

## An Alternative Method for Biological Production of Silver and Gold Nanoparticles

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Major purpose of this study was to explore an alternative and simple method for silver and gold nanoparticles bio-production by *Escherichia coli*. In the previous (routine) technique for nanoparticles bio-production, after culturing the bacteria, the bacterial culture supernatant was treated with aqueous solutions of silver nitrate and chloroauric acid ions. To perform bio-synthesis of silver and gold nanoparticles by the alternative method, unlike the routine one, the bacteria were subjected to the toxic metal ions at their MICs (minimal inhibitory concentration) immediate after inoculation to the medium. Bio-production of nanoparticles by these two methods was monitored through visible spectrophotometry, transmission electron microscopy (TEM) and X-ray diffraction analysis (XRD). The results showed that silver and gold nanoparticles were produced by both types of the routine and alternative techniques but the rate of bio-production was relatively more by the alternative one. Moreover, this type of bio-production is simpler, faster and cleaner than the previous one.

**Key words:** Bio-production, Silver nanoparticles, Gold nanoparticles, Alternative method.

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Nanoparticles synthesis is mostly done by physical and chemical methods. Although these techniques are simple and somehow fast, but remaining of the toxic reagents on the surface of the resulted nanoparticles is one of their deficiencies for medicinal applications. So there is a growing need to develop a clean, non-toxic and environmental friendly method for the aim of metal nanoparticles synthesis. Recently, the third strategy was used for nanoparticles production which is named green or biological method (Brust *et al.*, 2002, Mandal *et al.*, 2005, Raveendran *et al.*, 2003). In this way, by using some plant extracts or microorganisms, nanoparticles are produced. Generally, bio-production of metal nanoparticles by microorganisms takes place in order to remove harmful effects of the toxic metal ions that are

introduced to the microorganisms culture media (Sun *et al.*, 2002). There are two types of microbial detoxification of the metal ions: extracellular and intracellular. Extracellular bio-production of metal nanoparticles, by using cell free extract, has more commercial applications and is simpler than the intracellular one (Pourali *et al.*, 2013).

Moreover, bio-production of metal nanoparticles takes place by either active or passive mechanisms. In the active mechanism, enzymatic reaction is the most known process and in the passive one, nanoparticles are synthesized by non-enzymatic reactions (Sun *et al.*, 2002). It is shown that some especial functional groups of the organic materials such as carboxyl, amide, aldehyde and ketone that are present in the amino acid residues, extracellular polymers and surface of the cell walls are involved in the non-enzymatic bio-reduction of the metal nanoparticles (Maneerung *et al.*, 2008). In both of the active and passive methods of extracellular bio-production

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of metal nanoparticles, after production of the microbial biomass, the cell free extract is used for the bio-reduction process. By this way, enzymes or other such active components that were secreted during the cell reproduction in the culture medium are responsible for metal ions bio-reduction (Pourali *et al.*, 2014). To date, all the accessible research papers deal with this method. By this technique, the microbe itself does not seem to be active in the bio-reduction process but some research articles such as the work of Parikh et al in 2008 reported that the genes that are named silE, silP and silS were subjected in silver resistant of *Morganella* sp. This report can be the molecular evidence of silver resistance in bacteria (Parikh *et al.*, 2008). Through the different data there still remains one simple question that is if we know that microorganisms are very precise and do not use their energy when it is not necessary and if the microorganisms have some genes for detoxification of the toxic metal ions, why don't we expose the microorganisms to these toxic ions from the initial step of the inoculation?? In order to answer this question, we have assayed an alternative method for extracellular bio-production of gold and silver nanoparticles, in the present paper. By this technique, the tested bacteria were subjected to the toxic metal ions immediate after inoculation to the medium and bio-production of the nanoparticles was determined by several strategies.

## MATERIALS AND METHODS

### Bacterial strain

*Escherichia coli* (PTCC 1330) was purchase from Persian Type Culture Collection (PTCC), Iran. The strain was stored in Nutrient broth (Merck, Germany) medium contained 15% glycerol at -15°C until the experiments were started (Pourali *et al.*, 2012).

### Determination of the minimal inhibitory concentration (MIC) by broth microdilution method

*Escherichia coli* strain was grown in Nutrient agar (Merck, Germany) medium and the obtained bacteria were used for further experiments. A bacterial single colony was suspended in 1 mL of normal saline and the optical density of the suspension was adjusted to 0.08 at wavelength of 600 nm (corresponding to McFarland's standard

No 0.5). In order to determining MICs of the silver nitrate (Sigma-Aldrich, USA) and chloroauric acid (Sigma-Aldrich, USA) 1 molar solutions a sterile 96-well microtitre plate was used. 50  $\mu$ L of Nutrient broth and 50  $\mu$ L of the bacterial suspension that was prepared above, was added to each well that already containing 50  $\mu$ L of two-fold serially diluted of the silver nitrate in one row and chloroauric acid in the other one. The negative control wells were prepared with culture medium containing 1 molar of silver nitrate or chloroauric acid solutions and sterile culture medium and the positive control well was prepared with culture medium containing bacterial suspension. The plate was incubated at 37°C for 24 hours. The MICs were the lowest concentration of silver nitrate and chloroauric acid where no viability was observed after 24 hours (Mourey *et al.*, 2002)

### Biosynthesis of silver and gold nanoparticles

Two sterile flasks of 30 mL Nutrient broth medium were used and 100  $\mu$ L of the bacterial suspension that its optical density was adjusted to McFarland's standard No 0.5 was transferred to each of them. Then, for silver nanoparticles production, silver nitrate solution at its MIC concentration was added to one flask and for gold nanoparticles production, chloroauric acid solution at its MIC concentration was added to another one. The flasks were incubated in the shaker incubator at 200 rpm, 37°C for 24 hours in dark condition. The negative controls were two flasks containing sterile Nutrient broth with the same concentration of silver nitrate and chloroauric acid solutions, respectively. These flasks were also incubated in the same conditions. Bio-production of silver and gold nanoparticles was observed through the changes in the color of the **broth medium** from yellow to dark brown and yellow to pink-purple, respectively. These color changes were due to the surface plasmon resonance (SPR) and oscillation of the conducting electrons on the surface of the silver and gold nanoparticles. Hereinafter in this paper this strategy is named alternative method for bio-production of silver and gold nanoparticles<sup>7</sup>. For better results, the usual strategy for extracellular bio-production of silver and gold nanoparticles was performed regarding to our previous published data. Briefly, 100  $\mu$ L of the bacterial suspension that its optical density was adjusted to McFarland's standard No 0.5 was

transferred to the 60 mL of sterile Nutrient broth medium and incubated in the shaker incubator at 150 rpm, 37°C for 24 hours. After that, the cell free extract was obtained through the centrifugation (6,000 rpm, 10 min), divided in to two flasks and challenged with the obtained MIC concentration of the silver nitrate and chloroauric acid solutions, separately. The flasks were incubated in the shaker incubator at 200 rpm, 37°C for 24 hours in dark condition. Furthermore, the negative controls were two flasks containing sterile Nutrient broth with the obtained MIC concentration of silver nitrate and chloroauric acid solutions, respectively. Hereinafter in this paper this strategy is named routine method for bio-production of silver and gold nanoparticles (Saifuddin *et al.*, 2009).

#### Measurement the bacterial cell viability

This test was run only for the alternative method and the test flasks contained the bacteria and the metal ions at the same time. After nanoparticles bio-production, a loop full of each of the flasks was transferred to the sterile Nutrient agar medium and the plates were incubated at 37°C for 24 hours and formation of the colonies was detected. The positive control was a streak plate of *Escherichia coli*.

#### Characterization of nanoparticles

##### Visible spectral analysis

In order to determine the bio-reduction of silver and gold nanoparticles took place, all the color changed flasks that were obtained from both types of the alternative and routine methods were examined through the visible spectrophotometer from 300 to 700 nanometer (nm) wavelengths. The sterile Nutrient broth medium was used as a blank (Pourali *et al.*, 2014).

##### Transmission electron microscope (TEM)

Sizes and morphologies of the produced silver and gold nanoparticles that were obtained from both types of the alternative and routine methods were analyzed by using Ziess Leo 910 TEM that worked at 80 kV accelerating voltage and Gatan SC1000 camera (Pourali *et al.*, 2014).

##### X-ray diffraction analysis (XRD)

The produced silver and gold nanoparticles solutions that were obtained from both types of the alternative and routine methods were first Freeze-dried and then studied by Philips automatic X-ray diffractometer with Philips PW 1830 X-ray generator. The diffracted intensities for

samples were obtained from 30° to 80° 2 $\theta$  angles (Pourali *et al.*, 2014).

## RESULTS

### Determination of the minimal inhibitory concentration (MIC) by broth microdilution method

The MIC results for silver nitrate and chloroauric acid were 0.3318 g/L and 1.327 g/L, respectively. These concentrations were used for production of silver and gold nanoparticles by alternative and routine methods.

### Biosynthesis of silver and gold nanoparticles

The ability of silver and gold nanoparticles production by *Escherichia coli* (PTCC 1330) was verified. In this step, the color of all the nanoparticles containing flasks that were obtained by both of the alternative and routine methods changed from yellow to dark brown and pink-purple for silver and gold nanoparticles, respectively. Figure 1 shows the changed color of the reaction mixtures that were obtained from the alternative method in contrast to the controls.

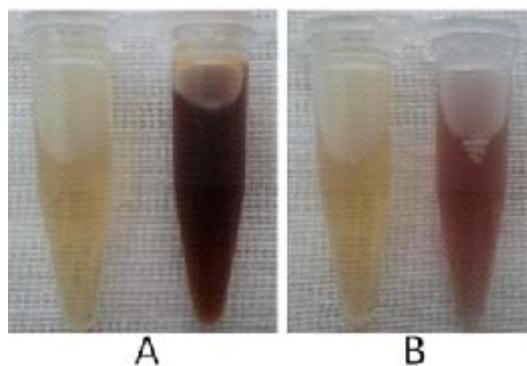
#### Measurement the bacterial cell viability

No bacterial growth was detected in all of the inoculated plates. In the positive control plate, formation of the bacterial colonies was seen.

#### Characterization of nanoparticles

##### Visible spectral analysis

The resulted visible spectra for the color changed flasks proved the formation of the silver and gold nanoparticles that were produced by both the routine and alternative methods. Results



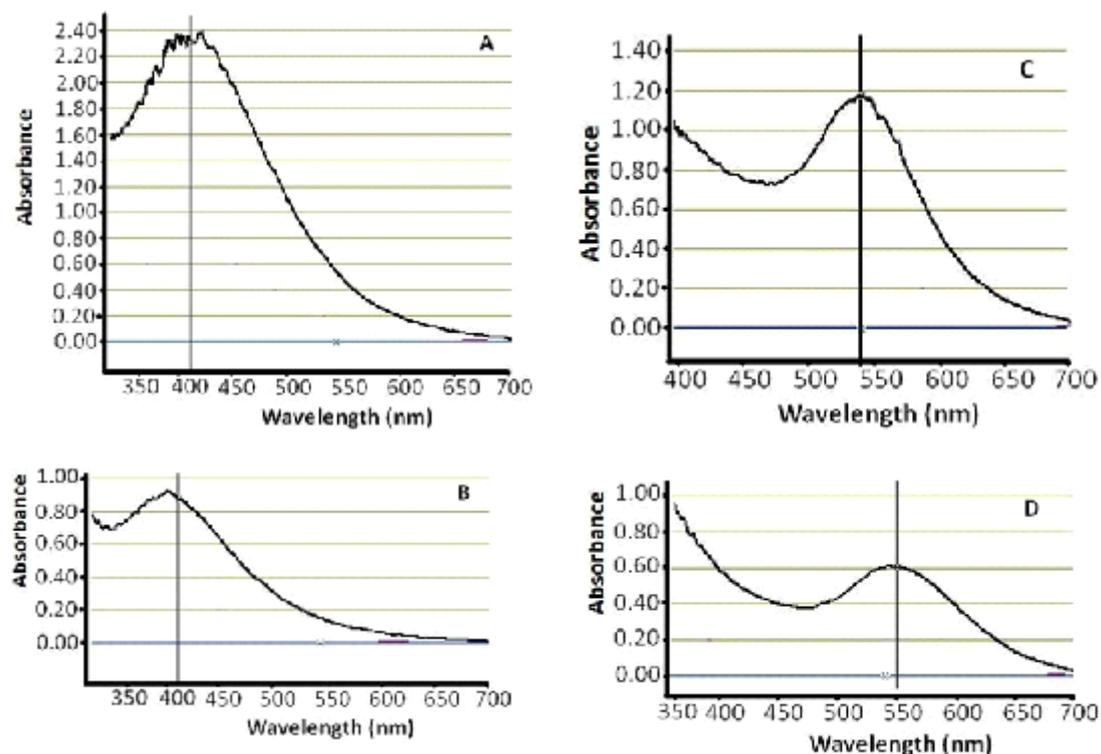
**Fig. 1.** The changed color resulted from the alternative method for silver (A-the right micro tube) and gold (B-the right micro tube) nanoparticles in contrast to the controls (the left micro tubes)

indicated that the flasks containing silver nanoparticles had maximum absorption peaks around 410-450 nm and the flasks containing gold nanoparticles had maximum absorption peaks around 510-550 nm attributed to the surface plasmon resonance band (SPR) of the silver and gold nanoparticles, respectively. Figure 2 shows visible absorption spectra of the silver and gold nanoparticles produced by *Escherichia coli* by the alternative (A & C) and routine (B & D)

strategies. The results show the maximum absorption peaks for the alternative method is higher than the routine one at the same wavelength.

#### Transmission electron microscope (TEM)

TEM analysis revealed that the obtained silver and gold nanoparticles from both of the alternative and routine methods were spherical in their shapes and their average sizes were around 5-100 nm. Figure 3 shows the TEM images of the obtained silver and gold nanoparticles from both of the alternative and routine methods.



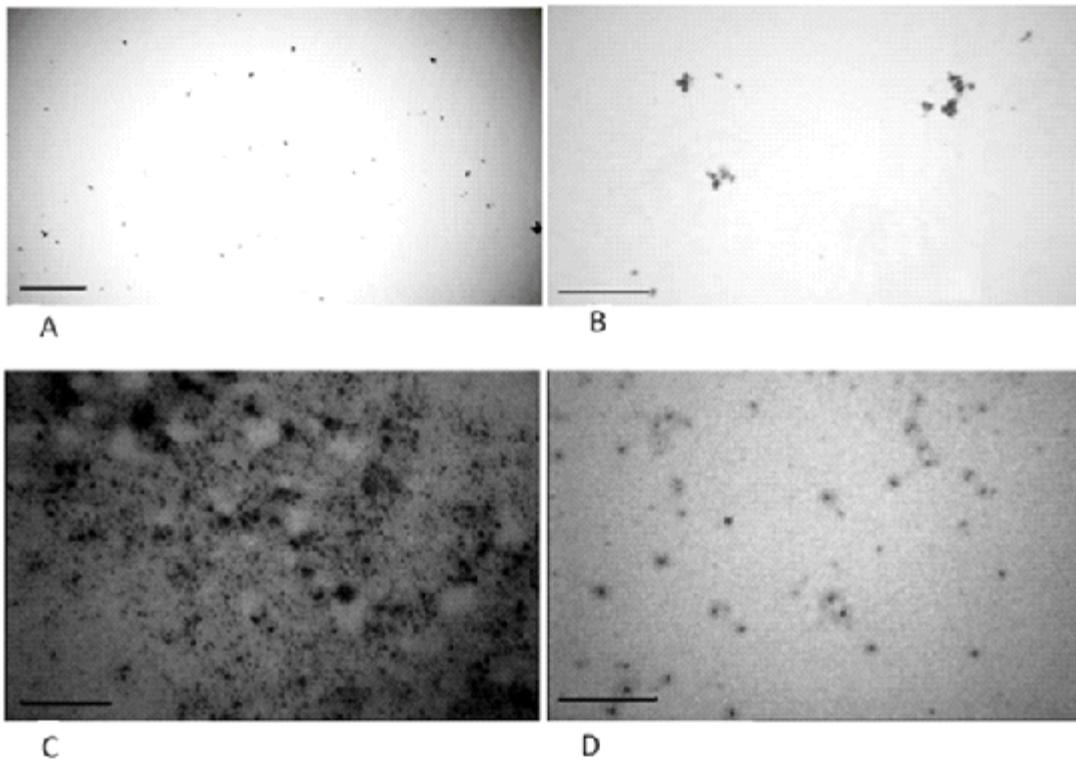
**Fig. 2.** Visible absorption spectra resulted for silver and gold nanoparticles produced by the routine and alternative methods. A: Silver nanoparticles- alternative technique. B: Silver nanoparticles- routine technique. C: Gold nanoparticles- alternative technique. D: Gold nanoparticles- routine technique

As the results show, the density of the nanoparticles that was obtained from the routine method (Figure 3 A & B) is lower than the nanoparticles that was obtained from the alternative one (Figure 3 C & D).

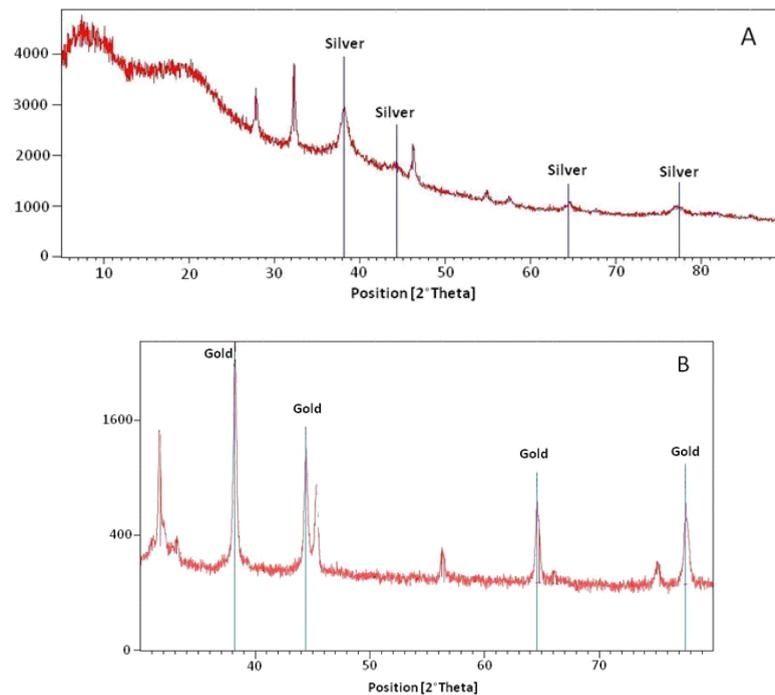
#### X-ray diffraction analysis (XRD)

XRD results showed the presence of the clear Bragg peaks at  $2\theta$  values of  $38.181^\circ$ ,  $44.18^\circ$ ,  $64.49^\circ$ ,  $77.16^\circ$  and  $81.75^\circ$  for silver nanoparticles and

$2\theta$  values of  $38.2071^\circ$ ,  $44.4179^\circ$ ,  $64.6041^\circ$  and  $77.5742^\circ$  for gold nanoparticles that were resulted from the alternative method. Same results were obtained from the routine strategy. These patterns proved the formation of the elemental silver and gold in the bacterial **culture media**. Figure 4 indicated the XRD results for silver (Figure 4A) and gold nanoparticles (Figure 4B) that were produced by the alternative method.



**Fig. 3.** TEM images resulted for silver and gold nanoparticles produced by the routine and alternative methods. A: Silver nanoparticles- routine technique. B: Gold nanoparticles- routine technique. C: Silver nanoparticles- alternative technique. D: Gold nanoparticles- alternative technique (Scale bars 150 nm)



**Fig. 4.** XRD results for A: Silver nanoparticles and B: Gold nanoparticles produced by the alternative method

## DISCUSSION

Recently the use of the green method for bio-production of the non toxic metal nanoparticles is emerged. There is different available data about multicellular and unicellular organisms with the ability of nanoparticles bio-production (Narayanan *et al.*, 2010). Almost all of these reports deal with the extracellular bio-production of nanoparticles and some of them have reported the intracellular one (Murali *et al.*, 2003). Green approach of nanoparticles synthesis has some advantages such as being easily scaled-up, without releasing toxic by-products in nature and is not a time consuming manner. Although this approach is named active or biological method, in the extracellular technique the exact function of the live cells is not clear. In this approach, after biomass production, the supernatant (i.e. cell free extract) is encountered with the metal ions and nanoparticles formation was detected (Pourali *et al.*, 2014).

In some previous data, it is reported that formation of the nanoparticles or water insoluble matters is a defense mechanism by that bacteria will overcome such harmful ions (Murali *et al.*, 2003). Some available data has reported the presence of the different electron shuttles and enzymes in and out of the bacterial cells that are responsible for the metal reduction process. It is reported that NADH and NADH-dependent enzymes are the most important factors and may be responsible for the bio-reduction of metal ions into metal nanoparticles (Senapati *et al.*, 2005). So according to the method that was used and the hypothesis that was explained for the extracellular bio-production of nanoparticles by microorganisms, there is still some confusion about the exact mechanism of nanoparticles synthesis. Actually, in the extracellular method, the biomass itself was not attacked by the toxic metal ions and so would not secret extracellular polymers, enzymes and etc as the defense mechanism (Pourali *et al.*, 2014). The exact mechanism leading to the formation of metal nanoparticles is difficult to find. In present research, we have used an alternative way for nanoparticles formation. In this approach, immediate after inoculation of the bacteria to the culture medium, the metal ions at their MICs were

brought in to the culture medium and the formation of the nanoparticles was observed approximately after 18 hours. As the results showed, the formation of the silver and gold nanoparticles was observed by changing in the color of the culture medium (Figure 1). Surprisingly, unlike the previous method (i.e. routine one), the color changed reaction mixtures did not have any bad odor and were transparent. The bad smell and opaqueness of the media containing nanoparticles in the routine method was due to the bacterial growth and their secondary metabolites such as the volatile fatty acid. In the solution of the nanoparticles developed by the alternative technique no bacterial growth was detected. It seems that in the case of the alternative strategy, the bacterial cells are actively produced nanoparticles in the first hours of the inoculation and metal ions were actively converted to the nanoparticles as a bacterial defense mechanism. After formation of the nanoparticles the cell viability and reproduction was decreased because of the toxic effects of the metal ions and the produced nanoparticles. In the routine method for nanoparticles bio-production by bacteria at least 24-48 hours are needed because the first step is to culture the bacteria and after the biomass is formed, the cell free extract is used for nanoparticles formation. But the alternative method needs only half hours in contrast to the routine one due to it does not need time for culturing bacteria. In our results, visible spectral analysis for both of the alternative and routine methods confirmed the formation of the silver and gold nanoparticles but as the data shows the spectral peaks for both of the silver and gold nanoparticles that were obtained from the alternative method was higher than the routine one (Figure 2). The TEM images showed the silver and gold nanoparticles were formed in both of the reaction mixtures but the density of the nanoparticles that were produced by the alternative strategy was higher than the routine one (Figure 3). Finally, XRD analysis confirmed the formation of the silver and gold nanoparticles by both of the two strategies.

## CONCLUSION

In conclusion as the results showed, formation of the nanoparticles by both of the two strategies is possible but by the use of the

alternative method bio-production is faster, cleaner and the produced nanoparticles have higher densities in contrast to the routine method. In future additional studies are needed for exploring the exact mechanism of this type of bio-reduction.

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