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RESEARCH PAPER

Mycosynthesis and characterization of selenium nanoparticles using standard penicillium chrysogenum PTCC 5031 and their antibacterial activity: A novel approach in microbial nanotechnology

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ABSTRACT

Objective(s): This study deals with mycosynthesis and characterization of selenium nanoparticles (SeNPs) using the Penicillium chrysogenum PTCC 5031 and evaluating their antibacterial activity.

Materials and Methods: The formation of SeNPs was confirmed with the color change from pale yellow to orange. Tyndall effect also confirmed the formation of colloidal systems through the samples. The SeNPs were characterized using different analytical techniques including photon correlation spectroscopy (PCS), Scanning Electron Microscope (SEM), Atomic Force Microscope (AFM), Energy Dispersive X-ray (EDX), X-ray diffraction (XRD) and Fourier Transform Infrared (FT-IR) analysis.

Results: Our findings revealed that SeNPs were fairly uniformed with good monodispersity and the lesser aggregation of particles in pH value of 7 with the average hydrodynamic size of 24.65 nm, polydispersity index (PdI) of 0.392 and zeta potential of -34 mV. The SeNPs revealed antibacterial activity against gram positive bacteria including Staphylococcus aureus, and Listeria monocytogenes with the zone of inhibition (ZOI) of 10 and 13 mm, respectively.

Conclusion: The results of this study provided a potential solution to the growing need for the development of cost-effective and eco-friendly ways of nanoparticle synthesis to overcome the microbial resistance and control the infectious diseases. However, further investigations are required to demonstrate the efficacy of SeNPs through in vivo models.

Keywords: *Antibacterial activity, Biosynthesis, Nanobiotechnology, Selenium nanoparticles*

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INTRODUCTION

Nanotechnology is emerging as a rapidly growing field that deals with design, synthesis, characterization, and application of materials, devices, or other structures in the sub-100 nm range [1, 2] These nano-sized structures involve nanotubes, nanowires, nanofibers, and nanoparticles (NPs). Nanotechnology has created new opportunities for advances in many medical and biological applications such as cancer treatment, tissue regeneration, biosensing, medical imaging and diagnosis, etc due to their novel properties that differ from their bulk materials [3-6]. One of the favorite researches in nanoscience

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is the synthesis of metal NPs. Generally, there are physical, chemical, and biological approaches for synthesis of NPs. Noticeably, physical and chemical approaches may require high energy and also produce toxic byproducts affecting the humans and the environment, while biological approach is a single step for NPs synthesis with non-utilization of toxic chemicals, inexpensive equipment and eco-friendly [7, 8]. The biological approach due to their biocompatible nature has been attractive for the researchers compared to conventional physical and chemical methods for synthesis of NPs [9-14]. Besides, metal NPs due to their unique properties could be conjugated to biological compartments including monoclonal antibodies, peptides, tumor markers, etc to target receptors on cancer cells [15, 16].

Microbial nanotechnology is an expanding research arena on the basis of the capability of microorganisms to convert metal ions to their nano-sized structures [17]. Microbial mediated preparation of NPs are carried out through two intra- and extracellular pathways [18]. In the intracellular pathway, NPs are fabricated within the cell and further downstream processes are required to release the NPs from inside the cell. Hence, it is of interest to find the microorganisms that could produce NPs extracellularly. The extracellular biosynthesis of NPs by using fungi has been frequently reported [18, 19]. However, some of them are pathogenic [20]. Therefore, to explore non-pathogenic fungi for biosynthesis of NPs could help us to use these NPs for pharmaceutical applications. Selenium is a dietary nutrient trace element that plays a role in many aspects of health in humans. The US National Academy of Sciences recommended 55 µg of Se for daily intake in adults [21]. Moreover, SeNPs showed favorable biological activities such as antioxidant, antimicrobial and anticancer activities. Moreover, SeNPs exhibited protective effects against cardiovascular diseases [8] as well as alcohol-induced oxidative challenge [22]. SeNPs exhibited similar potential of increasing the selenoenzymes such as glutathione peroxidases compared to other selenium supplements such as L- selenomethionine [23]. In a study, oral administration of SeNPs (50 µg/kg/day) represented considerable anti-atherosclerotic activity in apolipoprotein E deficient (ApoE−/−) mice fed a high-fat diet. The anti-atherosclerotic activity of SeNPs was found as potent as the group treated with atorvastatin (10 mg/kg/day) after 8 weeks of treatment [24].

Biogenic synthesis of SeNPs have attracted attention during recent years owing to the excellent physicochemical and biological properties of biogenic SeNPs. Sonkusre (2020), reported biosynthesis of SeNPs using *Bacillus licheniformis* and stated that biogenic SeNPs at the concentration of 2 µg/mL induced necroptosis in cancerous LNCaP-FGC cells, without any toxic effect on the normal human red blood cells. In addition, according to the histopathological findings, 50 mg/kg of biogenic SeNPs exhibited significantly lower toxicity compared to the 5 mg/kg of L-selenomethionine through oral administration in mice [23]. Fan et al (2020), reported phytosynthesis of SeNPs using *Hibiscus sabdariffa* leaf extract and represented protective effects of SeNPs in streptozotocin (STZ) induced diabetes rats by attenuating oxidative damage induced by diabetes, especially in the testicular tissue [21]. Menon et al (2020) reported biosynthesis of SeNPs using aqueous extract of cow urine as reducing and stabilizing agent. The fabricated SeNPs exhibited excellent antibacterial activity against *Klebsiella* sp. with a ZOI of 10.5 ± 02.8 mm at a concentration of 100 µg/mL [8]. Anu et al (2020), reported *Cassia auriculata* leaves extract-mediated synthesis of SeNPs which induced significant cytotoxicity in leukemia cells in a dose-dependent manner with IC_{50} value of 7.01 µg/mL after 120 h of incubation, whereas the IC50 was found to be 109.13 µg/mL in normal vero cells representing far less cytotoxicity in normal vero cells [7]. The promising advantages of biogenic SeNPs on the one hand, and the exceeding need to explore eco-friendly and non-pathogenic microorganisms with high efficacy for preparation of SeNPs in the field of microbial nanotechnology on the other hand, motivated us to design the current study to explore the capability of *P. chrysogenum* PTCC 5031 for green synthesis of SeNPs and evaluating their preliminary antibacterial activity against both gram positive and gram negative bacteria.

MATERIAL AND METHODS

Materials

The lyophilized vial of *P. chrysogenum* PTCC 5031 was purchased from Iranian Research Organization for Science and Technology, Tehran, Iran. Furthermore, yeast extract, sucrose, sodium selenite (Na2SeO3, 5H2O), and other chemical reagents were purchased from Merck, Germany.

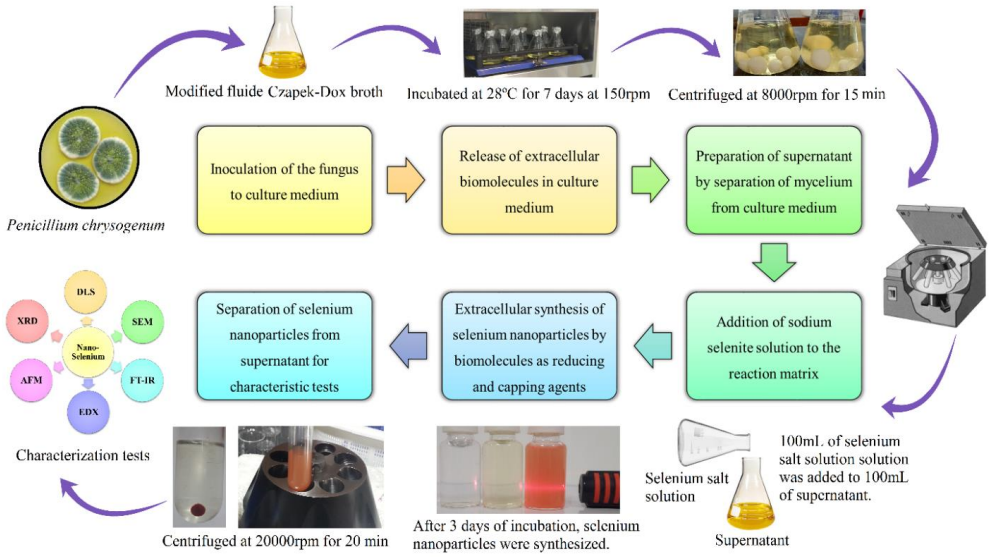

Fig 1. A schematic illustration representing the procedures of SeNPs synthesis

Biosynthesis and characterization of SeNPs

The fungus *P. chrysogenum* PTCC 5031 was cultured on modified fluid Czapek-Dox broth incorporating 21g sucrose and 3g yeast extract in 1000 mL distilled water and incubated on a rotary shaker (JAL TAJHIZ®, JTSL 40, Iran) at 150 rpm for ten days at 28°C. Further, to separate the mycelia from the supernatant, the culture was centrifuged at 8,000 rpm by centrifuge (HETTICH®, ROTINA 380R, Germany) for 15 minutes. Then, 100 mL of sodium selenite solution with the concentration of 1 mM was added to 100 mL of supernatant and consequently was adjusted for pH values of 6, 7, 8 and 9 separately, and after that incubated again for 72 h at 28°C. Tyndall effect was pursued as macroscopic observation to confirm the formation of colloidal systems through the samples [25]. The fabricated SeNPs were separated from the supernatant at 20000 rpm for 20 min using ultracentrifuge (Beckman, L90k, US). The SeNPs were washed thrice with deionized double distilled water to improve the purification of the NPs. Size of SeNPs was determined by a Zetasizer Nanoparticle Analyzer using Zetasizer 3600 at 25°C with a scattering angle of 90° (Malvern instruments, UK). The morphology of SeNPs was determined by SEM (MIRA3 model, Czech Republic) operated at 15 kV coupled with EDX analysis, and also AFM (JPK, NanoWizard II model, Germany) under ambient conditions in non-contact mode by employing silicon nitride tips with varying resonance frequencies at a linear scanning rate of 0.5 Hz. The XRD was executed using PW3050/60 on the fabricated SeNPs with XPERT-PRO at 30 kV and 100 mA and the spectrum was recorded by CuKα radiation with wavelength of 1.5406 Å in the 2θ range of 20°–80°. The FT-IR spectrum was conducted over the wavelength range of 400-4000 cm-1 to identify the conjugated biomolecules to the surface of NPs by mixing the synthesized NPs with potassium bromide at 1:100 ratio and compressed to a 2-mm semitransparent disk for 2 min (Agilent, Cary 630 model, US) [25, 26]. The procedures of SeNPs synthesis were illustrated in Fig 1 in brief. Moreover, the yield of SeNPs synthesis was calculated using the Inductivity Coupled Plasma-Mass Spectrometry (ICP-MS) (Hewlett-Packard, HP4500, US).

Antibacterial activity of SeNPs

Well diffusion assay was used for SeNPs antibacterial efficacy against reference bacteria including *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Listeria monocytogenes* ATCC 7644. Initially, the inoculum of each bacterial isolate was adjusted to 0.5 McFarland standard (106 CFU/mL). Consequently, the inoculum was transferred and spread on the Mueller Hinton Agar (MHA) plate containing a 6 mm-diameter well at the center. Then, 100 µL of SeNPs stock dispersing in deionized water (1 mg/mL) was transferred in each well. Finally, the primed plates were incubated at 37°C for 24 hr.

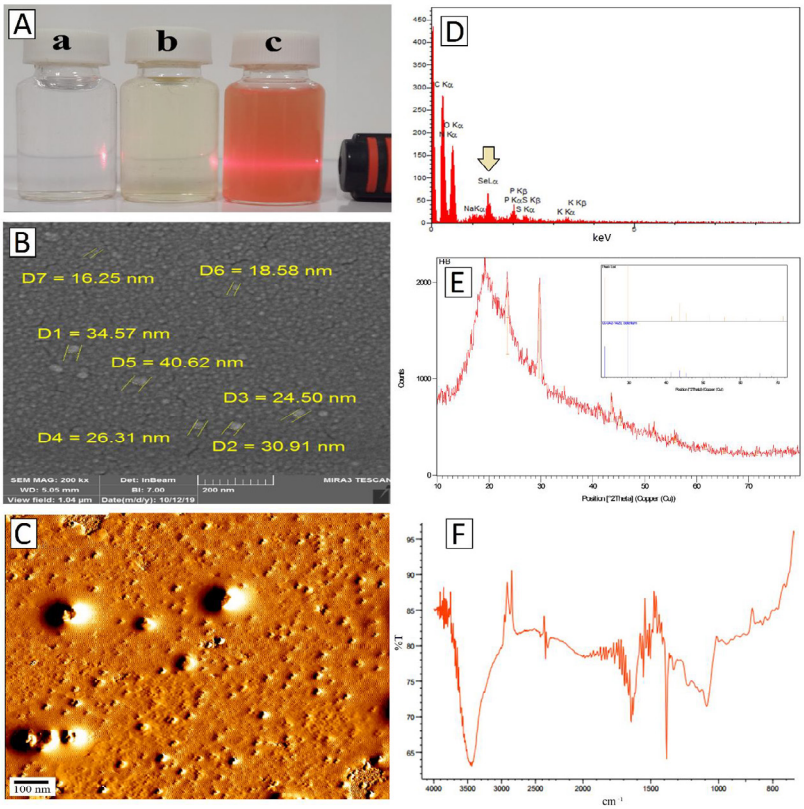

Fig 2. A) The optical photograph of color change from colorless [(a) sodium selenite solution] and pale yellow [(b) fungal supernatant] to orange [(c) colloidal SeNPs], and visible laser beam path due to the Tyndall effect (c); B) SEM image of SeNPs; C) AFM image of SeNPs; D) EDX spectrum of SeNPs; E) XRD spectrum of SeNPs; and F) FT-IR spectrum of SeNPs

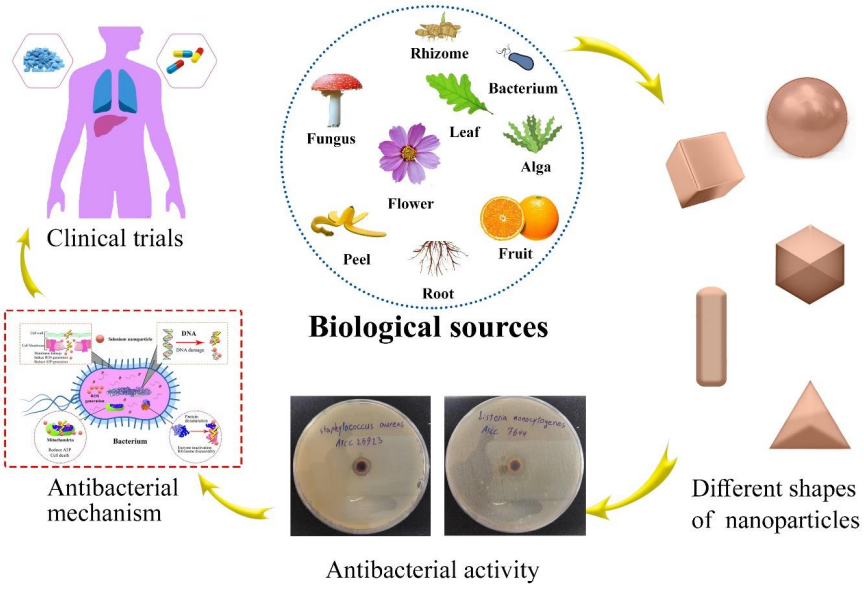

Fig 3. A schematic illustration representing the interface of nature, nanotechnology and biological activities

RESULTS AND DISCUSSION

Mycosynthesis of SeNPs

Addition of sodium selenite solution to the supernatant of *P. chrysogenum* PTCC 5031 led to the appearance of orange color after 72 h of incubation suggesting the formation of SeNPs, while no color change was observed in control sample under the same conditions (Fig 2A). The appearance of orange color has been stated in similar studies resulting from the reduction of selenium oxyanions (SeO32-) to elemental selenium (Se0) [27, 28]. The studies stated that this color alteration was attributed to the surface plasmon vibrations excitation of SeNPs in colloidal systems [28, 29]. In addition to color alteration, the Tyndall effect was used to confirm the colloidal nature of the sample. Tyndall effect is a phenomenon confirming the colloidal system by using a laser beam that illuminated its path through the colloidal sample [26]. Fig 2A depicted the Tyndall effect through the colloidal sample containing SeNPs, whereas in the control sample no Tyndall effect was seen. In the biological approach for synthesis of metal NPs, it is believed that biomolecules act as reducing and stabilizing agents [30]. Fig 3 illustrated the interface of nature, nanotechnology and biological activities. As shown in Fig 3, different biological sources for biofabrication of SeNPs with different sizes and morphologies. During the bioprocess, the biomolecules reduce the metal ions to their elemental nano-forms and surround the fabricated nanostructure acting as stabilizer. Impressively, the conjugated biomolecules on the surface of nano-sized particles have an influence on the biological activity of these nanostructures. In the present study, we evaluated the preliminary antibacterial activity of mycofabricated SeNPs, however, future *in vivo* studies are required before translating this laboratory setting investigation for clinical applications.

Characterization of SeNPs: Analytical investigations

The SeNPs were fabricated in pH values of 6, 7, 8 and 9. To reach the best pH for SeNPs fabrication, the PCS analysis was used to evaluate the hydrodynamic diameter sizes in each pH value. The PCS analysis represented the average hydrodynamic size of SeNPs to be 55.76, 24.65, 99.39, and 39.41 for the pH values of 6, 7, 8 and 9, respectively (Fig 4). As shown in Fig 4B, the SeNPs in pH value of 7 were fabricated in monodisperse manner and displayed a narrow peak, while Fig 4A, 4C, and 4D depicted formation of polydisperse SeNPs in pH values of 6, 8 and 9, respectively. Additionally, the PdI values were found to be 0.75, 0.392, 0.717, and 1 for the pH values of 6, 7, 8 and 9, respectively. The range of PdI is from 0.01 to 1. The values of over 0.7 refer to polydisperse samples, whereas the lower values indicate monodisperse samples [26]. The PdI of 0.392 as an output of PCS analysis showed good SeNPs monodispersity and the lesser aggregation of particles in pH value of 7. Moreover, the zeta potentials were found to be -33.8, -34.0, -27.2 and -28.1 for the pH values of 6, 7, 8 and 9, respectively (Fig 5). Hence, it was concluded that the surface of SeNPs was negatively charged in all tested pH values. The zeta potential of higher than +30 mV or lower than -30 mV indicates the electrostatic stability of a colloidal sample [26]. The zeta potential of -34.0 mV for SeNPs in pH value of 7 supplies the repulsive force as an electrostatic stabilization. In addition to electrostatic stability, it was demonstrated that secreted biomolecules from microorganisms surround the NPs and provide steric stability [25, 26]. According to the above findings, the SeNPs obtained from pH value of 7 was selected for further analytical investigations. The average yield of conversion of selenium oxyanions to elemental selenium and fabrication of SeNPs were found to be 26.81% for the SeNPs obtained from pH value of 7. The morphology of the SeNPs was investigated by SEM and AFM which showed spherical shaped SeNPs below 100 nm (Fig 2B and 2C). Besides, the EDX results depicted that SeNPs displayed an absorption peak at 1.4 keV indicating the presence of the elemental selenium (Fig 2D). Moreover, Fig 2E depicted the XRD spectrum that confirmed the formation of SeNPs with highly crystalline nature (Ref. code. 00-042-1425). The FT-IR spectrum of SeNPs exhibited the absorption peaks at 1088.38, 1412.66, 1632.57 and 3440.33 cm-1 in the region of 450 to 4000 cm-1. The band at 1088.38 cm-1 corresponds to the C–N stretching vibration of the amine. The band at 1412.66 cm-1 corresponds to the N–H stretching vibration of primary amides. In addition, the peaks at 1632.57 and 3440.33 cm-1 were assigned to C–C and O-H stretching, respectively (Fig 2F). The FT-IR findings represented the presence of functional groups on the surface of SeNPs. These functional groups are attributed to the conjugated biomolecules to the surface of SeNPs acting as reducing and stabilizing agents [26].

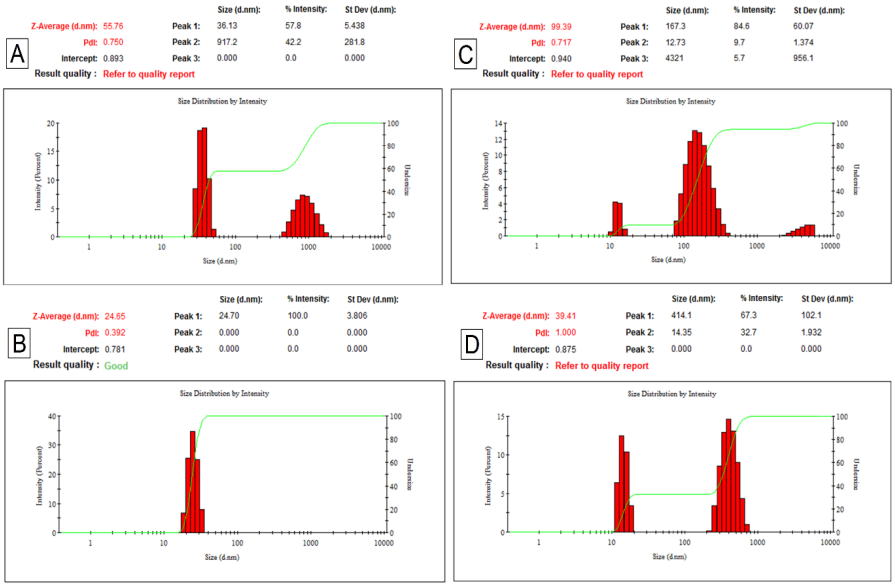

Fig 4. PCS of SeNPs obtained from pH values of 6 (A), 7 (B), 8 (C) and 9 (D)

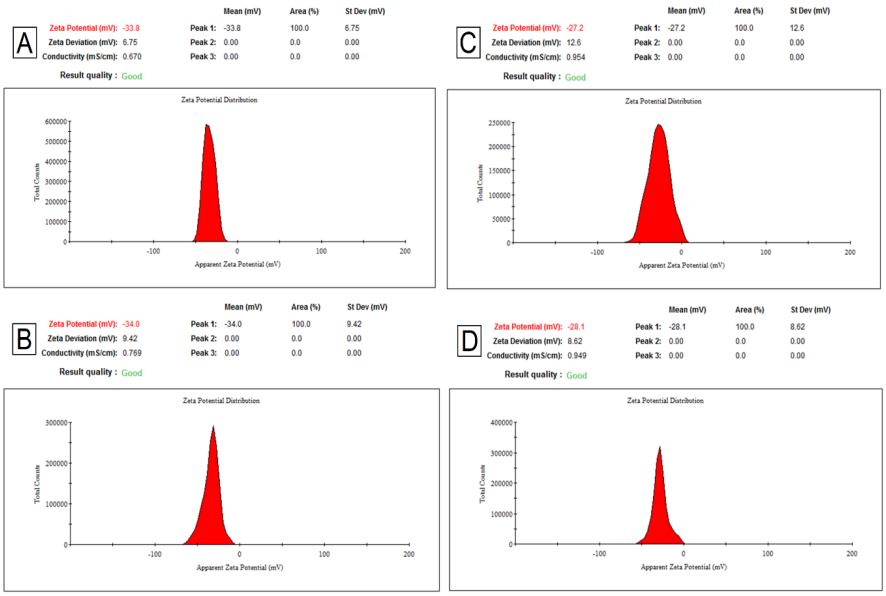

Fig 5. Zeta potential of SeNPs obtained from pH values of 6 (A), 7 (B), 8 (C) and 9 (D)

Antibacterial activity of SeNPs

The development of multidrug resistant (MDR) pathogens at an alarming rate on the one hand [31], and the promising capacities of nanotechnology with unique and remarkable properties of nanomaterials on the other hand provided a novel arena to explore innovative nano-based materials as an alternative to antibiotics to combat MDR pathogens [32]. In the current study, the *P. chrysogenum*-mediated fabricated SeNPs showed antibacterial activity against gram positive bacteria including *S. aureus* ATCC 25923, and *L. monocytogenes* ATCC 7644 through well diffusion assay with ZOI of 10 and 13 mm, respectively. However, no ZOI was found against gram negative bacteria including *E. coli*

ATCC 25922, *S. typhimurium* ATCC 14028, and *P. aeruginosa* ATCC 27853 (Fig 6). In support of our study, Gunti et al, prepared SeNPs using aqueous fruit extract of *Emblica officinalis* and reported the higher antibacterial activity of SeNPs against Gram-positive bacteria (*S. aureus* MTCC 96, *Enterococcus faecalis* MTCC 439 and *Listeria monocytogenes* MTCC 657) compared to Gram-negative bacteria (*E. coli* MTCC 4) with minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of around 9.16 and 19.83 µg/mL against *S. aureus*, 16.17 and 33.17 µg/mL against *E. faecalis*, 33.17 and 53.5 µg/mL against *L. monocytogenes*, and 59.83 and 97.5 µg/mL against *E. coli*, respectively [33]. Likewise, Tran et al, reported synthesis of polyvinyl alcohol-stabilized SeNPs and stated strong growth inhibition activity of SeNPs against *S. aureus* at a concentration as low as 1 µg/mL, while no growth inhibition was found against *E. coli* at all concentrations tested [34].

Alternatively, Kokila et al, phytosynthesized SeNPs and reported their antibacterial activity against *S. aureus,* and *E. coli* with ZOI of 8 and 7 mm, respectively [35]. Similarly, Guisbiers et al, stipulated that SeNPs affect *S. aureus* in a more efficient way than *E. coli* with the inhibition rate of 63% and 46% after 24 hours of incubation, respectively [36].

Particle size, morphology, surface charge, surface chemistry and hydrophilicity are important parameters that have influence on disruption of microbial cell membranes [37, 38]. The higher antibacterial activity of SeNPs against gram positive bacteria compared to gram negative bacteria may be attributed to the strong electrostatic repulsion between SeNPs and bacterial membrane charge [33].

Tran et al, proposed that because of the significantly lesser membrane negative surface charge in gram-positive bacteria compared to gram negative bacteria, the deposition of SeNPs on the surface of gram positive bacteria and inducing bacterial damage is more possible. Thereby, the gram negative bacteria tend to resist to SeNPs [34].

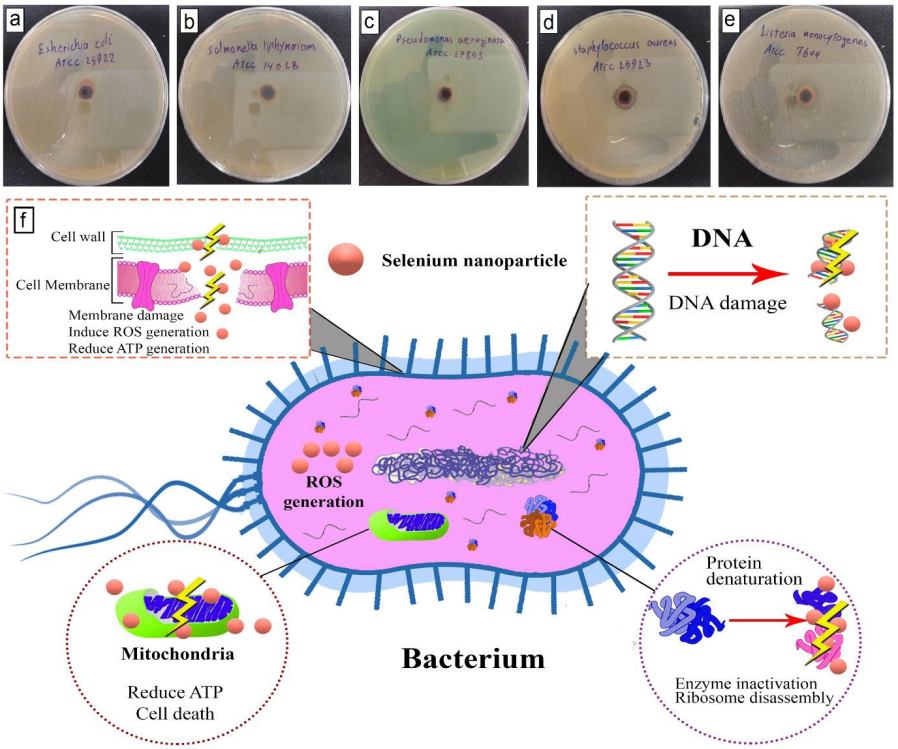

Fig 6. Antibacterial activity of SeNPs against a) E. coli ATCC 25922, b) S. typhimurium ATCC 14028, c) P. aeruginosa ATCC 27853, d) S. aureus ATCC 25923, and e) L. monocytogenes ATCC 7644; f) a schematic illustration representing the proposed mechanisms of antibacterial activity of SeNPs

Guisbiers et al, stated that a possible mechanism for penetrating the SeNPs inside the bacteria is chemisorption. In gram negative bacteria, the outer membrane contains lipopolysaccharides, linked by a covalent bond to the cell's peptidoglycan by Braun's lipoprotein. In gram positive bacteria the cell wall contains a thicker peptidoglycan membrane with no outer lipopolysaccharide membrane. So, it seems that SeNPs penetrate much more easily into the gram positive bacteria by chemisorption [36].

Fig 6f represented a schematic illustration of proposed antibacterial mechanisms of SeNPs. The SeNPs disturb the bacterial cell wall and membrane and enter inside the cell with a frequent overgeneration of reactive oxygen species (ROS), interfering with respiratory sequence and ATP synthesis, protein denaturation, inhibition of enzyme activity, DNA damage, etc which all together result in a systematic failure of the intern metabolism leading the cell death [33, 37].

CONCLUSION

Microorganisms due to the rich biodiversity are biofactories that serve as an eco-friendly, simple and cost-effective method for the synthesis of NPs. In the present study, a simple, fast and non-toxic method was used for the synthesis of SeNPs without any additive capping and/or stabilizing agents. The yield of fabrication of SeNPs was found to be 26.81%. Impressively, according to antibacterial investigations, the mycofabricated SeNPs represented considerable antibacterial activity against gram positive bacteria. It is of highly important to note that future studies should be conducted to evaluate the synergistic effects of biogenic SeNPs in conjugation with other antibiotic agents to fight MDR pathogens. In addition, extensive clinical translational studies are required to provide a safety profile of biogenic SeNPs for the humans and the environment.

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