

Eco-friendly synthesis of gold nanoparticles by gold mine bacteria *Brevibacillus formosus* and their antibacterial and biocompatible studies

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Abstract: Nanotechnology is recently emerging in various fields especially in biomedical applications as many pathogenic organisms are gaining resistant against available antibiotics. In the present study, we have synthesized biocompatible therapeutic gold nanoparticles using *Brevibacillus formosus* isolated from the Hutti gold mine, India. Gold nanoparticles (AuNPs) synthesised by the reduction of gold chloride (HAuCl₄) using bacterial metabolites as reducing and stabilizing agents. The synthesized AuNPs are characterized by using UV-vis spectroscopy, Fourier transfer infrared spectroscopy (FTIR), Dynamic light scattering (DLS) and Transmission electron microscopy (TEM). The UV-vis spectrum of the AuNPs showed peak around 535 nm and presence of functional groups on AuNPs were confirmed by FTIR analysis. The size and shape of synthesized AuNPs were found to be spherical with an average particles size of 5-12 nm. A DLS result confirms the formation of monodispersed AuNPs. AuNPs were showed good antibacterial activity against gram positive *Staphylococcus aureus* and less activity against gram negative *E. coli*. Further, biocompatibility of biogenic AuNPs was verified by incubating with chicken RBCs and showed no hemolysis. The outcome of the study clearly indicates that as synthesized AuNPs are biocompatible and show good antibacterial activity could be effectively utilized in biomedical applications.

Key Words: Antibacterial activity, Eco-friendly synthesis, Gold nanoparticles, DLS, TEM.

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I. INTRODUCTION

There is an increasing industrial demand for the development of eco-friendly methods for the synthesis of metal nanoparticles due to their potential applications in the biomedical and pharmaceutical fields. Traditionally, nanoparticles are being synthesized by chemical and physical methods with controlled sizes and required chemical purity. However, physical methods consumes high level of energy during the synthesis process and in chemical methods use of plenty of toxic chemicals as well as production of hazardous wastes during the synthesis process limits their applications in medical fields. Hence, the development of eco-friendly and biocompatible process for AuNPs synthesis is required. Biological process by using microorganisms is emerging due to its normal experimental process and reduced use of toxic chemicals. Hence, it has been suggested as an alternative to chemical and physical methods [1]. Microbial synthesis of nanoparticles is eco-friendly and has greater advantages over other methods since it takes place at relatively at ambient temperature and pressure [2]. A number of microorganisms reported for the synthesis of AuNPs including actinomycetes, archaea, fungi and bacteria[3]. However, bacteria is considered potential candidate for the synthesis of nanoparticles due to the ease of handling and manipulation of genetic material [4]. Bacteria can survive in all kinds of environmental conditions, for example, low and high temperatures, alkalinity, acidity and high salt concentrations. Recently, all researchers in the world are trying to find out new extremophiles that can produce pure gold from its compounds. Some bacteria have developed the ability to resistant to foreign metal ions or metals; even at high metal ion concentrations some of these organisms can survive and grow[5]. The organism which resides in gold mines would be having more capability to resistant against soluble gold toxicity and produce gold nanoparticles in efficiently. Hence, recent days increasing demand for the screening of bacteria with tolerance to both insitu toxicity and efficient nanoparticles production. Most of the previous reports for the synthesis of AuNPs used bacterial cultures collected from culture collection centres and isolated from soil. For example, *Klebsiella pneumoniae* [6], *Streptomyces fulvissimus* [7], *Lactobacillus plantarum* [8] and *Deinococcus radiodurans* [1]. In recent research, reported for the synthesis of AuNPs by a *Arthrobacter*

isolated from Andalian gold mine, Iran [9]. In previous studies, bactericidal silver nanoparticles were synthesised by using *Lactobacillus spp* [10].

Biologically synthesised AuNPs are non toxic and biocompatible, which leads to use of these AuNPs in biomedical purpose including photothermal therapy, drug delivery, tumor imaging, sensing and antibacterial agents [11]. In recent days, most of the pathogenic bacterial strains have developed resistance through genetic mutations which accounts for considerable threat to mankind. Now, nonomaterials have emerging as novel antibacterial agents owing to their large surface area to volume ratio, physical and chemical properties increase their contact with pathogenic bacteria and their ability to penetrate inside the cell [12]. Biologically synthesised metal nanoparticles tested for their antibacterial potential [13][14].

In the present study, we have isolated several bacteria from gold mines and screened for AuNPs synthesis. Among different bacterial cultures one bacterium, *Brevibacillus formosus* having more potential for AuNPs synthesis. The synthesized AuNPs were characterized by UV-visible spectroscopy, FTIR, DLS and TEM analysis. These AuNPs studied for their antibacterial activity and biocompatibility with RBCs.

II. MATERIALS AND METHODS

2.1. Isolation of bacteria

Soil samples collected from the Htti gold mines, Karnataka state, India and bacteria isolated by serial dilution plating method. The samples were inoculated onto nutrient agar plates and incubated at 37 °C for 24 h. The colonies obtained after incubation were further sub cultured and preserved under 4 °C for further use.

2.2. Screening for AuNPs synthesis

The different cultures obtained from the above procedure inoculated into sterile nutrient broth and incubated for 24 h at 37 °C. Then the cultures were centrifuged to separate cells at 8000 rpm for 10 min and obtained cell free supernatants were used for AuNPs synthesis [15]. Bacterial supernatants were mixed with 1 mM Hydrogen tetrachloraurate (HAuCl₄) and the resulting experimental solutions were heated in a microwave oven. The control tube which lacked cell free supernatant incubated under the same experimental conditions. The tubes which observed formation of ruby red color were selected for further study. Among different cultures, based on the reduction efficiency, we have selected potential isolate for further synthesis, characterization and applications studies of AuNPs. After synthesis, nanoparticles were separated from the colloidal suspensions by centrifugation at 14,000 rpm for 10 min to use characterization and applications studies.

2.3. Identification of potential bacteria

Active strain for AuNPs synthesis was identified based on 16S rRNA gene sequencing analysis. For this the bacterial DNA was isolated according to the previously reported protocol [16]. After DNA sequencing, the obtained results were subjected to BLAST analysis to compare with the sequence similarities.

2.4 Characterization of AuNPs

Synthesis of AuNPs was confirmed by using UV-visible spectroscopy by measuring the spectra from 400 to 700 nm. Functional groups in the synthesised AuNPs were studied by FTIR equipped with a horizontal attenuated total reflectance. For this the samples were dried and analysed for the presence of functional groups recording with wavelength 4,000 - 400 cm⁻¹ at resolution 4 cm⁻¹. To study the dispersity and size distribution of AuNPs in colloidal suspension DLS was used. The size, shape and distribution of AuNPs were characterized by TEM analysis. Samples prepared for TEM analysis by adding a drop of synthesised gold colloidal suspension on a 200 mesh carbon coated copper grid and dried before observations.

2.5. Antibacterial activity

Antibacterial property of AuNPs was studied by agar well diffusion method [17]. The synthesised AuNPs were investigated against gram positive bacterium *Staphylococcus aureus* and gram negative bacteria *E.coli*. The pathogenic bacterial cultures obtained from Microbial type culture collection centre (MTCC), Chandigarh. The pathogenic bacteria cultures were uniformly spread on a solidified MHA plates and wells were made by using a cork borer. Each well was filled with different concentrations (50,100,150 and 200 µg/ml) of biogenic AuNPs against the pathogenic bacteria. The plates were incubated at 37 °C for 24 h and observed for clear zone of inhibition around the well. We have prepared separate control plate for each pathogenic bacterium and wells were filled with tetracycline (positive control), saline (negative control) bacterial supernatant and HAuCl₄.

2.6. Hemocompatibility assay

Hemolytic assay was performed according to earlier reports [18]. To perform haemolytic assay chicken blood was collected and separated RBCs by centrifugation at 1,500 rpm for 10 min. Supernatant was removed

and RBCs were washed repeatedly with sodium saline (0.85%). The pellet was making up to 20 ml with saline and 1 ml of RBCs was mixed with two different concentrations of AuNPs (150 and 200 µg/ml). Sodium saline was used as negative control and distilled water was used as positive control. All samples were incubated at 37 °C for 4 h and after incubation samples were centrifuged at 14,000 rpm for 10 min to remove AuNPs. The resultant supernatant was measured at 540 nm in UV-vis spectroscopy and percentage of hemolysis was calculated by using the following formula-

$$\% \text{Haemolysis} = \frac{\text{Absorption of test sample} - \text{Absorption of negative control}}{\text{Absorption of positive control} - \text{Absorption of negative control}} \times 100$$

III. RESULTS AND DISCUSSIONS

3.1. Visual observation and UV-visible spectroscopic analysis

Different bacterial cultures isolated from gold mines selected for the synthesis of AuNPs. Among different isolates one strain was selected as an active species based on intensity of color change from yellow to red and UV-visible spectrum. There was no color change observed in a control tube indicates the synthesis of AuNPs occurs only in the presence of biomolecules. A sharp and high intense peak observed in the UV- visible spectrum at 535 nm clearly indicates the formation of monodispersed AuNPs as shown in Fig.1.

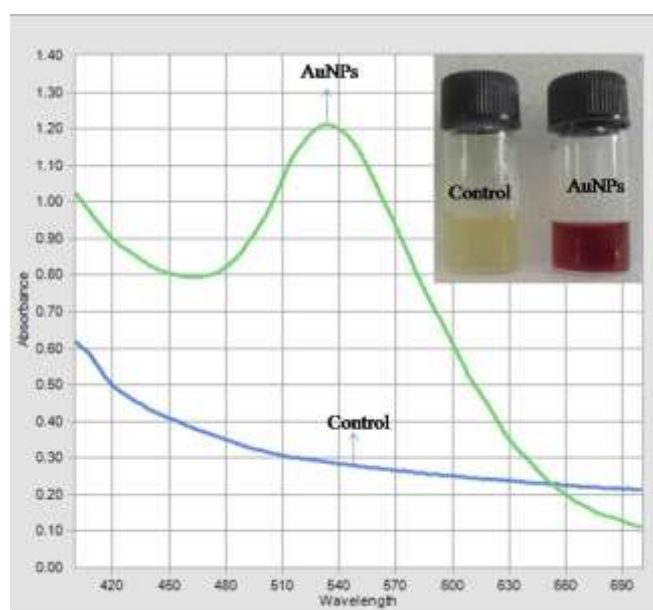


Figure (1): 1 UV-visible spectrum of biogenic gold nanoparticles and inset photo colloidal solution of AuNPs.

The exact position of the peak depends on dielectric constant of medium and particles size. The peak of AuNPs usually has a range of 520-560 nm in colloidal solution depending on the dispersity and size of the nanoparticles. With increasing the size of the AuNPs, the peak move towards longer wavelength and the sharp peaks indicates the uniform size and broader peaks indicates polydispersity of synthesised AuNPs in colloidal solutions [19]. In the present report, a single sharp high intense peak was observed is an evidence for spherical shaped AuNPs formation. Synthesis of AuNPs by using bacteria has been previously reported by several researchers obtained the UV- visible spectrum between 530-560 nm.

3.3. FTIR studies

Identification of biomolecules responsible for the synthesis and stabilization of AuNPs was analyzed by FTIR studies. FTIR studies show the absorption peaks at 3358, 1635, 657, 611 and 563 cm^{-1} (Fig. 2) for biosynthesized AuNPs by *Brevibacillus formosus*.

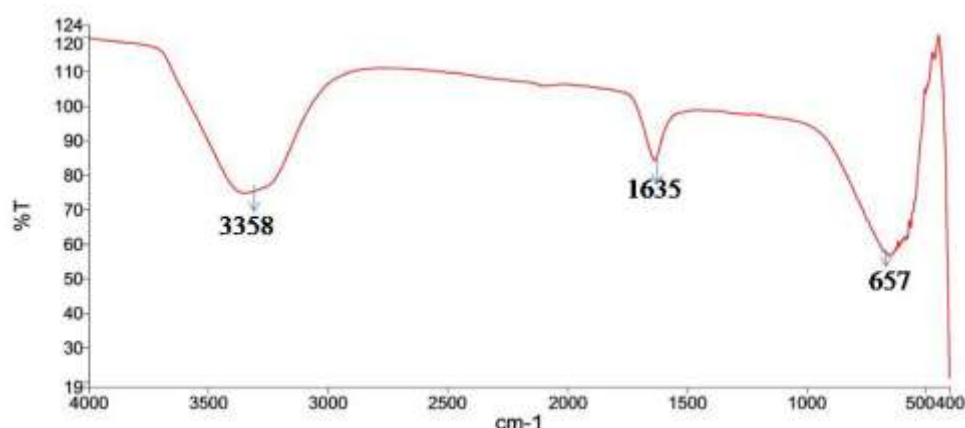


Figure (2): FTIR peaks of biogenic gold nanoparticles

A strong broad peak at 3358 cm^{-1} confirms the involvement of N-H stretch in the reduction of gold ions to gold nanoparticles [6]. The strong narrow peak at 1635 cm^{-1} confirms the role of amide group involved in the reduction, most probably from proteins present in the cell free supernatant [20]. A narrow peak at 657 cm^{-1} there may be corresponds to amino groups or aromatic groups from bacterial supernatant [21]. The presence of the above mentioned functional groups confirms the involvement of biomolecules in the reduction and stabilization of biologically synthesised AuNPs. This study also helps in further functionalization of biogenic AuNPs with other molecules for biomedical applications. The obtained result proves that the functional groups bind to biosynthesized metal nanoparticles and formed capped AuNPs to prevent aggregation [22]. It is found that the cell free supernatant of *Brevibacillus formosus* performs both reduction and stabilization of AuNPs.

3.4. Dynamic light scattering analysis

The DLS analysis is used to measure the shell thickness of stabilizing or capping molecules of the metallic particles along with their original size of the metallic core [23]. The size and distribution of AuNPs synthesized by *Brevibacillus formosus* is shown in Fig. 3.

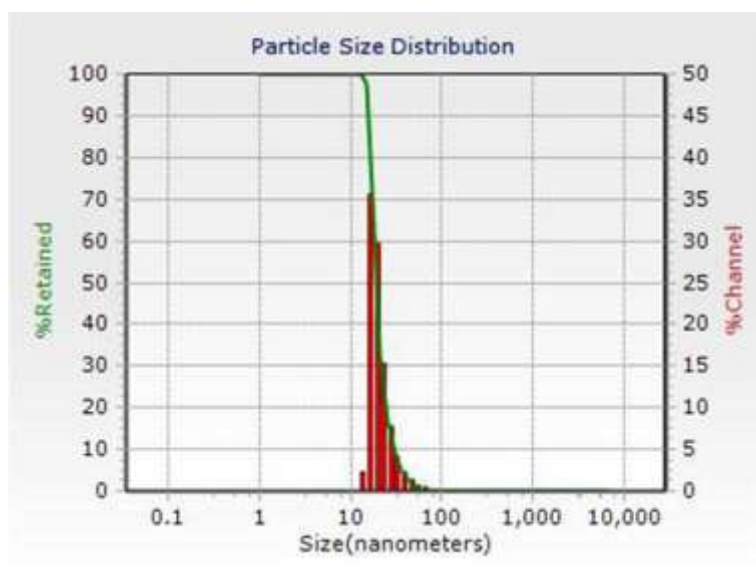


Figure (3): Size distribution of gold nanoparticles.

As shown in the figure the synthesized AuNPs are highly monodispersed and average size of nanoparticles was ranges from 15 to 30 nm and majority of the nanoparticles have the size of 19 nm, which matched with the size of AuNPs observed by TEM analysis. Synthesis of small and monodispersed AuNPs by biological methods has been difficult task in nanobiotechnology. The particles size obtained by the DLS analysis is different because it gives the particles average size. The nanoparticles size obtained from XRD, TEM and DLS is different, because of variation in principles used for analysis. The larger particles size observed by DLS analysis is due to the biomolecules enveloping the core of the AuNPs [24].

3.5. TEM analysis

The size, shape and morphology of AuNPs synthesised by *Brevibacillus formosus* was measured by TEM analysis. The results are shown in the Fig. 4, obtained from two different magnifications of AuNPs deposited on a copper grid. The synthesised nanoparticles are spherical and uniform size distribution with particles size ranging from 5-12 nm. Most of the synthesized AuNPs were not aggregated and are not in contact with each other. All the nanoparticles are spherical and small in size indicates the efficient formation of AuNPs by *Brevibacillus formosus*. This type of monodispersed spherical nanoparticles synthesised only when the supernatant has efficient reducing and stabilizing property [22].

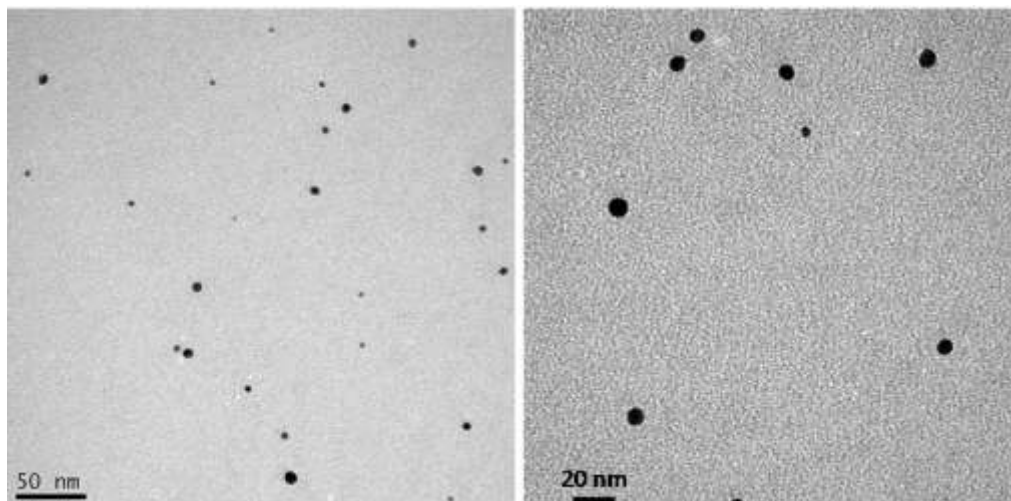


Figure (4): TEM image of gold nanoparticles synthesised by *Brevibacillus formosus*

3.6. Identification of Active bacteria

Identification metabolically active isolate for AuNPs synthesis was characterized by 16S rRNA gene sequences similarity with related bacterial species. After the analysis of gene sequences of the active strain, which was 99 % matching with *Brevibacillus formosus*. The gene sequence results were deposited in Genbank and obtained accession number KX853207.

3.7. Antimicrobial activity of AuNPs

The antibacterial property of the biogenic AuNPs against the tested pathogens was assessed on the basis of zone of inhibition. From the obtained results (Fig. 5 and Table 1), it has been observed that the AuNPs exhibited high antibacterial activity against Gram positive bacteria (*S. aureus*) compared to Gram negative (*E.coli*) bacteria.

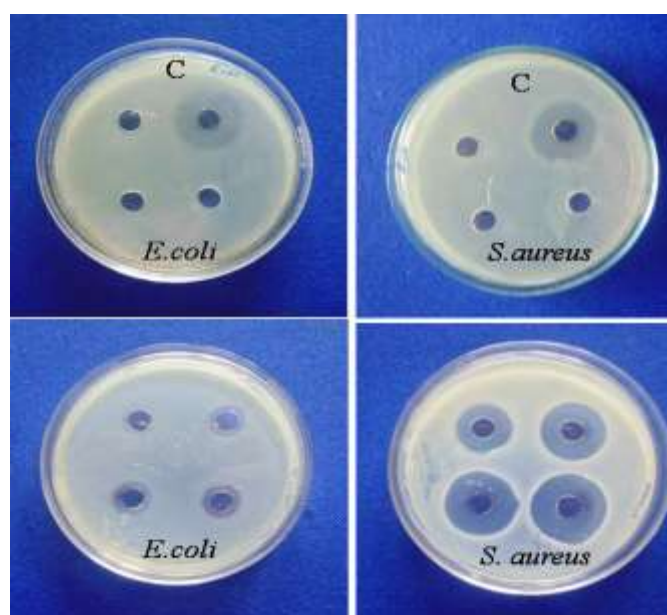


Figure (5): Inhibition zones of AuNPs for pathogenic bacteria

The antibacterial activity was measured on the basis of the zone of inhibition around the well. For the tested organisms, no zone of inhibition was observed for bacterial supernatant and H₂AuCl₄ and saline. It is suggested from the previous reports, AuNPs may attach to the bacterial surface membrane and release gold ions which may disrupt the permeability of bacterial cell membrane and DNA replication [25].

Table 1: Inhibition Zone of AuNPs

Bacteria	Inhibition zone at different concentration of AuNPs (µg/ml)			
	50	100	150	200
<i>E. coli</i>	-	7 mm	10 mm	11 mm
<i>Staphylococcus aureus</i>	20 mm	24 mm	26 mm	28 mm

3.8. Hemolytic Assay

Since most of the biomedical applications of AuNPs are based on systemic administration, tests on their interaction with RBC are more important.



Figure (6): Hemolytic activity biogenic gold nanoparticles.

Lysis of RBCs leads to cause several pathological conditions in *in vivo*, so evaluation of biocompatibility of AuNPs with blood is necessary by *in vitro* assays. In the present study investigated the cellular effect of biogenic AuNPs on RBCs. As shown in Fig. 6 AuNPs at different concentrations (150 and 200 µg/ml) exhibited no significant hemolysis. The Biogenic AuNPs exhibited unobservable hemolysis which is not detected by UV-visible spectrometry. This experiment allows hemocopatibility to be evaluated in a short time, as well as serving as a rapid, precise model for evaluating toxicity [26]. This result confirms biogenic AuNPs are biocompatible and non toxic can be used in biological and medical applications including drug delivery, labelling, photothermal therapy, tumor imaging and antibacterial therapy etc.

IV. CONCLUSION

In the present study, a eco-friendly approach for the synthesis of AuNPs using *Brevibacillus formosus* was successfully developed. This method is efficient, easy, eco-friendly and is the best option for the synthesis of metal nanoparticles. Our study suggests that the organism which resides in gold mines would be having more capability to resistant against soluble gold toxicity and produce AuNPs in efficiently. The synthesized AuNPs were characterized by using UV-Visible spectroscopy, FTIR, DLS and TEM analysis. A peak observed in the UV- visible spectrum at 535 nm clearly indicates the formation of AuNPs and FTIR peaks confirms the presence of reducing and stabilizing agents in biogenic AuNPs. DLS and TEM analysis confirms formation of monodispersed AuNPs size ranges from 5-12 nm. The synthesised AuNPs observed highest antibacterial activity against gram positive bacteria than the gram negative bacteria and no toxic effect on RBCs. Therefore, bacterial mediated AuNPs could be used for antibacterial and other biomedical applications.

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