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Short Communication

Endogenic mediated synthesis of gold nanoparticles bearing bactericidal activity

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ABSTRACT

The present investigation aimed to synthesize gold nanoparticles using *Pseudomonas fluorescens* 417 inhabiting *Coffea arabica* L. Biologically synthesized gold nanoparticles were polydispersed in nature and characterized using hyphenated techniques such as UV-visible spectrophotometry, which ascertained characteristic peaks between 450 nm and 650 nm. Fourier transform infrared analysis predicted the functional groups present in the cell-free supernatant that mediated the synthesis and stabilization of gold nanoparticles. The crystalline nature of the gold nanoparticles was analyzed with X-ray diffraction techniques that displayed the Bragg's diffraction intensity. Transmission electron microscopy revealed the size of nanoparticles ranging from 5 nm to 50 nm, with most of them bearing a spherical shape. The study also revealed the bactericidal activity of synthesized nanoparticles against a panel of clinically significant pathogens. Maximum activity was observed against *Pseudomonas aeroginosa* followed by *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae*. The results obtained in the present investigation are promising for ecofriendly approaches for synthesis of gold nanoparticles bearing bactericidal activity that can act as an alternative to combat drug-resistant pathogens.

1. Introduction

Use of nanomaterials can be traced from ancient times, but in recent times, their application and importance has increased [1]. Availability of technical resources and advances in scientific domains has led to emergence of nanotechnology and application of nanomaterials [2]. These nanomaterials have superior properties compared to their bulk counterparts. In recent years, nanomaterials have become a subject of interest among the scientific community, with many applications being explored. However, strict regulations have resulted in a decline in the use of these nanomaterials in biomedical applications. Their synthesis protocols involve the use of toxic materials, generate a lot of heat, and often require sophisticated infrastructure, which are barriers for many studies [3]. In order to overcome the limitations posed by these conventional methods, there has been a growing demand to develop ecofriendly and rapid synthesis of nanomaterials with the desired size and shape. Consequently, researchers have developed biogenic principles to synthesize nanomaterials by using biological resources such as plants and microorganisms or their products [4]. The use of biological entities is linked to their phyto- and bioremediation activities, and their ability

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to act as nanofactories to synthesize nanoscaled structures [5].

Most biogenic nanomaterials synthesized using diverse microbial flora are metallic nanomaterials such as silver and gold nanoparticles. Use of silver in therapeutics is well documented but the introduction of silver nanoparticles has resulted in expansion of its applications [6]. Silver nanoparticles are used as semiconductors, nanodevices, cosmetics, and biosensors, and in recent years, silver nanoparticles have been considered as potent antimicrobial agents against drug-resistant microorganisms [7]. Similarly, gold has been used for centuries in curing various aliments, and recently gold nanoparticles have demonstrated significant advances in medicine such as drug delivery, biocatalysts, and biolabeling [8]. Among the biological entities, the use of microorganisms in the synthesis of nanoparticles has successfully competed with conventional methods in synthesizing size-dependent nanomaterials. Microorganisms can synthesize nanomaterials in aqueous solutions, and the nanomaterials can be easily separated, thus becoming ecofriendly and cost-effective [9]. Microorganisms are an inexhaustible resource that can be preserved and reused, unlike plants, which can only be used once [10]. This causes an imbalance to plant diversity, especially among endangered species, and gives microorganisms an advantage over their plant counterparts [10]. Among the microbial community, plant-associated symbionts, "endophytes", have made a significant impact by secreting diverse secondary metabolites with biological activities. Although there has been significant research on endophytes, interference of endophytes in synthesizing nanomaterials is at an early stage and can generate major advances [11]; hence, the present emphasis on isolation of endophytic bacteria inhabiting *Coffea arabica* L. in Southern India. Isolated endophyte was characterized to reveal its close affinity to *Pseudomonas fluorescens*. Upon treatment with the metal salt gold chloroaurate, the endophytes were capable of rapid extracellular synthesis of gold nanoparticles bearing bactericidal activity against a panel of test pathogenic microorganisms. Here, we report an ecofriendly approach for synthesis of bactericidal nanoparticles without using any toxic elements.

2. Materials and methods

2.1. Isolation of endophytes

Healthy plants of *C. arabica* L. were collected, washed under running tap water, and subjected to sequential surface sterilization by immersion in 3.15% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 1 minute. Tissues were subsequently washed with double-distilled sterile water and dried using sterile blotter sheets. The outer tissue of surface-sterilized plant segments was excised using a sterilized scalpel, cut into 0.5–1.0-cm blocks and placed on the surface of nutrient agar supplemented with 250 μg/mL cycloheximide and incubated for 48 hours to observe colonies of endophytic bacteria [12,13]. Sterility checks were performed by transferring aliquots of final rinse water onto nutrient agar, which served as a control plate.

2.2. Screening of endophytes for synthesis of gold nanoparticles

Endophytic bacteria were cultured in nutrient medium supplemented with 1 mM gold chloroaurate and incubated at 37 °C until visible growth was observed. Colonies growing abundantly on this medium were subjected to large-scale fermentation for 72 hours under optimized conditions as described by Baker et al. [8]. The fermentation broth was centrifuged at 10,000 g at 4 °C for 5 minutes, and the supernatant was assessed for synthesis of gold nanoparticles by applying 1 mM gold chloroaurate and incubating until a color change was observed. Samples were drawn periodically and monitored using UV-visible spectrophotometry to confirm the synthesis of gold nanoparticles by recording the spectra between 200 nm and 800 nm using a Shimadzu double beam spectrophotometer (Shimadzu Corp., Kyoto, Japan).

2.3. Characterization of gold nanoparticles

Biophysical characterization of gold nanoparticles was carried out by precipitating gold nanoparticles, washing with sterile deionized water, drying under vacuum, and subjecting them to Fourier transform infrared spectroscopy (FTIR), which was carried out with a JASCO FT-IR 4100 (Jasco, Easton, MD, USA) at a resolution of 4 cm^{-1} to predict the functional group of biomolecules in the supernatant responsible for reducing metal salts and stabilizing gold nanoparticles. Diffraction pattern of the gold nanoparticles were studied by X-ray diffraction (XRD) analysis by coating the dried gold nanoparticles on a grid and recording the spectra with a Rigaku Miniflex-II Desktop X-ray diffractometer operating at a voltage of 30 kV (Rigaku, Tokyo, Japan). Size and morphology of gold nanoparticles was analyzed using transmission electron microscopy (TEM). Aliquots (~500 μL) of gold nanoparticles were transferred onto carbon-coated copper TEM grids and scanned using a JEOL JEM-2100 (Jeol, Akishima-Shi, Japan). The microscope was operated at a voltage of 120 kV with a Bioten objective lens. Subsequently, the particle size was ascertained using a Gatan CCD camera (Gatan, Pleasonton, CA, USA) [8].

2.4. Bactericidal activity of gold nanoparticles

Bactericidal activity was evaluated by well diffusion, disc diffusion and microbroth dilution assays. Prewarmed Mueller–Hinton agar plates were seeded with 10^6 colony forming units (CFU) suspensions of selected test bacteria such as *Pseudomonas aeruginosa* (MTCC 7903), *Escherichia coli* (MTCC 7410), *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 121), and *Klebsiella pneumoniae* (MTCC 7407) which were swabbed uniformly. Using a sterile cork borer, a 10-mm diameter area of agar was removed and 50 μL of 10 mg/mL gold nanoparticles was added to the well, and simultaneously, the sterile agar disc was impregnated with gold nanoparticles and placed onto the agar and incubated at 37 °C for 24 hours. After incubation, the zone of inhibition was measured and interpreted with gentamicin (1 mg/mL) as standard.

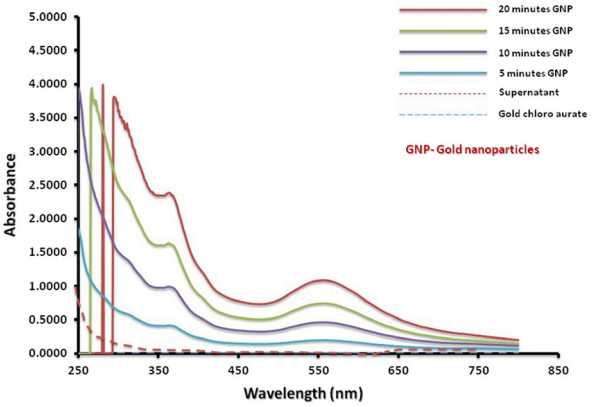

Fig. 1. UV-visible spectrum of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417.

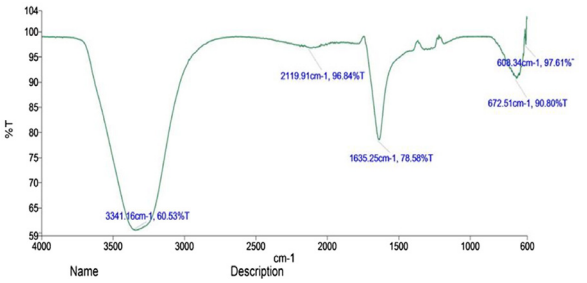

Fig. 2. Fourier transform infrared spectroscopy analysis of biosynthesized gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417.

Microbroth dilution assay was performed using 25–100 μg/mL gold nanoparticles suspended in sterile saline. Each aliquot was suspended in test tubes with 10 mL Muller–Hinton broth seeded with 150 μL test bacteria (5×10^6 CFU/mL), and incubated at 37 °C on a shaker (150 rpm) for 20–24 hours. Absorbance at 600 nm was subsequently measured to quantify bacterial growth. Positive and negative controls were maintained to distinguish the activity of the gold nanoparticles based on the optical density (OD) of the controls compared with the test pathogens treated with gold nanoparticles [14].

CFU assay was performed as described by Sondi and Salopek-Sondi [15]. Test bacteria were added to the medium and poured onto a sterile Petri dish and allowed to solidify. Different concentrations of nanoparticles (10–100 μg/mL) were added to the surface of the media under sterile conditions and spread. All the plates were incubated at 37 °C for 24 hours and results were observed. One control plate was kept without adding nanoparticles.

3. Results and discussion

Our results contribute to the growing scientific knowledge on endophytes playing an emerging role in synthesis of nanoparticles. Most studies of the use of endophytes for synthesis of nanoparticles have been conducted on fungal endophytes. The present study investigated isolation of bacterial endophytes for synthesis of nanoparticles. When cell-free extract was treated with gold chloroaurate at a ratio of 9:1, the color of the reaction mixture turned from yellow to ruby red within 5 minutes of incubation. The ruby red color intensity increased with time and the reaction was completed within 20 minutes incubation, with no further change in color. Synthesis of nanoparticles was confirmed by UV-visible spectra with absorption peaks between 450 nm and 650 nm (Figure 1). These characteristic peaks are due to surface plasmon resonance of nanoparticles [8,10]. Control solutions such as cell-free supernatant and gold chloroaurate neither developed ruby red color nor exhibited any characteristic peaks. This indicated that biomolecules present in the supernatant mediated synthesis of gold nanoparticles when treated with gold chloroaurate. Similar results have been observed with extracellular synthesis of gold nanoparticles using endophytes isolated from the medicinal plant *Bauhinia variegata* L. Among the isolates screened, *Penicillium citrinum* was capable of synthesizing nanoparticles compared to the other isolates [16]. It was also observed that various parameters such as alkaline pH and elevated temperature influenced the synthesis of nanoparticles. There was variation in synthesis when pH was varied between alkaline and acidic. Similarly, temperature < 80 °C exhibited proportionately less intense peaks and even the color of the reaction mixture was light brown. These results are similar to other studies that confirmed the synthesis of nanoparticles under the influence of different parameters [8,17].

3.1. Biophysical characterization of synthesized gold nanoparticles

Synthesized gold nanoparticles were subjected to biophysical characterization with hyphenated techniques. FTIR analysis measured the biomolecules present in the supernatant that mediated formation of gold nanoparticles. Different vibrational stretches were observed (Figure 2) that corresponded to various functional groups. For instance, in the present study vibrational stretch at 3341.16 corresponded to hydroxyl groups [18], 1635.25 to carbonyl groups [19], and 672.51 to aromatic groups. As stated in earlier reports, biomolecules interact via these functional groups and mediate the reduction process to synthesize and stabilize nanoparticles. An XRD profile (Figure 3) displayed peaks at $2\theta = 38°$, 44°, 64° and 78° assigned to the (111), (200), (220), and (311) planes of a faced center cubic lattice of gold, which indicated that the synthesized nanoparticles were crystalline in nature. The XRD spectrum in the present investigation was inconsistent with earlier studies. Transmission electron micrographs exhibited polydispersity of nanoparticles with sizes ranging from 5 nm to 50 nm (Figure 4). Morphological analysis of nanoparticles revealed different shapes, varying from spherical to triangular and hexagonal. These results are in accordance with earlier reports of biosynthesis of gold nanoparticles from microbial flora [20,21].

Table 1
Bactericidal activity of gold nanoparticles via disc diffusion assay.

Sample no.	Test pathogens	Zone of inhibition (mm)	
		Gold nanoparticles	Gentamicin (1 mg/mL)
1	*Bacillus subtilis* (MTCC 121)	11.00	34.00
2	*Escherichia coli* (MTCC 7410)	13.00	29.66
3	*Klebsiella pneumoniae* (MTCC 7407)	12.00	18.33
4	*Pseudomonas aeruginosa* (MTCC 7903)	22.00	24.00
5	*Staphylococcus aureus* (MTCC 7443)	15.00	24.66

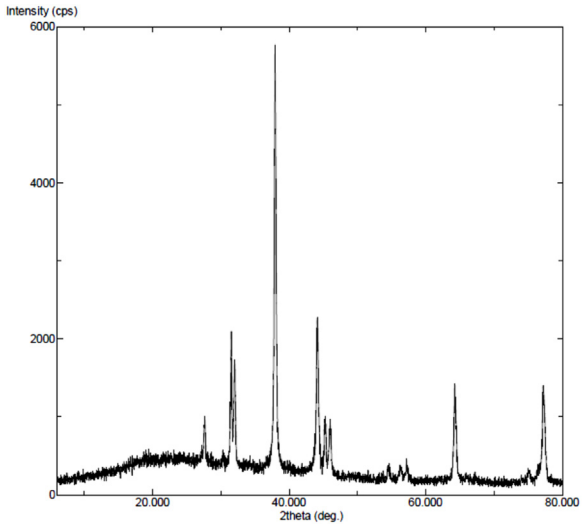

Fig. 3. X-ray diffraction analysis of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417.

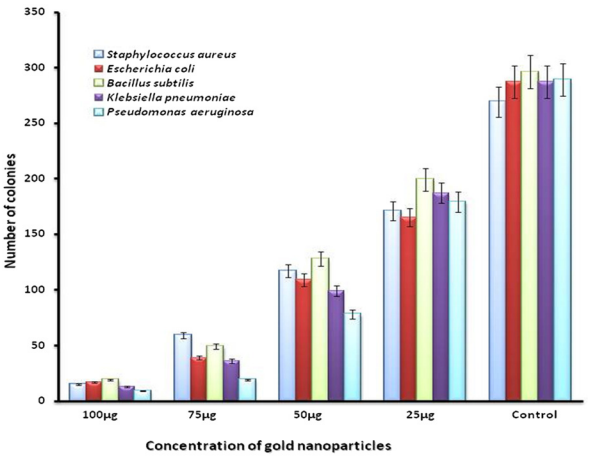

Fig. 5. Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using colony forming unit assay.

3.2. Bactericidal activity of synthesized nanoparticles

Synthesized gold nanoparticles were assessed for bactericidal activity against a panel of significant pathogens and activity was measured as degree of inhibition via disc diffusion assay (Table 1). Varying degrees of inhibition were observed with the maximum zone of inhibition obtained against *P. aeruginosa* (MTCC 7903), followed by *E. coli* (MTCC 7410), *S. aureus* (MTCC 7443), *B. subtilis* (MTCC 121), and *K. pneumoniae* (MTCC 7407). Similar results were found by broth dilution assay, with a marked decrease in OD of broth seeded with different test pathogens against the increase in concentration of gold nanoparticles (Figure 5). Bactericidal activity was confirmed with the CFU plate method. The number of viable colonies gradually decreased as the concentration of gold nanoparticles increased from 0 μg/mL to 100 μg/mL (Figure 6). Bactericidal activity of synthesized gold nanoparticles was interpreted and validated with gentamicin as a standard. Earlier studies demonstrated the bactericidal activity of biosynthesized gold nanoparticles that acted on pathogens with different modes of action [11]. For instance, the nanoparticles bound to the bacterial cell wall and caused pitting, which resulted in loss of cellular content, and in most cases, interfered with vital components like thiol groups, which suppressed replication [11]. Gold nanoparticles have been shown to have greater activity against Gram-positive bacteria compared to Gram-negative bacteria [22]. In the present investigation, the highest activity

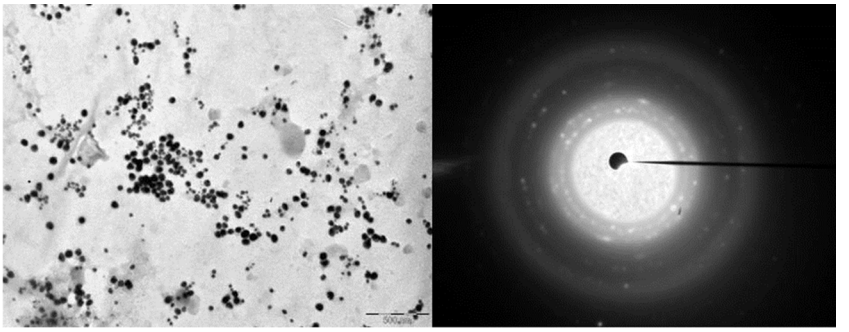

Fig. 4. Transmission electron micrographs of nanoparticles synthesis by *Pseudomonas fluorescens* strain CA 417.

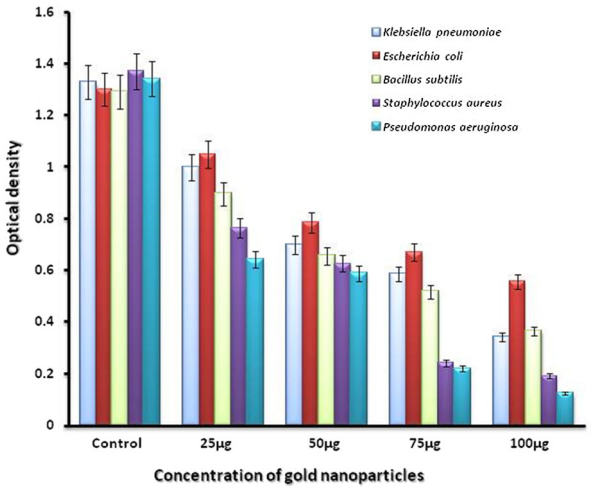

Fig. 6. Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using broth dilution.

was against Gram-negative *P. aeruginosa*, which is a clinically important as well as environmental pathogen. Our results confirm the bactericidal activity of gold nanoparticles against both Gram-positive and -negative bacteria.

4. Conclusion

Our results contribute towards ecofriendly and rapid synthesis of gold nanoparticles from endophytic *P. fluorescens* CA 417 inhabiting *Coffea arabica* L. The synthesized gold nanoparticles displayed bactericidal activity against clinically important pathogens.

Conflict of interest

The authors confirm that there is no conflict of interest.

Acknowledgments

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