

# Green Synthesis of Gold Nanoparticles by a Metal Resistant *Arthrobacter nitroguajacolicus* Isolated From Gold Mine

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**Abstract**—Biosynthesis of gold nanoparticles would benefit from the development of clean, nontoxic and environmentally acceptable procedures concerning microorganisms from bacteria to fungi and even algae. Actinobacteria are soil bacteria which have the enormous ability as biotechnological tools. In this paper, we reported the biosynthesis of gold nanoparticles by a member of *Arthrobacter* genus isolated from Andaliyan gold mine in north-west of Iran. This metal resistance strain obtained from an acidophilic region ( $\sim$ pH 5.6). The UV-vis and XRD spectra of the aqueous medium containing the strain and 1 mM HAuCl<sub>4</sub> for 24 h, demonstrated the formation of gold nanoparticles. TEM micrographs showed intra-extracellular production of gold nanoparticles with spherical shape and average size of 40 nm. The result of morphological and molecular tests revealed that the isolate was belonged to *Arthrobacter* and has 100% similarity in 16SrRNA gene sequences to *Arthrobacter nitroguajacolicus*.

**Index Terms**—Actinobacteria, *Arthrobacter nitroguajacolicus*, biotechnology, gold nanoparticles, HAuCl<sub>4</sub>.

## I. INTRODUCTION

GOLD nanoparticles having special electrochemical, optical, and magnetic features properties and therefore are considered as probes for molecular diagnostics [1], [2], catalyst in chemical reactions and pollution monitoring [3], drug and gene delivery [4], [5], cancer treatment [6], biosensors for diagnosis of tumor tissues [7], and determination of microorganisms [8]. Nanoparticles can be produced by chemical and biological processes. In chemical processes, the growth of nanoparticles is difficult, expensive and result to environmental pollution. But nanoparticle production by microorganisms is cheap and less environmental pollution is generated [9], [10].

There are various publications on the application of microorganisms in nanoparticle production. *Pseudomonas* [11] and

*Lactobacillus* [12] are studied for production of gold nanoparticles. Actinobacteria are one of the most habitants of soils. They have various biotechnological applications, including nanoparticle production. Ability of an alkaloresistant actinomycete (*Rhodococcus*) [13] and a thermophilic actinomycete (*Thermomonospora*) [14] in production of gold nanoparticles was reported. The gold nanoparticles produced by these bacteria possessed monodispersity. However, potential of other members of actinobacteria for this purpose has not considered.

Iran harbors hotspots in microbial diversity and some new species of actinobacteria were isolated and reported from soils of Iran [15]–[18]. However, there is no report on the screening of Iran's soils to find actinobacteria capable of gold nanoparticle production. In this survey, actinobacteria isolated from copper and gold mines are studied for production of gold nanoparticles.

## II. MATERIALS AND METHODS

### A. Culture Media

Dichloroacetic acid No.1 agar (g/l) (Yeast extract 10, Glucose 5, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 1.5, K<sub>2</sub>HPO<sub>4</sub> 1, Dichloroacetic acid 0.7, MgSO<sub>4</sub> 0.2, Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·5H<sub>2</sub>O 0.01, ZnSO<sub>4</sub>·7H<sub>2</sub>O 2, agar 15, pH 7.2) [19] and humic acid agar (g/l) (humic acid 1, Na<sub>2</sub>HPO<sub>4</sub> 0.5, KCl 1.73, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01, CaCO<sub>3</sub> 0.02, vitamin B solution 1 ml (50 mg each of thiamin, riboflavin, niacin, pyridoxin, calcium D-pantothenate, inositol, *p*-aminobenzoic acid, 25 mg biotin and 100 ml distilled water), agar 18, pH 7.2) [20] are used for the isolation of actinobacteria.

MGYP broth (g/l) (malt extract 3, glucose 10, yeast extract 3, peptone 5, sodium carbonate 10, pH 7.2) [13] was used for biomass production needed for producing of nanoparticles. Brain heart infusion broth [19] was used to produce biomass needed for identification of the selected isolates.

### B. Sampling and Isolation

From different natural, agricultural, and industrial regions of Azerbaijan Province, northwest of Iran, 7 soil samples were collected. The samples were taken from a depth of  $\sim$ 25 cm. Appropriate serial dilution of the soil samples were cultured on dichloroacetic acid No.1 and humic acid agar and were incubated at 28 °C for 5–7 days [15]–[18]. The isolates were subcultured in ISP2 medium [21] and kept at  $-80$  °C after addition of 30% glycerol.

### C. Primary Screening of Gold Nanoparticle Producers

**Culture Condition:** Appropriate concentration of cell suspension (ca.  $10^7$ – $10^8$  cells per ml) was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of GYMP medium. The

Manuscript received July 28, 2014; accepted November 20, 2014. Date of publication January 13, 2015; date of current version May 29, 2015. Asterisk indicates corresponding author.

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Digital Object Identifier 10.1109/TNB.2014.2377232

flasks were incubated at 28 °C with 200 rpm for 60 hours. The broth was centrifugated at 3500 *g* for 20 minutes and the pellet (biomass) was collected under aseptic conditions [11], [12]. The amount of 3 g of the biomass was added to 250 ml Erlenmeyer flasks containing 50 ml of sterilized 1 mM HAuCl<sub>4</sub> solution with pH 7 and were incubated at 28 °C with 200 rpm for 24 hours. The flasks that showed color changes from yellow to purple red were selected [13], [14].

**Spectrophotometric Analysis of the Culture Broth:** The purple red culture broths were centrifugated at 6000 *g* for 7 minutes and the pellet was collected and washed with de-ionized water three times. To 0.1 g wet weight of bacterial biomass, 500  $\mu$ l of lysis buffer was added and the suspension was frozen in liquid nitrogen after vortexing. After a short time, the samples were taken out of liquid nitrogen and were incubated in hot water bath (60 °C) to destroy the bacterial cells. It was centrifugated at 6000 *g* for 10 minutes and absorbance of the supernatant was measured at 200–800 nm by UV-visible spectrophotometer with a resolution of 0.72 nm (Shimadzu, UV Pharma spec 1700, Japan). HAuCl<sub>4</sub> solution (1 mM) was used as control. Increasing of the absorbance at 540 nm was considered as the presence of gold nanoparticles [14].

**Transmission Electron Microscopy of the Bacterial Cells:** The biomass samples showed increasing absorbance at 540 nm were chosen. The amount of  $\sim$ 1 g of biomass was prefixed for 3 h in 2% glutaraldehyde in 0.1 mM cacodylate buffer at 4 °C. After washing with cacodylate buffer, they were fixed with 2% osmium tetroxide in distilled water for 3 hours at 4 °C. Then, the cells were dehydrated at room temperature with 50%, 70%, 80%, 90%, and 100% ethanol for 15 minutes. Since ethanol is poorly miscible with epoxy resins, propylene oxide was used as a linking agent; the dehydrated cells were kept in propylene oxide for 30 min and then placed in a mixture of propylene oxide and epoxy resin for 1 hour. Embedding was performed using the mixture of resin (Epon 812) and DDSA (dodecenyl succinic anhydride) and MNA (nadic anhydride) fixator and accelerator (dimethyl amino methyl phenol). This mixture was polymerized for 2 days at 60 °C. Thin sections (100 nm) were achieved using an ultramicrotome (OMU3 C. Reichert, Austria) and was mounted on TEM copper grades using drop method. The sections were dyed with uranyl acetate 2% in sterile distilled water for 10 minutes and analyzed by a transmission electron microscope (Zeiss, EM208S, Germany [22]).

#### D. Secondary Screening of Gold Nanoparticle Producers

The positive solutions from primary screening section were analyzed by X ray diffraction (XRD) [13]. To determine the metal type deposited in the bacterial cells, the biomass was separated from HAuCl<sub>4</sub> solution by centrifugation at 4500 *g* for 7 minutes. The amount of  $\sim$ 0.1 g of wet bacterial weight was washed three times by deionized water. The samples were dried at 50 °C for 24 hours. The desiccated samples were analyzed by a XRD instrument (PHILLIPS, PHI-5300ESCA-X-ray, Netherlands) [13]. The results were interpreted using Xpert Highscore software.

#### E. Identification of the Active Strain

The selected active isolates were taxonomically identified by phenotypic and genotypic studies including study of microscopic and macroscopic morphology, analysis of



Fig. 1. After 24 h incubation of the actinobacterial isolate in HAuCl<sub>4</sub> medium, the color was changed from yellow to red (left) purple (right).

diaminopimelic acid and 16S ribosomal RNA (rRNA) gene sequencing. The cultural properties of the isolates were evaluated according to the guidelines of the ISP as described by Shirling and Gottlieb [21].

Biomass of the isolates for chemical and molecular systematic studies was obtained by cultivation for 6 days in shake flasks (200 rpm) using brain-heart infusion broth. The cells were harvested by centrifugation and washed twice with deionized water.

**Molecular Analysis of the Selected Isolate:** The 16S rRNA gene of the selected isolates was amplified by polymerase chain reaction using bacterial 16S rRNA gene primers. The forward primer was AF (5' AGAGTTTGATCCTGGCTCA 3'), and reverse primer was AR (5' AAGGAGGTGATCCAGCCG 3'). The gene was cloned into plasmid vector pTZ57R/T and was transferred to *Escherichia coli* DH5 $\alpha$  competent cells. To ensure the presence of 16SrRNA gene in the isolates, the plasmids were extracted and amplified by the above mentioned primers. The plasmids were digested by *HindIII* and *SacI*, sequenced and analyzed by GenBank and EzTaxon [23]. Clustal X. 2.1 program [24] was used to estimate evolutionary distance, and similarity values were used to reconstruct the phylogenetic tree by MEGA version 6 software package [25].

### III. RESULTS

#### A. Isolation of Actinobacteria and Primary Screening of Gold Nanoparticle Producers

Among 7 soil samples, 15 isolates were obtained. One isolate was able to change the color of culture medium from yellow to purple red. The color of the reaction solution turned from pale yellow to red after 48 h (Fig. 1). The red color of the supernatant indicated the extracellular formation of gold nanoparticles by the bacteria.

UV-vis spectroscopy demonstrated the reduction of the gold ions to gold nanoparticles during exposure to actinobacterial cells. It is well known that gold nanoparticles absorb electromagnetic waves in the visible region of the spectrum ( $\sim$ 520 nm) because of the excitation of surface plasmon vibrations that giving gold nanoparticles striking colors in the media. Fig. 2 shows the UV-vis absorption spectra recorded from uninoculated (A) and inoculated (B) HAuCl<sub>4</sub> solutions. The spectrum (B) shows absorption in the spectral window of 400–800 nm [13].

#### B. Secondary Screening of Gold Nanoparticle Producers

Existence of gold nanoparticles was confirmed by X-ray diffraction (XRD) analysis of the biomass and the result was shown in Fig. 3, based on data provided by X Pert Highscore software. The XRD spectrum resulted in four intense peaks in the spectrum, (38.269), (44.600), (64.678), (77.549), and

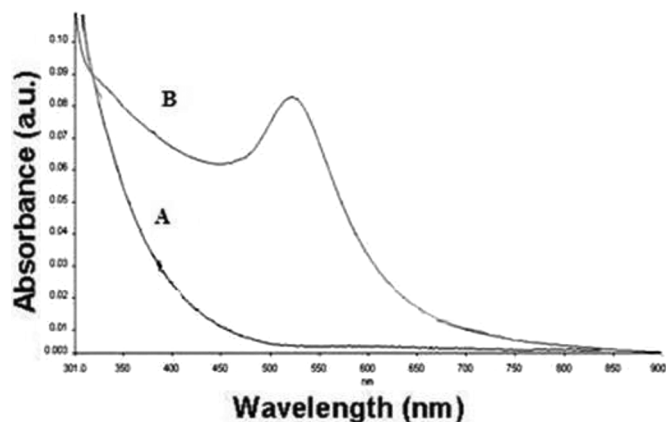


Fig. 2. Spectrophotometric analysis of  $\text{HAuCl}_4$  medium at wavelengths of 200–800 nm. (A) Control: uninoculated  $\text{HAuCl}_4$  medium (B) inoculated  $\text{HAuCl}_4$  medium by the actinobacterial isolate after 24 h incubation.

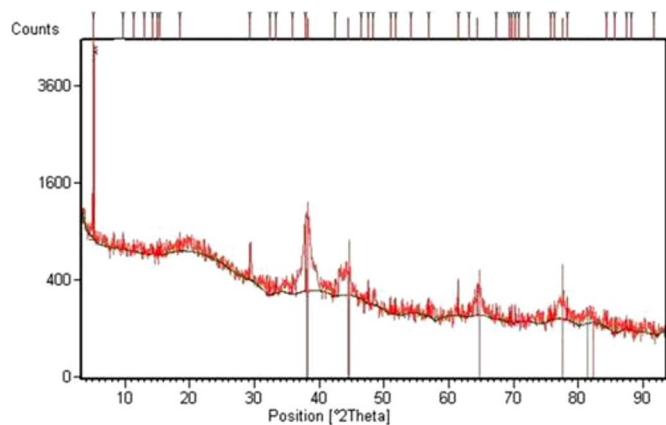


Fig. 3. XRD analysis of biofilms produced by the actinobacterial cells.

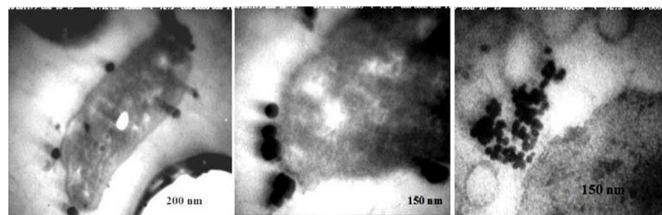


Fig. 4. TEM electron microscope image of the actinobacterial cell isolated from Andalian gold mine (magnitude 40 nm).

(82.352) which agree to the Bragg's reflection of gold nanoparticles [26].

Size and location of the gold particles in the cells was determined by transmission electron microscopy and the results were shown in Fig. 4. The average size of nanoparticles was 40 nm for isolate. As seen, gold nanoparticles can be seen inside and outside the cells.

### C. Identification of Gold Nanoparticles Producers

The results of morphological, chemotaxonomical, and molecular identification tests on the premium nanoparticles producer were revealed that its characteristics were consistent with its classification in the genus *Arthrobacter* [27]. The analysis of cellular constituents of the isolate was revealed the presence of the lysine in the cell wall, similar to other members of *Arthrobacter* genus. After analysis of 16S rRNA gene sequence of the isolate, the highest similarity (100%) was

observed to *Arthrobacter nitroguajacolicus*, which deposited in Genbank with accession number HQ597008.

## IV. DISCUSSION

Actinobacteria have a vast ability in production of secondary metabolites like antibiotics. It was recently reported that the alkalothermophilic rare actinobacterium, *Thermomonospora* sp. was able to reduce gold ions to nanoparticles extracellularly with monodispersity [14]. Furthermore, intracellular biosynthesis of gold nanoparticles with the size range of 5–15 nm was demonstrated by the alkalotolerant actinomycete of *Rhodococcus* genus [13].

In conducted reported studies, among the significant items for industrial production of gold nanoparticles by microorganisms is the pace of bacterium in reduction of aurum ion. In the case of bacterium *Thermomonospora*, the produced nanoparticles are extracellular and have a high transmittance. Unfortunately, *Thermomonospora* has a low pace in reduction of aurum ion and it takes 120 hours to accomplish this reaction [14]. The bacterium *Rhodococcus* reported by Ahmad *et al.* has a rapid growth and can reduce aurum ion in 24 hours. But the stored nanoparticles are intracellular and the extraction process of nanoparticles from the inside of cells is costly and destroys the producing bacterium [13]. The *Arthrobacter nitroguajacolicus* isolated from gold copper mine is able to produce gold nanoparticles both extra and intracellularly. So the process is simpler for downstream production. Gold nanoparticle biosynthesis in *Arthrobacter nitroguajacolicus* is a rapid process and 24 hours is needed. The extracellular synthesis of nanoparticles is a great advantage, since there is no need to an extra step for release of the gold nanoparticles.

## V. CONCLUSION

In this study we demonstrated the extra- and intracellular synthesis of gold nanoparticles gold by *Arthrobacter nitroguajacolicus* isolated from the soil of Andalian gold mine. The average size of nanoparticles is 40 nm and the size of gold nanoparticles outside the cell is often larger than the nanoparticles stored inside the cell. This non-pathogen bacterium is also able to synthesis gold nanoparticles within 24 h. Further research is needed to understand the biochemical and molecular mechanism of nanoparticle synthesis by the cell filtrate in order to achieve better control over the size and dispersity of nanoparticles.

## ACKNOWLEDGMENT

The authors thank Mr. Ahad Mokhtarzadeh for his assistance.

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