledge School of Chemical Sciences, Mahatma Gandhi University, Kottayam for the help and support for the SEM analysis of samples.

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Received 2012-03-21
Accepted 2013-01-03