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Antibacterial and antibiofilm activity of biogenic Se-Au nanoparticles against most prevalent pathogenic bacteria

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Abstract

Luminescent bacteria, having reductase enzymes, can reduce metal ions as nanoparticles. In this study, the synergic effect of biosynthesized selenium nanoparticles (SeNPs), using *Pseudomonas putida* KT2440, carrying *lux* AB gene with biogenic gold nanoparticles (AuNPs) is assessed. The SeNPs were first purified and then characterized using different techniques, including UV-visible and Fourier Transform Infrared (FTIR) spectroscopy, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Energy Dispersive x-ray (EDX) spectroscopy analyses. The antibacterial and antibiofilm activities of SeNPs were also evaluated with and without AuNPs, at 12.5, 25, 50, and 100 mg l^{-1} concentrations. The SEM images showed that biogenic SeNPs were spherical attached to the surface of the producing bacteria. The FTIR spectroscopic results revealed the presence of protein molecules on the surface of the synthesized SeNPs. It is found that purified SeNPs have proper antibacterial and antibiofilm activities against several potential human pathogenic bacteria, depending on the NPs concentrations. It was also found that the presence of AuNPs enhances the antibacterial activity of SeNPs. Our results showed that adding SeNPs improved the antibacterial activity of AuNPs against *Bacillus subtilis* (71%), *Staphylococcus aureus* (55%), *Staphylococcus epidermidis* (52%), *Escherichia coli* (47%), and *Pseudomonas aeruginosa* (26%). Also, the antibiofilm activity of SeNPs improved by about 26% and 37% against *E. faecalis* and *S. aureus*, respectively. In conclusion, the mixture of biosynthesized SeNPs and AuNPs, using luminescent bacteria as Se-Au nanoparticles is more effective and can be applied as an antimicrobial agent against multi-antibiotic resistant pathogenic bacteria.

Keywords: Antibacterial activity, biogenic NPs, luminescent bacteria, pathogenic bacteria, Se-Au NPs
Classification numbers: 2.05, 4.02, 5.08, 5.11, 5.13

1. Introduction

In the last two decades, nanoparticles of various metals - have played an important role in a variety of science and technology fields [1, 2]. Selenium is a semi-conductor and a trace element in humans and other mammals and a crucial element in activating glutathione peroxidase [2–4]. Selenium nanoparticles have attracted extensive attention in many fields due to their high bioactivity, low cytotoxicity, good human tolerability, and antioxidant and anticancer activity [5–8].

Although various physical, chemical, and biological methods have been used to synthesize nanoparticles,

physicochemical methods are not recommended due to the high cost, energy consumption, and toxic by-product generation [9–12]. Biological methods apply the reductase of organisms to convert metal salts to nanoparticles. This method is low-cost, environmentally friendly, and free of hazardous by-product generation [1, 13]. Furthermore, biomolecules cover the nanoparticles surface in biological methods, preventing their aggregation leading to enhancing their stability [12]. SeNPs synthesized by bacteria are almost spherical with 10 to 700 nm size [14–17].

Selenium NPs are found to exhibit high antibacterial, antibiofilm and antifungal activities. SeNPs biosynthesized by *Lysinibacillus* have shown good antibacterial activity against Gram-positive and Gram-negative bacteria [15]. Several works have studied the antibiofilm activity of SeNPs against various bacteria, such as *S. aureus*, *P. aeruginosa*, *P. mirabilis*, *E. coli*, and *K. pneumoniae* [18, 19]. Also, the antifungal activity of SeNPs has been investigated against *Aspergillus fumigatus*, *Candida albicans*, *Pyricularia grisea*, *Colletotrichum capsici*, and *Alternaria solani* [20].

The prevalence of multidrug-resistant bacteria and their nosocomial infections is increasing [21–24], which kill about 700,000 people worldwide every year. According to the World Health Organization (WHO) prediction, this number will reach 10 million in 2050 [25]. Metal NPs can be promising candidates for developing new low-cost and more effective antibacterial agents [21–24]. Also, combining different metal ions and surface decorations of some nanoparticles can have synergistic effects, improving microbial killing and leading to the development of more effective antibacterial agents [26, 27].

In this research, SeNPs were biosynthesized using a luminescent bacterium which contained *lux* operon with 3 aldehyde reductase genes to empower the reduction ability of the synthesis. Also, the synergic effects of SeNPs and biogenic AuNPs on different pathogenic bacteria are investigated. These particles were chosen to study synergic antimicrobial effects since, up to our knowledge there are no reports on the cytotoxicity of AuNPs in human cells [26].

2. Materials and methods

2.1. Medium and bacterial strains

The genetically engineered luminescent bacterium, *Pseudomonas putida* KT2440, carrying *lux* AB gene, was prepared from laboratory stock. Other bacterial strains including *Bacillus subtilis*, *Escherichia coli*, *Enterobacter faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* were denoted from the Microbial Division of Ghaem Hospital, Mashhad University of Medical Sciences. The AuNP biosynthesis was performed using *Pseudomonas putida* KT2440 as described in our previous work. The biosynthesized AuNPs were shown to be spherical with a size between 10 to 50 nm [28]. All the chemicals used in this research were of analytical grade and were sourced from Merck.

2.2. Biosynthesis of selenium nanoparticles

P. putida KT2440 was employed for SeNPs biosynthesis. The genetically luminescent bacterium was first cultured in nutrient broth and incubated at 30 °C for 24 h at 150 rpm. Then, 1 ml of cell suspension with 0.1 absorbance at 600 nm was inoculated to 99 ml nutrient broth containing 100 mg L^{-1} Se^{4+} and incubated at 150 rpm at 30 °C for 24 h [15, 29].

2.3. Purification of selenium nanoparticles

The cell suspension was centrifuged at 4000 × *g* for 10 min, after the exposure time. The supernatant containing selenium cations was discarded and the pellets containing bacterial cells and SeNPs were transferred to a porcelain mortar, frozen with liquid nitrogen and then ground. As most of the synthesized SeNPs were anchored to the bacterial cell wall or located at the periplasmic space, the resulting slurry was first transferred to a test tube and then ultrasonicated gently at 100 W for 5 min. The obtained solution including the cell debris and SeNPs was centrifuged three times at 10000 × *g* for 5 min by 1.5 M Tris-HCl containing 1% SDS (pH = 8.3) and water, respectively. Furthermore, the pellets were washed with octanol-deionized water at 1:2 ratio at 2000 × *g* for 5 min and then kept at 4 °C for 24 h. After 24 h, the produced SeNPs were observable at the bottom of the test tube. Then, the upper and lower phases were removed and the NPs were successively washed with chloroform, ethyl alcohol and deionized water. Then, the obtained solution was centrifuged at 8000 × *g* for 10 min after each step. Finally, the suspension was centrifuged at 12000 × *g* for 10 min and the pellet and the supernatant were analyzed by ICP-OES (Spectro ARCOS) [29].

2.4. Characterization of selenium nanoparticles

2.4.1. UV-visible spectroscopy. The turning color of the mixture to red indicated the completion of the reaction. The absorbance spectrum of the suspension was obtained in the range of 200 to 700 nm using a spectrophotometer (Shimadzu UV 1700) [30, 31].

2.4.2. TEM and SEM-EDX. The position of the synthesized NPs in the bacterial cells was determined by employing SEM (LEO 1530VP model). Additionally, the elemental nature of NPs was determined through EDX analysis. TEM (Zeiss LEO 912AB model) was used to evaluate the purification quality and determine the size of the synthesized SeNPs.

2.4.3. FTIR spectroscopy. Identifying biomolecules that may play a key role in SeNPs synthesis was carried out through FTIR spectroscopy analyses using KBr pellet methods [32]. To conduct the analysis, 30 ml of purified SeNPs was dried at 60 °C for 24 h in an oven (Memmert, Germany). The obtained

powder was then examined using the Thermo Nicolet Avatar 370 FTIR model [30, 31].

2.5. Preparation of Se-Au NPs

To evaluate the synergistic effect of Se-Au NPs on specified bacteria, 10 ml of purified SeNPs at a concentration of 100 mg l^{-1} were mixed with 10 ml of purified AuNPs at the same concentration. The reaction mixture was stirred for 15 min.

2.6. Inhibition of bacterial growth

The main purpose of this study has been to evaluate the inhibitory effect of purified SeNPs and Se-Au NPs at 12.5, 15, 50, and 100 mg l^{-1} concentrations on potential human pathogenic bacteria *E. coli*, *S. aureus*, *S. epidermidis*, *B. subtilis*, and *P. aeruginosa*, using microdilution method [33, 34]. First, 100 µl of bacterial culture (0.5 McFarland) was added to each well of the microtiter plate containing 150 µl of different concentrations (12.5, 25, 50, and 100 mg l^{-1}) of SeNPs and Se-Au NPs, separately. The microtiter plate was incubated for 24 h at 37 °C and 150 rpm. Then, the absorbance of each well was determined at 630 nm by an ELISA microtiter plate reader (Stat Fax® 2011). The same experiments were performed in triplicate with NPs without bacterial cells and bacterial cells without NPs as negative and positive controls for each experiment, respectively. The growth inhibition percentage (GI%) for each concentration of SeNPs and Se-Au NPs was calculated in comparison with positive control using equation (1).

$$GI\% = 100 - (A/B \times 100) \quad (1)$$

where A and B are the optical density at 630 nm wavelength in the presence and absence of an antibacterial agent, respectively.

2.7. Inhibition of biofilm formation

The inhibitory effects of purified SeNPs and Se-Au NPs on forming biofilms by clinical *E. faecalis* and *S. aureus* isolates were determined using the microtiter plate test based on crystal violet dye [35]. After overnight incubation of the bacterial culture at 37 °C, a dilution (0.5 McFarland) of the bacterial suspension was prepared in the Luria Bertani medium. This suspension was then exposed to different concentrations of SeNPs and Se-Au NPs (12.5, 25, 50, and 100 mg l^{-1}) on the microtiter plate. After 24 h of incubation at 37 °C, the liquid suspension of each well was discarded and the wells were washed three times with PBS. Then, the bacterial cells sticking to the bottom of the wells were stained by crystal violet dye (0.2% w/v) and the microtiter plate was incubated for 30 min at 37 °C. Finally, after washing the wells with deionized water and drying, ethanol (96%) was added to each well and the absorbance was measured at the wavelength of 590 nm by an ELISA microtiter plate reader. The process was performed in triplicate at different times. NPs without bacterial cells and bacterial cells without NPs were considered as negative and positive controls for each experiment, respectively. The

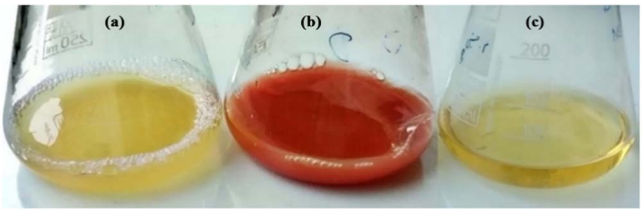

Figure 1. (a) *P. putida* KT2440 with no selenium oxide. (b) SeNPs synthesized by *P. putida* KT2440. (c) Selenium oxide with no bacteria.

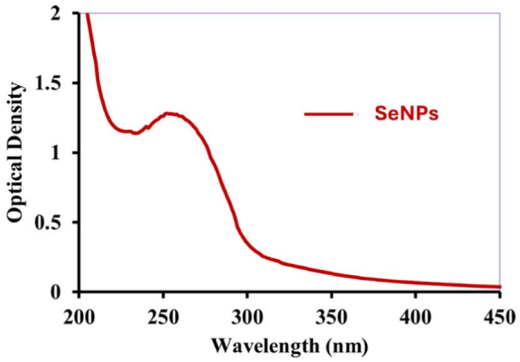

Figure 2. The UV-visible spectrum of SeNPs synthesized by *P. putida* KT2440.

percentage of biofilm inhibition (BI%) was calculated in comparison with the positive control using equation (2):

$$BI\% = 100 - (A/B \times 100) \quad (2)$$

where A is the optical density at 590 nm wavelength in the presence of an antibacterial agent and B is the optical density at 590 nm wavelength in absence of the antibacterial agent.

3. Results and discussion

Biological methods for the synthesis of nanoparticles are currently known with more eco-friendly options which is developing. In the biosynthesis of metal NPs, metal ions initially attach to the surface or inside bacterial cells and then the trapped metal ions are reduced to nanoparticles by bacterial reduction enzymes [36]. The enzymatic reduction of metal ions to nanoparticles is the most feasible for nanoparticle synthesis in bacterial cells [22]. Mukherjee *et al* reported that nitrate reductase plays a key role in the biosynthesis of silver nanoparticles by *Bacillus clausii* [37]. It has been demonstrated that NADH and NADH-dependent nitrate reductases are effective in silver nanoparticle synthesis by *Bacillus licheniformis* [38]. In this study, SeNPs were synthesized by *P. putida* KT2440, genetically engineered luminescent bacteria containing *lux* operon with three aldehyde reductases to ensure the reduction capacity.

Figures 1a and 1b show the bacterial mixture of *P. putida* KT2440 before and after the synthesis of SeNPs. The bacteria medium started to turn red after 6 h, and the process was completed after 48 h (figure 1). The duration of the process indicates that the biosynthesis of SeNPs by *P. putida* KT2440

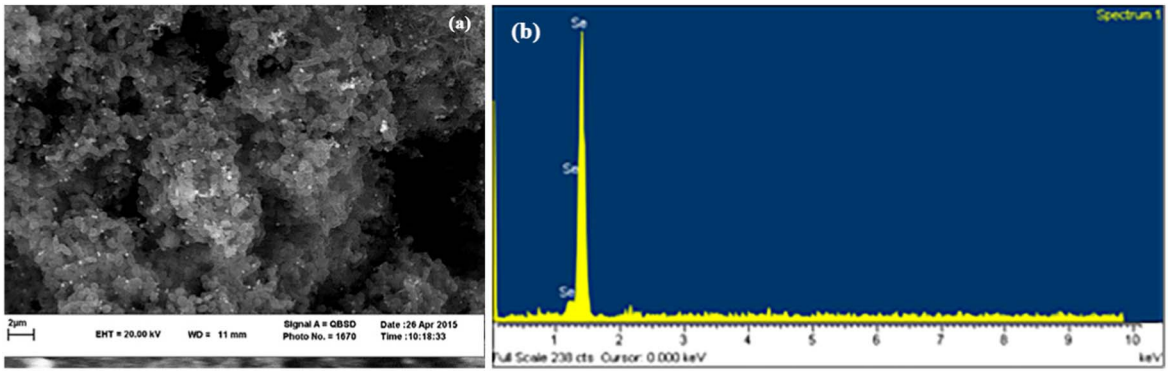

Figure 3. (a) The SEM micrograph of *P. putida* KT2440 with the synthesized SeNPs. (b) EDX analysis.

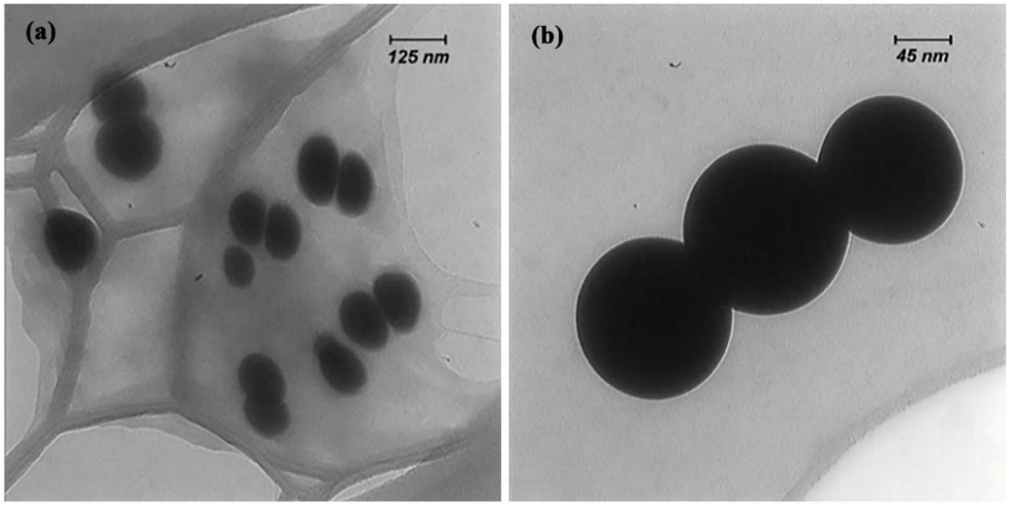

Figure 4. (a),(b) The TEM micrograph of purified SeNPs synthesized by *P. putida* KT2440 at different sample locations.

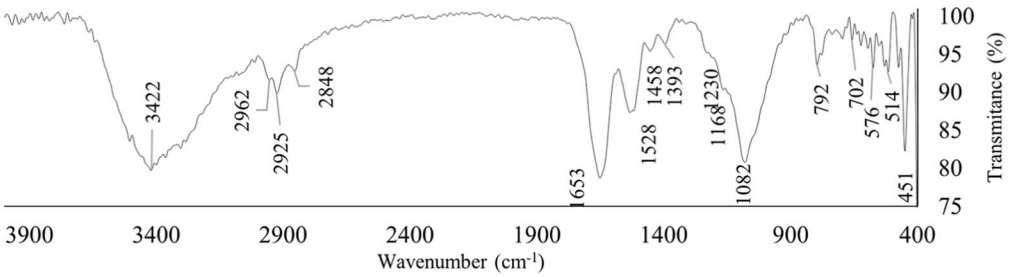

Figure 5. The FTIR spectrum of purified SeNPs.

is rather a slow process. Figure 1(b) indicates the synthesized SeNPs in the presence of bacterial cells, and figure 1(a) shows the medium with bacteria and no Se ions. Figure 1(c) shows the control mixture in which no color change is observed, indicating that the synthesis of SeNPs does not occur without bacterial cells.

UV-visible spectra of the SeNPs synthesized by *P. putida* KT2440 are shown in figure 2, with the maximum absorption at 250 nm indicating the SeNPs existence in the solution. Zhang *et al* have reported that the maximum absorption of SeNPs synthesized by *Pseudomonas alcaliphila* was at 240 to 290 nm wavelengths [4]. Ramamurthy *et al* synthesized SeNPs using UV-visible spectroscopy, with the maximum absorption reported at 200–400 nm [39].

SEM image of *P. putida* KT2440 after SeNPs synthesis is given in figure 3(a) showing that the synthesized SeNPs (light points) anchored to the surface of the bacterial cells. The EDX analysis of the light points, as depicted in figure 3(b), confirmed the SeNPs elemental composition.

TEM micrographs of purified SeNPs are given in figure 4. The purified NPs are quite spherical with diameters between 90 to 120 nm. The TEM images also verified the success of the purification process, as no bacterial cells were observed in the images.

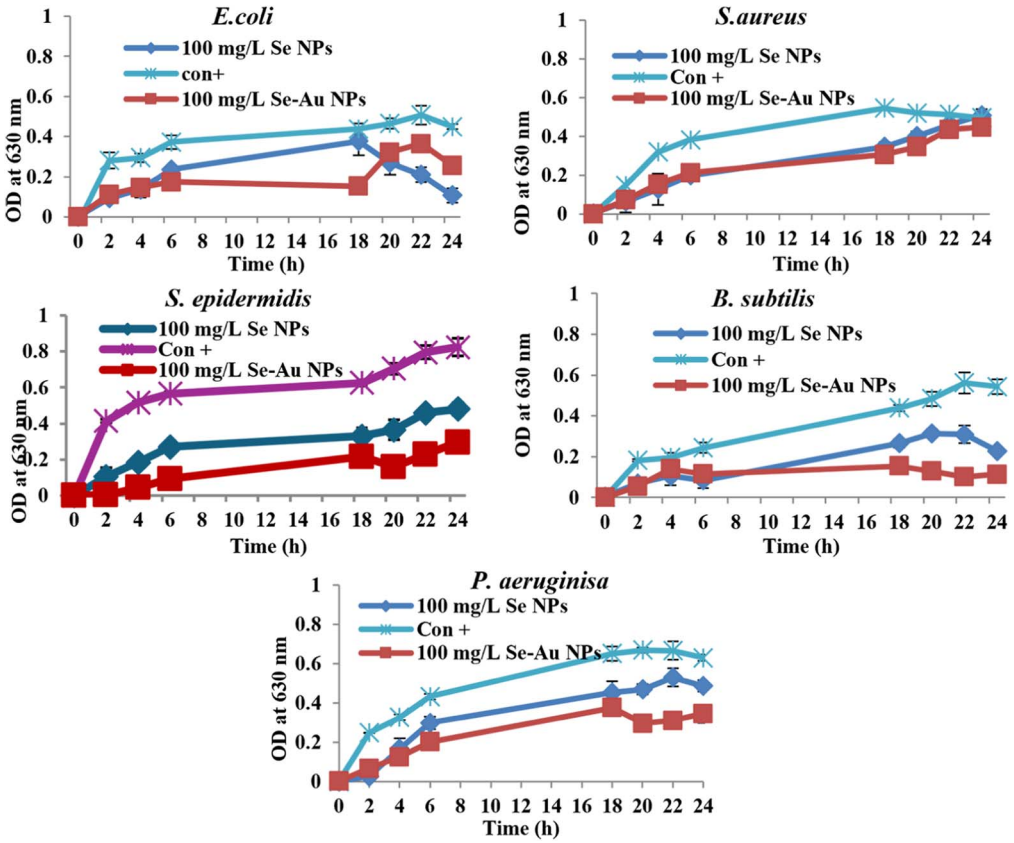

Figure 6. Growth curve of *E. coli*, *S. aureus, S. epidermidis*, *B. subtilis*, and *P. aeruginosa* at the presence of SeNPs and Se-Au NPs (100 mg l^{-1}).

Table 1. The antibacterial activity (GI %) of SeNPs, Se-Au NPs, and AuNPs at 12.5, 25, 50, and 100 mg l^{-1} against different pathogenic bacteria.

Bacteria	NPs	GI % at various NPs Concentration			
		100 mg. L^{-1}	50 mg. L^{-1}	25 mg. L^{-1}	12.5 mg. L^{-1}
E. coli	Se	42%	22%	17%	0
	Se-Au	47%	22%	20%	0
	Au	25%	23%	19%	15%
S. aureus	Se	37%	37%	31%	26%
	Se-Au	55%	47%	47%	41%
	Au	42%	42%	35%	26%
S. epidermidis	Se	40%	39%	35%	11%
	Se-Au	52%	44%	42%	38%
	Au	18%	17%	15%	13%
B. subtilis	Se	58%	31%	29%	29%
	Se-Au	71%	71%	68%	62%
	Au	35%	23%	11%	10%
P. aeruginosa	Se	0	0	0	0
	Se-Au	26%	20%	15%	2%
	Au	25%	22%	22%	15%

The FTIR spectrum of the purified nanoparticles powder is shown in figure 5. The broad band in the range of 3200–3600 cm^{-1} is assigned to −OH bonds where polysaccharides absorb strongly owing to their multiple > C–OH moieties. The peaks at 2962, 2925, and 2847 cm^{-1} indicate the presence of asymmetrical and symmetrical stretching vibration of $–CH_3$, $–CH_2$, and > CH- groups, present both in amino-acid side chains in proteins and polysaccharides. The peaks at 1652 and 1527 cm^{-1} are probably related to the amide I and amide II bonds, respectively. The peaks at 1458 and 1393 cm^{-1} reperesent various bending vibrations of methyl/methylene groups. The observed peaks in the range of

Table 2. Biofilm inhibitory effect (BI %) of SeNPs and Se-Au NPs at different concentrations against two biofilm-forming pathogenic bacteria.

Bacteria	NPs	BI % at various NPs Concentration			
		100 mg. L^{-1}	50 mg. L^{-1}	25 mg. L^{-1}	12.5 mg. L^{-1}
E. faecalis	Se	12%	7%	5%	3%
	Se-Au	38%	38%	38%	38%
S. aureus	Se	11%	11%	7%	1%
	Se-Au	48%	44%	44%	42%

940–1200 cm^{-1} are related to carbohydrates. Therefore, the presence of polysaccharides, together with proteins, in the capping layer of the SeNPs is likely high [40–43]. Husen *et al* proposed that proteins, phenols, alcohols or sugars are probably necessary for the synthesis of SeNPs. These functional groups act as reducing agents facilitating the process [44]. Safaei *et al.* biosynthesized SeNPs by *Halomonas elongate* who believe that hydroxyl, C=O, –NH and groups act as reducing agents facilitating NH_2 groups have a major role in SeNP synthesis [45]. Wang *et al* conducted SeNPs biosynthesis by *Proteus.* Their FTIR spectrum of SeNPs indicated the presence of protein, polysaccharides, and lipids which play a significant role in their stability [46].

A preferred feature of biological methods for metal nanoparticle synthesis is the stability and biocompatibility of the produced nanoparticles which is higher than for other conventional methods [29]. The stability of biogenic NPs is almost related to their capping layer which usually forms during the biosynthesis process [36, 47] and prevents their aggregation [36].

SeNPs exhibit good antibacterial activity due to their small size and high surface-to-volume ratio [48, 49]. Figure 6 shows the growth curve of *E. coli*, *S. aureus*, *S. epidermidis*, *B. subtilis*, and *P. aeruginosa* in the presence of 100 mg l^{-1} of Se and Se-Au NPs. The bacterial growth in the presence of NPs has decreased compared to the positive control which was examined with no NPs.

Table 1 shows the inhibitory effect (GI %) of SeNPs and Se-Au NPs on the examined bacteria after 18 h of exposure at 12.5, 25, 50, and 100 mg l^{-1} concentrations. Both SeNPs and Se-Au NPs showed antibacterial activities against Gram-negative and Gram-positive bacteria. Increasing the NPs concentration enhanced their antibacterial activities. Also, the inhibitory effect of AuNPs on different potential human pathogenic bacteria has been provided from our previous research and is presented in table 1 [28]. According to the obtained results, the mixture of SeNPs and AuNPs has improved the inhibitory effect.

Furthermore, Se-Au NPs improved the inhibitory effects of SeNPs. As demonstrated in figure 6, SeNPs had antibacterial activity against potentially pathogenic bacteria, including *B. subtilis*, *E. coli*, *S. epidermidis*, *S. aureus,* and *P. aeruginosa* with 58%, 42%, 40%, 37% and zero inhibitory effects, respectively. Moreover, Se-Au NPs enhanced the antibacterial activity of SeNPs against *P. aeruginosa*, *S. aureus*, *B. subtilis*, *S. epidermidis*, and *E. coli* by approximately 26%, 18%, 13%, 12% and 5%, respectively. Considering our results, there are several reasons which can explain the synergistic effect of selenium and gold nanoparticles. The strongest possibility is that SeNPs cannot easily enter the cell due to their relatively large size (90 nm). But when they are combined with gold nanoparticles, due to the smaller size of gold particles (10 to 50 nm), the rate of entering the cell increases and the antibacterial activity improves related to the effect on the intracellular components. This is more prominent in the case of *P. aeruginosa* because SeNPs have no effect on bacterial growth, and the addition of gold nanoparticles has decreased its growth [50].

Similar to our studies, research on AgNPs indicates that the antibacterial activity of AgNPs depends on their concentration [51]. However, Tran *et al* conducted a study demonstrating that SeNPs inhibit the growth of *S. aureus* up to 60 times higher than the untreated cells [24]. Furthermore, Soflaei *et al* found that the inhibitory effect of SeNPs on *Leishmania infantum* depends on NPs [52]. Grade *et al* evaluated the antibacterial activity of the Au-Ag alloy against *S. aureus* at different concentrations, revealing a significant inhibitory effect on *S. aureus* growth [27]. To discuss the mechanism of action of the nanoparticles utilized in this research, we compare them to silver nanoparticles as a model. Yoon *et al* studied the antibacterial effect of Ag NPs against *E. coli* and *B. subtilis.* They reported a higher sensitivity for *B. subtilis* to NPs, suggesting that the lower sensitivity of Gram-negative bacteria may be related to the presence of an outer membrane layer covered by lipopolysaccharide. This outer membrane is a strong barrier against NPs penetration [53]. Sondi *et al* studied the antibacterial activity of AgNPs on *E. coli* as a model of Gram-negative bacteria. Their observations showed that the treated *E. coli* cells were damaged due to NPs inducing pit formation on the cell wall. Furthermore, they found that the NPs accumulated inside the bacterial cells [54].

Table 2 shows the inhibitory effects of SeNPs and Se-Au NPs on biofilm formation. The results show that SeNPs had 12% antibiofilm activity against *E. faecalis* and *S. aureus*. Nonetheless, when these bacteria were treated with Se-Au NPs, the antibiofilm activity was enhanced up to 37% and 26% against *S. aureus* and *E. faecalis*, respectively. Shakibaie *et al* investigated the antibiofilm activity of SeNPs on *S. aureus*, *P. aeruginosa*, and *Proteus mirabilis*. Their results showed that SeNPs, ranging in size from 80 to 220 nm, inhibited biofilm formation by approximately 53%, 42%, and 34% for *P. mirabilis*, *S. aureus*, and *P. aeruginosa*, respectively [18]. In another study, Mirza *et al* synthesized SeNPs coated fabric cotton and evaluated their antimicrobial activity against *Candida albicans*, *S. aureus* and *E. coli*. Their findings demonstrated antibiofilm effects of 99%, 78%, and 58% against the mentioned bacteria, respectively [55].

4. Conclusion

In this study, SeNPs were successfully biosynthesized using genetically engineered luminescent bacteria in the monodisperse

state with a nearly spherical shape and a size ranging from 90 to 120 nm. The biogenic SeNPs are thought to be coated with proteins, which play a key role in SeNPs biosynthesis and stability. The antibacterial activity of the synthesized SeNPs and their synergistic effect with biogenic AuNPs on the most prevalent pathogenic bacteria were evaluated. The results showed that biogenic AuNPs enhance the antibacterial activity of SeNPs in the mixture, depending on their concentration. Also, Se-Au NPs exhibited significant antibiofilm activity against the potential human pathogenic bacterium, *S. aureus*, inhibiting biofilm formation by up to 48%. Considering the small size and high surface-to-volume ratio of SeNPs and AuNPs, they can be used as promising antibacterial and antibiofilm agents without cytotoxic effects at low concentrations. Further research is needed to find out their potential use in human treatments against multidrug-resistant bacteria commonly found in hospitals.

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