# Biosynthesis and antibacterial activity of gold nanoparticles coated with reductase enzymes

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Potential of luminescent bacteria in production of metal nanoparticles (NPs) has not been well evaluated up until now. These bacteria contain *lux* operon which is included of reductase enzymes, by increasing bacterial cell density, the expression of aldehyde synthetic enzymes elevate and enhance the yields of NPs synthesis. Therefore, extracellular synthesis of gold NPs (AuNPs) using natural occurring luminescent bacteria, VLA, VLB, VLC and genetically engineered luminescent bacteria, *Pseudomonas putida* KT2440 and *Pseudomonas fluorescence* OS8 have been successfully conducted. NPs were characterised and their antibacterial activity evaluated using microtitre plates at different concentrations against some hospital pathogenic bacteria. Biosynthetic AuNPs produced had maximum absorption at the ranges of 500–550 nanometre wavelengths. Transmission electron microscopy images showed particle sizes between 10 and 50 nanometres and confirmed the success of purification process. The NPs were spherical and the FTIR analysis showed the existence of biomolecules on surface of purified NPs that could be most probably related to reductase enzymes that are stabilised on NPs surfaces. Further investigation on antibacterial properties of these novel NPs which coated by reductase enzymes showed that any increase or decrease in antibacterial activity is dependent on NPs concentration.

**1. Introduction:** Among metal nanoparticles (MNPs), gold NPs (AuNPs) have more application in medicine, being used most frequently as biosensor [1, 2] and catalysts [3]. They are good candidate for drug delivery and cancer therapy due to their non-toxic effect on human cells and the easy functionalising with biological molecules [2].

Synthetic methods of MNPs are very different, usually classified in three categories: physical, chemical and biological methods [4, 5]. Physical methods are expensive and need a lot of energy [6]. Chemical methods, in addition to its high cost and energy consumption, produce toxics and harmful side material that is not suitable for some application [5]. In the case of metallic NPs, biological methods are most exciting and included of bacteria, fungi, plant extract and so on. Biological methods without producing harmful material reduce cost synthesis and energy consumption.

Bacterial cells due to their high growth rate, easy manipulation and possibility of modifying and controlling growth condition can be a suitable area for MNPs synthesis [7, 8]. Also in recent years with the widespread use of antibiotics in the various communities, multiresistance microbial strains have been developed. Treatment of infections caused with these resistant microorganisms is hard and requires multiple broader spectrum antibiotics with systemic side effects which leave irreparable damages and causing numerous deaths [9–11]. As development of resistant strains and their infections increases, need for a low cost, minimal side effects and strong new antibacterial agent are inevitable [11–14]. For this purpose MNPs are a good choice due to their high surface to volume ratio which provides higher possibility of contact with bacterial surface and their antibacterial nature [9–11].

In recent years using of bacterial cells for NP synthesis has attracted much attention because it is cheaper and has an easier purification procedure. As reducing factors, reduction enzymes of bacteria can play important role in intracellularly or extracellularly NP biosynthesis [15]. Luminescent bacteria have several such enzymes including: Luciferase, 3 aldehyde synthase [16]. Therefore, in this research the ability of luminescent bacteria (natural and genetically engineered) which are containing *lux* operon with reducing enzymes in production of AuNPs has been evaluated. NPs synthesised, and after purification their characteristics were determined. Then, the antibacterial and antibiofilm activity of biosynthetic AuNPs on some pathogenic bacteria was investigated.

## 2. Methods

2.1. Medium, salt of Au and bacterial strains: Tryptone, yeast extract and glycerol purchased from Q-Lab (Canada). Naturally occurring luminescent bacteria (VLA, VLB and VLC) isolated from an Iranian shrimp farm (data are not published) and after identification and genes sequencing (99% similarity to *Vibrio harveyi*) have been used in NPs synthesis. Genetically engineered luminescent *Pseudomonas putida* KT2440 (carrying plasmid pV1360 containing *luxAB* gene) and *Pseudomonas fluorescence* OS8 (OS8:KnmerRBSBPmerlux chromosomally *lux* marked with suicide plasmid pTCRKnmerRBSBPmerlux containing *luxCDABE* genes) were resuscitated from laboratory stock cultures. All other bacterial strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter*, *Klebsiella pneumoniae* and *Salmonella typhimurium*) were obtained from Microbiology Division of Ghaem Hospital of Mashhad University of Medical Science.

2.2. Biosynthesis of AuNPs: *P. putida* KT2440 and *P. fluorescence* OS8 genetically engineered bacteria were cultured in Luria-Bertani (LB) medium (containing 10 g tryptone, 5 g yeast extract and 10 g NaCl in 950 ml deionised water) and VLA, VLB, VLC, natural bioluminescent bacteria, cultured in seawater-based complete (SWC) medium (24 g sea salt, 5 g tryptone, 3 g yeast extract and 3 ml glycerol in 1 L deionised water) and incubated at 30°C, 150 rpm/min shaking for 48 and 72 h, respectively. After incubation, biomass centrifuged at 5000 rpm for 10 min and washed three times. Then, 1 g wet weight of biomass was added to 20 ml of 1 mM of $HAuCl_4$, pH adjusted at 7 by 1 N NaOH and incubated at 30°C, 150 rpm/min shaking. Additionally, 1 g from wet weight of




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bacterial biomass was inoculated to 20 ml of LB broth as bacterial growth control and 20 ml of 1 mM of $HAuCl_4$ with no bacteria used as NPs synthesis control. After 48 h incubation, the process was completed.

2.3. Purification of AuNPs: After completion of the AuNP synthesis process, the bacterial cells were removed from the solution interaction by filters with 0.22 micrometre dimensions. Then the filtered solution were centrifuged at 10,000 rpm for 10 min and the supernatant transferred for inductively coupled plasma optical emission spectrometry (ICP-OES), then the pellet was washed three times with deionised water and from pellet sent to the ICP-OES, Spectro ARCOS EOP instrument, for concentration determination.

2.4. Characterisation of AuNPs

2.4.1. Ultraviolet–visible (UV–vis) spectroscopy: Changing the colour of interaction solution from yellow to pink indicates that reaction is complete and the solution absorption were controlled from 300 to 700 nm wavelength by Shimadzu UV1700 spectrophotometer (Japan) with quartz cells of 1 cm path length.

2.4.2. Transmission electron microscopy: The colloidal forms of AuNPs after purification have been analysed by transmission electron microscopy (TEM) images of LEO 912AB instrument (Germany) to find out shape and size of NPs and confirming the purification process.

2.4.3. Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS): Bacteria with their produced NPs in aqueous solution were biologically prepared. After fixation of preparation on a filter paper without Au coating, SEM image captured by LEO-1530VP field-emission (Germany) followed by the EDS analysis which confirmed the NPs genus.

2.4.4. Fourier transform infrared spectroscopy: Dried powder of purified NPs was analysed by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra were performed on an Avatar 370 FTIR Thermo Nicolet spectrometer (USA) to identify NPs synthesis key molecules.

2.5. Antibacterial activity

2.5.1. Bacterial growth inhibition: To examine the minimum inhibitory concentration of NPs for *E. coli*, *Enterobacter*, *K. pneumonia*, *S. typhimurium*, *S. aureus*, *S. epidermidis*, *B. subtilis*, *L. monocytogenesis* and *Pseudomonas aeruginosa*, the bacterial cells were exposed to NPs, on 96 well flat bottom tissue culture plates. The bacterial suspension adjusted to 0.5 McFarland's standard and serial dilution was then prepared (0, 12.5, 25, 50 and 100 ppm) so that each well of microtitre plate filled with 150 µl of suspension with three replication of every concentration had $10^8$ CFU/ml bacterial cells. The microtitre plate incubated for 24 h at 37°C with 150 rpm shaking. After incubation, the optical density of bacterial cells was determined by microtitre plate reader (Stat fax® 2100, England) at 620 nm. The percentage of growth inhibition (GI %) of each treatment at various reaction conditions was calculated in comparison with its own positive control using the following equation: (see equation at bottom of the page)

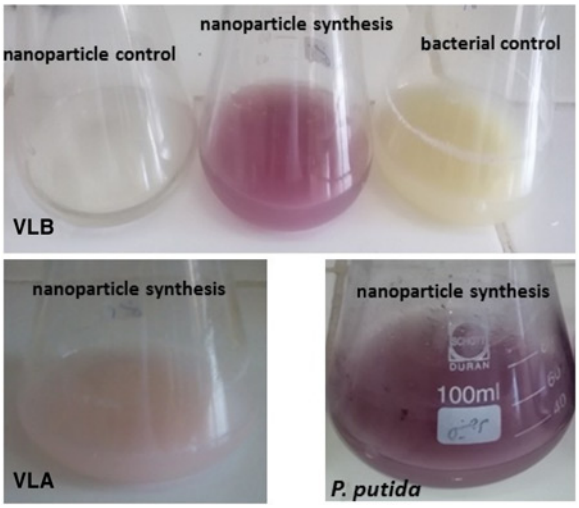


**Fig. 1** *Colour turning of mixture solutions affords synthesised Au nanaoparticles by luminescent bacteria, VLA, VLB and P. putida KT2440*

2.5.2. Biofilm formation: To examine biofilm inhibitory behaviour of AuNPs, *Enterobacter*, *K. pneumoniae*, *S. epidermidis* and *P. aeruginosa* were chosen according to their ability in biofilm formation. The bacterial cells were exposed to NPs on 96 well flat bottom tissue culture plates similar to Section 2.5.1. The plate was incubated for 24 h at 37°C. After incubation, the suspension of each well removed and the wells washed three times with deionised water. Adherence of bacteria cells to the bottom of wells was monitored by staining with crystal violet (0.2% w/v) following with incubation for 30 min at 37°C. Then suspension was washed three times with deionised water, and after drying 96% ethanol was added to wells and optical densities of stained adherent bacteria were defined with microtitre plate reader (Start fax® 2100, England) at 590 nm. The process was performed in triplicates on different times. The percentage of biofilm inhibition (BI %) of each treatment at various reaction conditions was calculated in comparison with its own positive control using the following equation: (see equation at bottom of the page)

**3. Results:** The visually colour changing of reaction mixtures from pale yellow to deep pink was began from 6 h incubation for *P. putida* KT2440, *P. fluorescence* OS8 and VLB bacteria and was completed after 48 h but about VLA and VLC bacteria, the colour changing to pale pink was began after 12 h incubation and after 72 h incubation the colour remained pale pink and reaction was completed. The reaction solution colour turning has been shown in Fig. 1.

SEM images from bacteria producing AuNPs are shown in Fig. 2. The light points are AuNPs, they are synthesised extracellularly and EDS analysis from these points that is not coated with Au has been shown in Fig. 3 and the sharp peak of Au confirms the existence of AuNPs without any impurities. The size of AuNPs biosynthesised in this Letter was about 10–50 nm with sufficient concentration and appropriate distribution.

The reaction mixture was purified by syringe filter dimension 0.22 µm smaller than bacterial size. The solution free bacteria

$$\text{percentage of growth inhibition (GI\%)} = 100 - \left(\text{OD}_{630} \text{ at the presence of antibacterial agent}/\text{OD}_{630} \text{ at the absence of antibacterial agent} \times 100\right)$$

$$\text{percentage of biofilm inhibition (BI\%)} = 100 - \left(\text{OD}_{590} \text{ at the presence of antibacterial agent}/\text{OD}_{590} \text{ at the absence of antibacterial agent} \times 100\right)$$

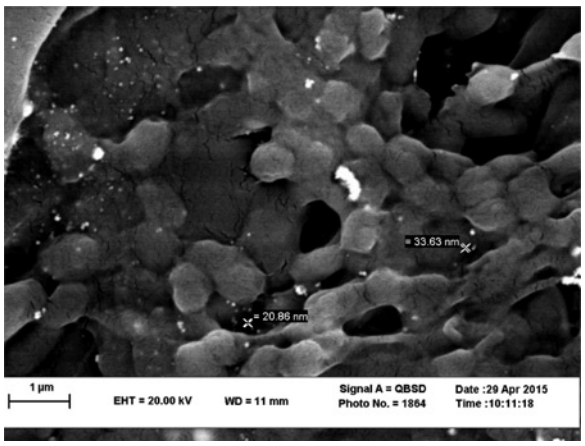


**Fig. 2** *Position of AuNPs in relation to their producing bacteria. The light points indicate AuNPs which are located extracellularly*

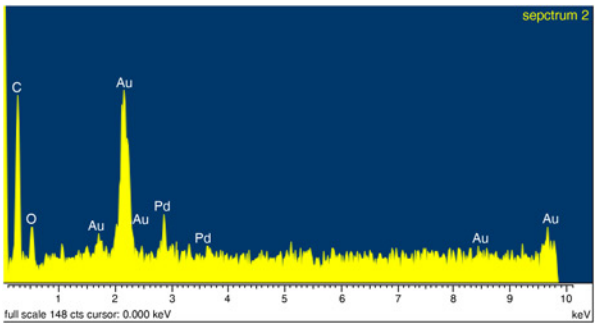


**Fig. 3** *EDS analysis of light points on SEM image*

was centrifuged for separating AuNPs from other component mixture which might affect the characteristics of NPs and their antibacterial properties. The ICP-OES analysis performed to find out convert index of Au cations compared with AuNPs that summarised as follows:

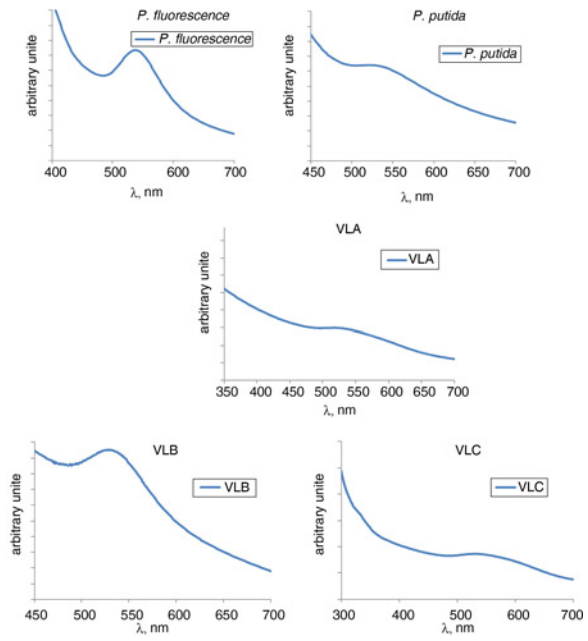


**Fig. 4** *UV–vis spectrum of purified AuNPs biosynthesised by different luminescent bacteria*

supernatant concentration (containing Au cations) 16 mg $l^{-1}$

pellet concentration (containing AuNPs, without Au cations) 116 mg $l^{-1}$

$$\text{convert yield} = [Au^0]/([Au^0] + [Au^+])$$

Their convert yield was calculated about 88%.

UV–vis spectrum from all of purified AuNPs showed maximum absorption at 500–540 nm indicating AuNP formation that are shown in Fig. 4.

The TEM images of purified AuNPs from *P. fluorescence* OS8 and *P. putida* KT2440 NPs revealed spherical particles with the sizes of 10–50 nm. In addition, TEM images have also confirmed that the purification process was successful in which no bacteria detected. The TEM images are shown in Fig. 5.

The FTIR analysis of dried powder of the purified NPs showed that the band at 3291 $cm^{-1}$ related to stretching vibration of hydroxyl and the 2925 $cm^{-1}$ is about stretching vibrations of first or secondary amines. The 1660 and 1078 $cm^{-1}$ band assigned to C=O, C–N or C–O stretching vibrations, respectively [17]. The FTIR spectrum is shown in Fig. 6.

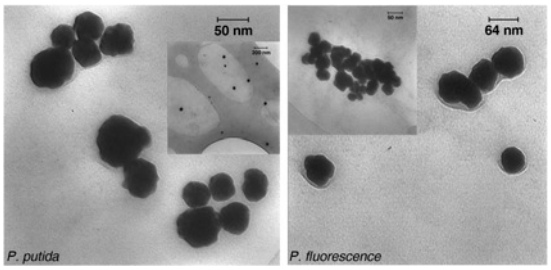


**Fig. 5** *TEM images of purified AuNPs synthesised by P. putida KT2440 and P. fluorescence OS8*

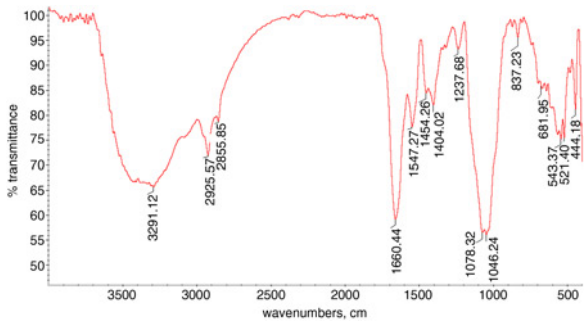


**Fig. 6** *FTIR spectrum of purified AuNPs of P. putida KT2440*

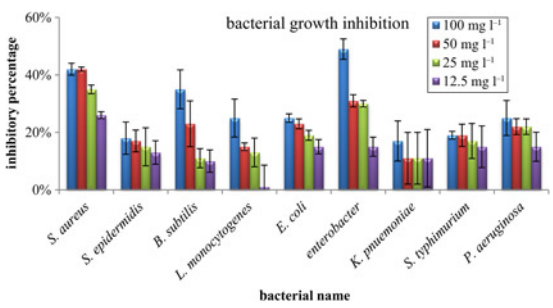


**Fig. 7** *Bacterial GI by different concentration of biogenic Au NPs (12.5, 25, 50 and 100 mg/l)*




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**Table 1** Bacterial GI (per cent) of various concentrations of AuNPs (mg $l^{-1}$)

| Bacterial name | AuNP concentration | | | |
|---|---|---|---|---|
| | 12.5 mg $l^{-1}$, % | 25 mg $l^{-1}$, % | 50 mg $l^{-1}$, % | 100 mg $l^{-1}$, % |
| *S. aureus* | 26 | 35 | 42 | 42 |
| *S. epidermidis* | 13 | 15 | 17 | 18 |
| *B. subtilis* | 10 | 11 | 23 | 35 |
| *L. monocytogenes* | 1 | 13 | 15 | 25 |
| *E. coli* | 15 | 19 | 23 | 25 |
| *Enterobacter* | 15 | 30 | 31 | 49 |
| *K. pneumoniae* | 11 | 11 | 11 | 17 |
| *S. typhimurium* | 15 | 17 | 19 | 19 |
| *P. aeruginosa* | 15 | 22 | 22 | 25 |

The percentage of bacterial GI by AuNPs biosynthesised in this Letter is shown in Fig. 7 and their results are summarised in Table 1. Increasing AuNPs concentrations increase bacterial GI.

The results of BI assay of AuNPs showed that with increasing concentration of NPs, biofilm formation decreases (Table 2, Fig. 8).

**4. Discussion:** Time of completing process showing that biosynthesis of AuNPs by luminescent bacteria have been a slow procedure. Changing in the colour of reaction mixture shows NPs synthesis and surface resonance plasmon feature that colour spectrum changing depends on NPs size [18, 19]. This colour reaction turning is similar to AuNP synthesis by *Rhodopseudomonas capsulata* and indicates extracellular synthesised NPs [20].

As shown in SEM images, AuNPs synthesis in this research is extracellular. The extracellular synthesis of NPs is more important due to their easier purification, reducing hazardous material production and reducing energy consumption [15]. The extracellular biosynthesis of AuNPs has also been reported using another bacteria such as *R. capsulata* [20], *Thermomonospora* sp. [18], *P. aeruginosa* [21], while in some other bacteria such as *E. coli* DH5$\alpha$ is synthesised intracellularly [19].

**Table 2** BI assay (per cent) of various concentrations of AuNPs (mg $l^{-1}$)

| Bacterial name | AuNP concentration | | | |
|---|---|---|---|---|
| | 12.5 mg $l^{-1}$, % | 25 mg $l^{-1}$, % | 50 mg $l^{-1}$, % | 100 mg $l^{-1}$, % |
| *Enterobacter* | 19 | 22 | 22 | 23 |
| *K. pneumoniae* | 29 | 32 | 33 | 33 |
| *S. epidermidis* | 14 | 15 | 16 | 23 |
| *P. aeruginosa* | 38 | 36 | 40 | 41 |

The obtained convert yield of NPs synthesis by *P. putida* KT2440 is suitable for a biological method for NPs synthesis and is a worthwhile result.

The maximum wave length of AuNPs relies on particle size and shapes [15, 22]. The maximum wave lengths of pink reactions were 517, 530 and 538 for *P. putida* KT2440, VLB and *P. fluorescence* OS8, respectively, and the size of *P. fluorescence* OS8 and *P. putida* KT2440 purified AuNPs NPs were 50 and 35 nm, respectively, indicating that when maximum wave length shifts to red range, particles size become smaller. The key impacts of NP size on its applications have been noted in several reports [23]. NPs with small size can rapidly be cleared from body while larger sizes accumulate in organs such as liver, spleen and bone marrow [24]. The size of microbiologically synthesised AuNPs could be optimised by adjusting culture conditions as Gericke and Pinches found that majority of the particles were predominantly spherical in shape, relatively uniform in size and <10 nm in diameter at a defined low pH (pH 3) [25]. In this research, results showed that luminescent bacteria have potential to produce AuNPs in sizes to be used for nanomedicine applications especially enzyme NPs.

The FTIR analysis confirmed protein existence on purified NPs surface which may play an important role in AuNPs synthesis, their stability or perhaps on their secretion. The layer around of these NPs in TEM image confirms this asseveration.

Reductase enzymes of bacterial cells play a fundamental role as reducing factor in NPs biosynthesis [15]. Naturally occurring luminescent bacteria carry *lux* operon to emit light, this operon included of three aldehyde synthesis genes encoding reducing factors that can play important roles in NPs synthesis. These gene expressions are controlling by quorum sensing so that with increasing bacterial cell density, pheromones production elevates expression of aldehyde synthesis enzymes [16, 26] resulting higher yields in NPs synthesis.

The information obtained from FTIR analysis and TEM images show the existence of a large amount of enzymes on NPs. The FTIR spectrum of reductase enzyme provided from SDBS [Spectral Database for Organic Compounds, SDBS website (http://www.sdbs.db.aist.go.jp)] compared with present spectrum showing that the peaks are very similar with little differences that confirm the extraction of producing enzyme with NPs that is shown in Fig. 9. Further investigations about these novel bioconjugated AuNPs are under consideration in our laboratories. We hope this research open a new window for preparation of supported enzymes on NPs and biochemical research.

We have to point out the importance of these findings because enzymes immobilised on NP showed a broader working pH and temperature range and higher thermal stability than the native enzymes. Also compared with the conventional immobilisation methods, nano-enzyme particles are easy to synthesise without using surfactants and toxic reagents. In addition, it is possible that co-immobilisation of multi-enzymes could be achieved on these NPs [27].

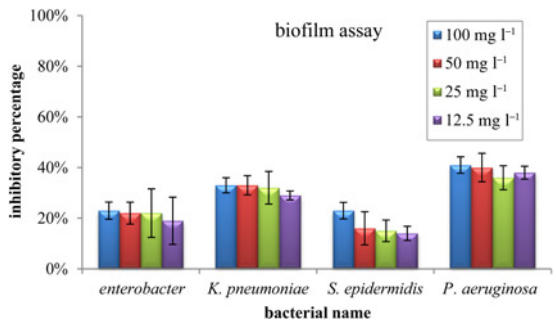


**Fig. 8** *Bacterial BI by different concentrations of biogenic Se NPs (12.5, 25, 50 and 100 mg/l)*

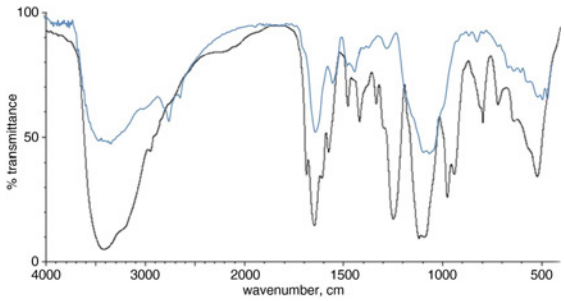


**Fig. 9** *Blue colour shows the FTIR of NPs and black colour shows the FTIR of reductase enzyme that has taken from SDBS*

Stability of purified NPs was considered, NPs produced by *P. fluorescence* OS8 had a stable state for 1 month while *P. putida* KT2440 and *Vibrio* luminescence B (VLB) could produce NPs with 3 month stability. Strains *Vibrio* luminescence A (VLA) and *Vibrio* luminescence C (VLC) were very stable, since after 1 year could preserve their colloidal states and had no precipitation while the longest period of NPs stability reported so far was 3 month [17, 20]. However, biosyntheses of NPs by VLA and VLC were slow and these low reaction speeds indicate the low ability of these bacteria in NPs synthesis and concentrations decreases that reduce the chance of NPs aggregation so enhance their stability.

NPs due to their small size and high surface to volume ratio exhibit good antibacterial activity [18]. Increasing AuNPs concentrations enhance bacterial GI as have been shown in Table 1. With Increasing NPs concentration from 12.5 to 100 mg/l, the growth of all of pathogenic bacteria decreased so increasing NPs concentration enhance the inhibitory effect of NPs. This linear relevance has been proven about silver NPs concentration and its bacterial GI [28].

Though in this research we have not studied the antibacterial mechanisms of AuNPs, however, with regard to other research, antibacterial mechanisms of present AuNPs could be due to one or more of following three categories: (i) NPs attaching to cell wall, membrane and surface respiratory enzymes that destroy cell integrity and ability of adenosine triphosphate synthesis; (ii) NPs penetrating into the cell, they bind to intracellular functional enzymes and inactivate them, in addition attaching to DNA and RNA molecule and denature them to stop transcription and cell division; and (iii) reactive oxygen generation, tightening both process and showing more destructive effects [29–31].

Inhibitory effect of AuNPs on biofilm formation of *Enterobacter*, *K. pneumonia*, *S. epidermidis* and *P. aeruginosa* up to concentration of 100 mg/l enhanced as shown in Table 2. Silver NPs inhibited biofilm formation of *P. aeruginosa* that is in agreement to decreasing biofilm formation by AuNPs in different concentrations [32].

**5. Conclusion:** According to the wide application of nanotechnology in various fields of science and human life, development of new synthetic methods with high efficiency, low-energy consumption and cost is necessary. The synthesis of AuNPs using luminescent bacteria would be a new step to more advance the science of nanotechnology according to green chemistry. In this Letter, the synthesis of NPs are extracellular and at room temperature that decrease the costs of purification process and energy consumption of NP synthesis. These NPs are spherical shape and about 10–50 nm size. Convert index of AuNPs synthesised by *P. putida* KT2440 calculated about 88%, that is, good yields but can be proved by changing various factors. Furthermore, the proteins on the surface of purified NPs are high that indicate extraction of NP synthesising or stabilising enzymes which could be used for easier synthesis of NPs without bacterial cells. There is a linear relevance between concentration of AuNPs and microbial GI which means that increasing the AuNPs concentrations cause an intensification in the microbial growth inhibitory effect of them. However, increasing AuNPs concentration beyond optimal concentrations enhances antibiofilm activity of these NPs.

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