

Screening of Antibacterial Activity of Selenium Nanoparticles Produced by Indonesian Marine Actinomycetes

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Abstract

Actinomycetes strains can reduce SeO_3^{-2} (selenite) and (or) SeO_4^{-2} (selenate) to Se^0 , which is less toxic by forming selenium nanoparticles (SeNPs). This study aimed to screen the actinomycetes strains capable of biosynthesizing and producing SeNPs with antibacterial activity. This study showed that the antibacterial properties of SeNPs were synthesized biologically by the three strains of actinomycetes, e.g., the strains BLH 5-21, BLH 5-22, and BLH 12-1. The nanoparticles synthesized by the three actinomycetes strains were active against the pathogenic tested bacteria: *Bacillus subtilis* BTCC B-612, *Staphylococcus aureus* BTCC B-611, *Micrococcus luteus* BTCC B-552, *Escherichia coli* BTCC B-609, and *Candida albicans* Y-033. The PSA and SEM images showed that the selenium nanoparticles from strain BLH 12-1 had a spherical shape, and their mean diameter was between 64-110 nm, with an average size of 98 nm. The biogenic selenium nanoparticles have exhibited promising application prospects in medicine, biosensors, and environmental remediation. These microorganisms might be explored as the potential of microbial nano factory for synthesizing metal nanoparticles.

Keywords: actinomycetes, selenium, nanoparticles, antimicrobial activities, UV-Vis, FTIR, PSA, SEM, XRD

Introduction

Selenium (Se) is a micronutrient mineral that is necessary for maintaining the health and growth of living organisms. Se must be obtained from food/diet because living organisms cannot produce it. Se deficiency causes several dangerous disorders such as cancer, neurological, muscle, and immune systems. At optimal doses, Se from organic or inorganic materials acts as an antioxidant, while at high doses, it shows toxicity. Because of concerns regarding Se toxicity have made researchers focus on Se nanoparticles (SeNPs) (Skalickova *et al.*, 2017).

SeNPs biosynthesis can be obtained from the bioconversion of microorganisms (e.g., bacteria, fungi, yeast), plant extracts, and algal extracts (Sharma *et al.*, 2009; Bisht *et al.*, 2022). Bioorganic natural compounds such as reducing

sugars, starch, enzymes, peptides, and proteins in such biomass can efficiently reduce the metal ions to zero-valent atoms, forming metal nanoparticles. Ranjitha and Ravishankar (2018) reported the simple and eco-friendly biosynthesis of SeNPs from *Streptomyces griseoruber* that showed good cytotoxic activity against the HT-29 cell line, suggesting that biogenic SeNPs could be a potential chemotherapeutic agent. Synthesized SeNPs are bactericidal and have been proven to be good alternatives for developing antimicrobial agents (Chudobova *et al.*, 2014). SeNPs have been studied for various medical applications and as a potential material for orthopedic implants (Wang & Webster, 2012). Studies indicate the ability of selenium compounds to inhibit the growth of bacteria and the formation of bacterial biofilms (Wang & Webster, 2012).

Microorganisms (bacteria, yeast, and fungi) play an essential role in the remediation of toxic metals by reducing metal ions. The method was considered attractive as microbial nano factories (Ghosh *et al.*, 2021). Nanoparticles synthesized by microbes prove promising for several biomedical and therapeutic applications due to their controlled biocompatible dimensions and unique properties.

Actinomycetes comprise diverse filamentous bacterial strains in different habitats, from high mountains to deep seas. Actinomycetes are shown to have an almost unlimited capacity for the production of secondary metabolites (such as antibiotics, antiviral and immunomodulators) with diverse chemical structures and biological activities as well as valuable enzymes. The ability of actinomycetes to biosynthesize metal nanoparticles such as selenium, silver, and gold has been previously reviewed (Yaradoddi *et al.*, 2022).

In the previous study, we reported two actinomycetes isolated from Lombok Island in Indonesia that had the potential to produce SeNPs (Ratnakomala *et al.*, 2018). This study aimed to screen the marine actinomycetes strains from Bitung, Indonesia that are capable of biosynthesizing and producing SeNPs with antibacterial activity. We analyzed SeNPs derived from actinomycetes to determine antibacterial activity against selected pathogens.

Material and Methods

Strain. This study used three strains of marine actinomycetes to biosynthesize SeNPs, i.e., strains BLH 5-21, BLH 5-22, and BLH 12-1. The inhibitory effect of SeNPs was studied by using three Gram-positive bacteria, *Bacillus subtilis* BTCC B-612 (= NBRC 3134 = ATCC 6633), *Staphylococcus aureus* BTCC B-611 (= NBRC 13276 = ATCC 6538), and *Micrococcus luteus* BTCC B-552; one Gram-negative bacteria, *Escherichia coli* BTCC B-609 (= NBRC 3972 = ATCC 8739), and a yeast *Candida albicans* Y-33 (= NBRC 1594 = ATCC 10231) as tested bacteria. The strains were obtained from the Biotechnology Culture Collection in Indonesia (WDCM 632). Working stock cultures were preserved at 80°C in 20% v/v glycerol.

Preliminary screening for antimicrobial activity. The preliminary screening for antimicrobial activity of actinomycetes isolates

was conducted using the double-layer agar method on Mueller Hinton Agar (MHA). The actinomycetes isolates were inoculated on ISP 2 agar plates and incubated at 28±2°C for seven days. The grown actinomycetes on ISP2 (International Streptomyces Project 2) plates were then cut with cork borer to make a piece of agar plug. The bottom layer of MH agar was then poured with soft MH agar inoculated with 10% of the test organisms (v/v) and allowed to solidify. The agar plug containing actinomycetes strain was placed on top of solidified soft agar inoculated with test microorganisms. The plates were left for 30 min at 4°C temperatures for supernatant diffusion and incubated at 37°C overnight for bacteria and at 28±2°C for 96 h for fungus. The inhibition of the test organism observed antagonism.

Secondary Screening for Producing SeNPs.

An inoculum of each actinomycetes strain was inoculated in ISP2 agar supplemented with 1 mM, 3 mM, 5 mM, or 10 mM concentration of selenium dioxide (SeO₂) (Sigma–Aldrich Chemical Co.) and incubated at 28°C for seven days.

Biosynthesis of SeNPs. One mL of fresh inoculum (OD₆₀₀, 0.5) of the strain BLH 5-21, BLH 5-22, and BLH 12-1 was inoculated into the sterile medium of 100 mL of ISP2 broth and then incubated in a shaking incubator (120 rpm) at 28°C for 48 hours. Stock SeO₂ is then added to the culture until it reaches a concentration of 3 mM SeO₂ in total volume and incubated again for the next 72 hours. As a control, flasks containing culture isolates growing in the SYP broth were prepared without SeO₂ and incubated under the same conditions. Biosynthesis of SeNPs was observed with UV-Vis Spectrophotometer by taking samples from an aliquot medium at different incubation periods (12 hours, 36 hours, and 72 hours). The cultures were centrifuged at 6,000 rpm for 15 minutes, and the resulting free cell filtrate was centrifuged at 14,000 rpm for 15 minutes for the biosynthetic SeNPs.

Antimicrobial activity of SeNPs. The SeNPs were tested against bacterial strains via the paper disc diffusion method described by Richtera *et al.* (2015). Forty µL (1 mg/mL) of the SeNPs solution was poured onto the paper disc using a sterile micro-pipette in the double-layer MHA plates. The inhibition zones were measured after

incubation at 37°C for 24 h. Culture supernatant without SeO₂ and standard antibiotics streptomycin were used as a control negative and control positive, respectively.

Characterization of SeNPs. Various instruments were used to characterize SeNPs for size, shape, and surface area. The absorption spectrum of particles obtained was measured using a UV-visible spectrophotometer at wavelengths between 300-600 nm. Functional groups found in SeNPs were analyzed using FT-IR spectroscopy. Structural characterization to determine information on particle size, crystal structure, and surface morphology was examined using a Particle Size Analyzer (PSA), X-ray diffraction (XRD), and Scanning Electron Microscopy (SEM).

Molecular Identification of Actinomycetes by 16S rRNA gene Sequencing. Genomic DNA was extracted as described by Saito & Miura (1963) and 16S rRNA gene was amplified, sequenced, analyzed, and constructed a phylogenetic tree according to Lisdiyanti *et al.* (2010).

Screening for antibacterial activity of SeNPs and measurement of minimum inhibitory concentration (MIC).

The examination of antibacterial activity utilized the microplate resazurin assay (MRA) technique in a sterile 96-well microtiter plate, adapted from Sarker *et al.* (2007). This approach relies on the bacterial reduction of the resazurin salt, resulting in the formation of a pink resofurin salt. Streptomycin served as the positive control, and medium without SeO₂ as the negative control. In each well of the 96-well plate, 50 µL of NB was added, excluding the first row. The first row received 100 µL of extract, and the second row received 50 µL of extract. Subsequently, 10 µL of resazurin was added to series 1-12 and mixed. Then, 150 µL was transferred from series A to series B and mixed, repeating this process until series H, where 150 µL was discarded. The plate was sealed with parafilm and incubated at 37 °C for 18-24 hours. Color changes were observed and captured using a camera, as described by Sarker (2007).

Result and Discussion

Growth of Actinomycetes on Selenium dioxide and Evaluation of Selenite Reducing Ability

The isolates actinomycetes were grown in the ISP2 medium by adding different concentrations of SeO₂ (0.5 mM, 1 mM, 3 mM, 5 mM, and 10 mM) to determine the toxicity of SeO₂ under aerobic conditions. Three marine actinomycetes strains showed good growth in a medium containing 3 mM SeO₂. The actinomycete strains BLH 5-21, BLH 5-22, and BLH 12-1 formed reddish colonies after seven days of incubation (Figure 1), which indicated its ability to reduce the toxic, colorless, soluble selenite (SeO₃⁻²) ions to a non-toxic, red elemental insoluble form of Selenium (Se⁰).



Figure 1. (A). The growth of actinomycetes isolates on SYP medium without SeO₂; (B). The growth of actinomycetes isolates on an agar medium containing SeO₂

The growth and capability of actinomycetes isolate to transform selenite to elemental Se were tested in SYP broth with the addition of 3.0 mM SeO₂ (Figure 2). The formation of the red cell suspension of elemental Se started after 12 h of exposure to selenite. All strains were able to reduce SeO₃⁻² to elemental Se. It was indicated by the formation of red cell suspension during 72 h incubation in SYP broth under aerobic conditions.

The color development has confirmed the synthesis of SeNPs. The incubation period needed to synthesize SeNPs in this study was 12-72 h. Similarly, Dhanjal and Cameotra (2010) and Ramya *et al.* (2015) reported that the optimal incubation period required to synthesize SeNPs was 48 h. The visual observation of the control Erlenmeyer flask incubated at 28°C for 12-72 h illustrated that no color change was noticed in the control flask, which contained culture broth without SeO₂ (Figure 2). On the other hand, a color change from colorless to insoluble red of elemental Selenium (Se⁰) was demonstrated in the culture broth of all the strains (after 12-72 h), which contained the tested culture broth and 3 mM SeO₂.

The cultivation of selected isolates on the SYP broth medium containing the SeO_2 ions changed the color of the culture medium to red color over the incubation time (Figure 2), demonstrating the ability of these isolates to synthesized SeNPs, converting toxic, dissolved, selenite ions colored into a Se^0 which is non-toxic, insoluble and red color.

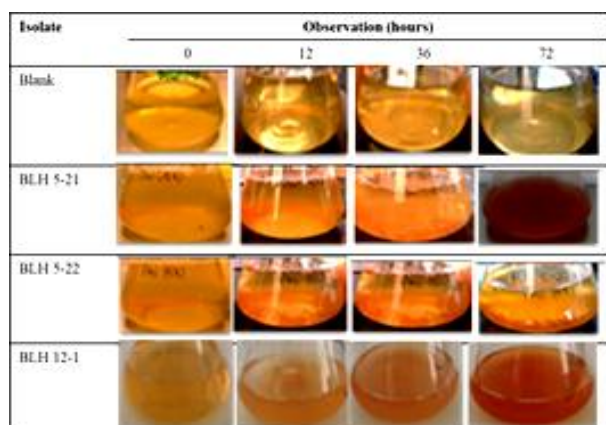


Figure 2. The color developments of the cultivation medium of the actinomycetes strain, which contains selenium dioxide (SeO_2)

Characterization of nanoparticles synthesized by actinomycetes BLH 12-1 isolate

UV-visible spectroscopy is considered a valuable tool for the structural characterization of nanoparticles. The UV-visible spectroscopic analysis of the SeNPs at different time intervals concluded that a maximum absorption of 320 nm was attained at 24 h of incubation (Figure 3). Dhanjal and Cameotra (2010) used UV-visible absorption spectra of SeNPs absorbed characteristic peak at 590 nm. In the UV-visible spectrum (Figure 3.a), a sharp and broad peak was observed at 320 nm, indicating the presence of SeNPs. The UV absorption spectrum of SeNPs obtained from BLH 12-1 isolate cultures showed characteristics of a peak at 320 nm; this corresponds to a particle size of 101.6 ± 9.8 nm (Lin & Wang, 2005). These might occur because of the reduction of metal ions by secondary metabolites inside the isolate BLH 12-1 cell. FTIR spectrum (Figure 3.b) of SeNPs synthesized by BLH 12-1 showed changes in the shape and peak position at 400-1,000 cm^{-1} . FT-IR spectroscopy was used to confirm the presence of functional groups involved in the biosynthesis of SeNPs. This study showed the presence of functional groups O-H, amide I, and C-O from the spectrum of nanoparticles obtained (Figure 3.b). The FTIR absorption spectrum of SeNPs shows different peak values

to identify various bonds responsible for the stability of the nanoparticle solution. The FT-IR spectrum of SeNPs synthesized by actinomycete BLH 12-1 showed sharp absorption peaks at 3,375 cm^{-1} and 1,641 cm^{-1} . A sharp and broad peak at 3375 cm^{-1} can be determined as an OH group. The peak at 1,641 cm^{-1} corresponds to the C = O amide group (Sharma *et al.*, 2019). From these two prominent peaks, there is a peak at 1,267 cm^{-1} , representing the C-O acid group.

Particle Size Distribution and Zeta Potential

This study showed the results of the particle size distribution pattern of the purified SeNPs determined by a Particle size analyzer (PSA), as illustrated in Figure 4A typical peak observed in the range between 64 nm and 110 nm, with an average size of 98 nm, were the most common particles (Figure 4A). Similar results were reported by Shoeibi and Mashregi (2017) that *Enterobacter faecalis* resulted in elemental Se of size <195 nm in diameter with an average size of 99 nm. The size of SeNPs by *Streptomyces* sp. ES2-5 was about 200 nm, even the size of larger SeNPs reached 500 nm (Tan *et al.*, 2016). Nano-size nanometers mean that particles have more expansive surfaces to interact with bacteria and cells.

Figure 4B shows that the SeNPs from BLH 12-1 are negatively charged, and the zeta potential value is around -16.38 mV. The negative electrostatic surface of SeNPs is essential in stabilizing nanoparticle suspension. According to Guisbiers *et al.* (2014), a zeta potential of more than 30 mV (more negative than -30 mV or more positive than 30 mV) is generally needed because it stabilizes nanoparticles in solutions that rely on electrostatic stabilization. If all particles in suspension have a significant negative or positive zeta potential, particles have little tendency to aggregate (Dhanjal & Cameotra, 2010). The surface electrostatic of nanoparticles is also essential in the interaction of nanoparticles with bacteria.

Many bacteria, including *E. coli* and *S. aureus* have a negative charge with different strengths. So, it is expected that there is a strong electrostatic force that can resist the negative charge of these bacteria (Dhanjal & Cameotra, 2010).

The synthesized SeNPs were characterized by scanning electron microscope (Figure 5). SeNPs show various sizes of spherical particles, and it is inferred that the well-dispersed and

spherical shape SeNPs produced from biosynthesized selenium dioxide by an actinomycete isolate BLH 12-1 (Figure 5). From the observations in the SEM image, it can be seen that the spherical structure is not uniform, and the SeNPs are diffused in the synthesis culture.

EDX microanalysis of nanoparticles separately shows the Se absorption peak of SeL α at 1,379 keV (Figure 6), according to the results of a study conducted by Tan *et al.* (2016). These confirm the existence of a sample. The EDX peaks also detected C, O, Na, P, and Cl, indicating that these elements are in the cell's cytoplasm.

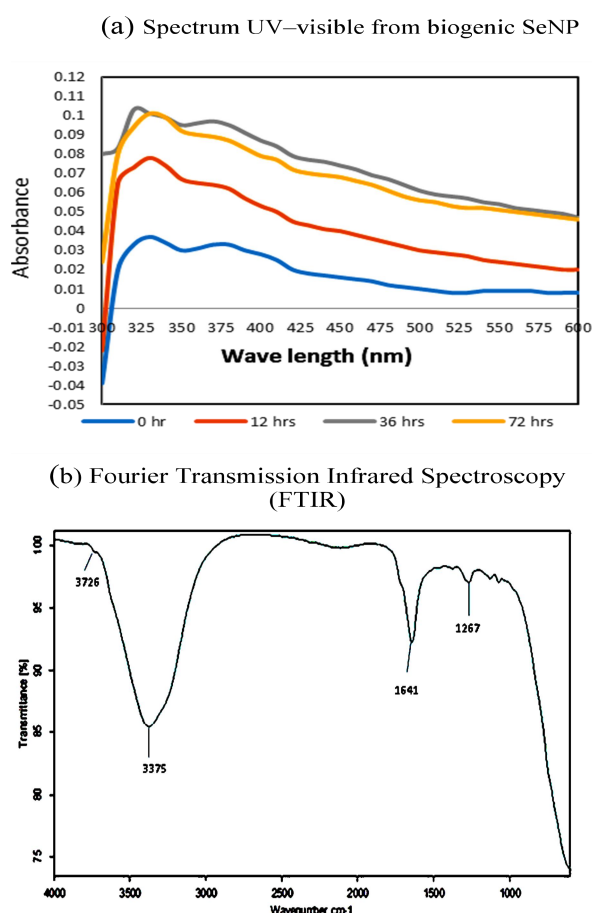


Figure 3. UV-vis spectrum for 72 h of incubation periods (a) and Fourier Transmission Infrared Spectroscopy (b) of Selenium nanoparticles synthesized by strain BLH 12-1.

Molecular identification of the BLH 12-1 strain

The BLH 12-1 strain was isolated in a sea sediment sample collected from Lembah Strait, Bitung, North Sulawesi, Indonesia. The phylogenetic analysis confirmed that this strain

belongs to the family *Micromonosporaceae* and the genus *Micromonospora*. Moreover, neighbor-joining phylogenetic analysis based on a 16S rRNA gene sequence revealed that the actinomycete BLH 12-1 strain belongs to the genus *Micromonospora* and is closely related to *Micromonospora tulbaghia* (99% sequence similarity) (Figure 7).

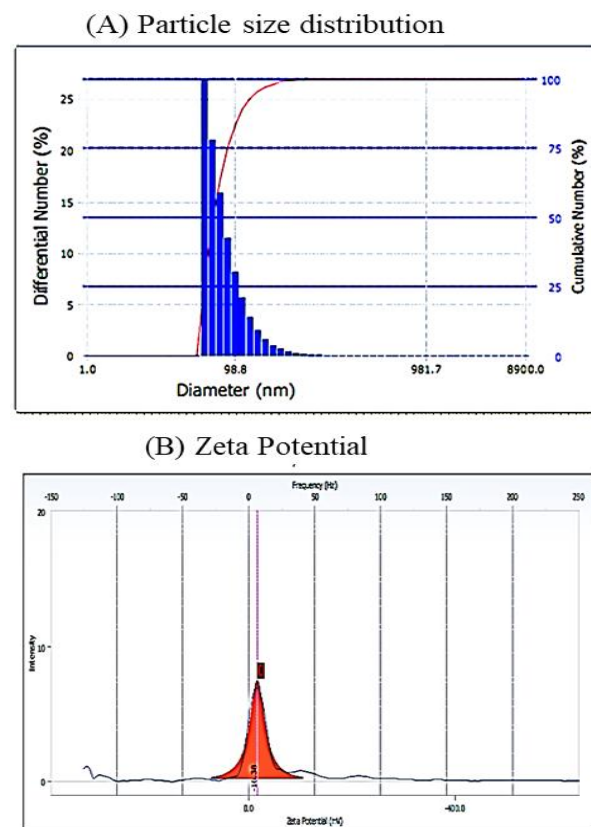


Figure 4. Particle size distribution and Zeta potential of SeNPs from BLH 12-1

Antimicrobial activity of Selenium Nanoparticle biosynthesis from actinomycetes isolates

SeNPs exhibit antibiotic properties against various pathogens. The antibacterial activity of SeNPs produced by actinomycete was studied against different Gram-positive and Gram-negative strains. The antibacterial activity was determined by the paper disc method described by Johnson and Raja (2015). All of the microbiologically synthesized SeNPs exhibited excellent antibacterial activity against the bacterial pathogens, both Gram-negative and Gram-positive (Table 1). The biogenic SeNPs produced by three marine actinomycetes isolates had antibacterial properties against three tested bacteria: *B. subtilis*, *S. aureus*, *M. luteus*, *E. coli*, and *C. albicans*.

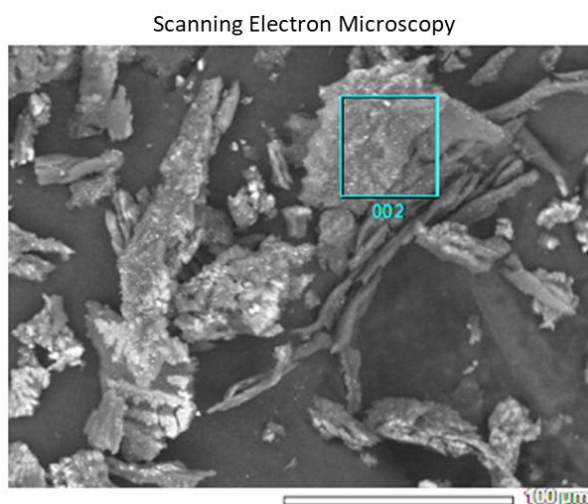


Figure 5. The observations in the Scanning Electron Microscopy show that the spherical structure is not uniform and spreads in the synthesis culture

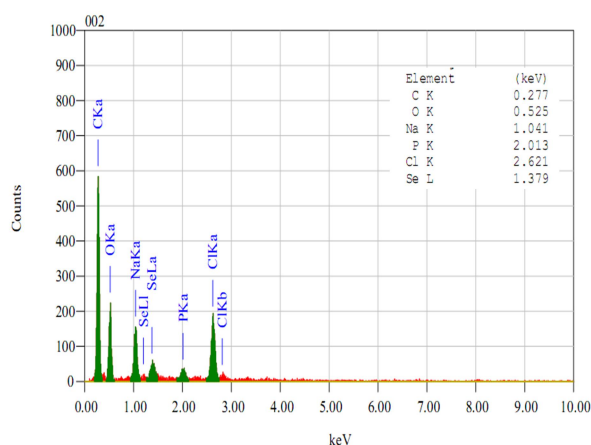


Figure 6. EDX spectrum of selenium nanoparticle particles synthesized by actinomycetes BLH 12-1, which showed the presence of Selenium

Table 1 shows the zone of inhibition against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, *M. luteus*), Gram-negative (*E. coli*), and yeast pathogens (*C. albicans*).

All SeNPs produced by selected actinomycetes have an inhibitory zone against test bacteria *B. subtilis*, *S. aureus*, *M. luteus*, *E. coli*, or *C. albicans*. The highest antimicrobial activity against *B. subtilis* was demonstrated by SeNPs produced by actinomycete BLH 5-21 with a Clear Zone Index (CZI) of 1.83. The highest antimicrobial activity of *S. aureus* with a CZI value of 4.5 was demonstrated by SeNPs synthesizing by actinomycete BLH 12-1 (Table 1). No inhibition was found in the culture supernatant, proving that the culture has no antibacterial activity.

The formation of the inhibition zone around the well was an indication of the antibacterial

activity of SeNPs (Figure 8) (Narasimha *et al.*, 2012).

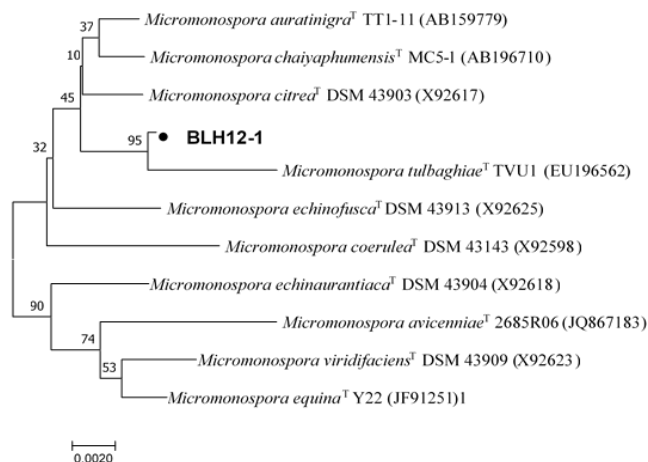


Figure 7. Phylogram derived from 16S rRNA gene sequences of isolate BLH 12-1 of marine actinomycete from Lembah Strait and their related strain, showing the relationship with type strain in a cluster node *Micromonospora*. Bootstrap values based on 1,000 replications shown at branch nodes.

Table 1. Antimicrobial activity of biogenic Selenium nanoparticles synthesized by actinomycetes strains

Code of Isolate		Clear Zone Diameter (mm)				
		Test microorganisms				
		<i>Bs</i>	<i>Sa</i>	<i>Ml</i>	<i>Ec</i>	<i>Ca</i>
BLH 5-21	Se 3 mM	17	32	11	11	-
	Streptomycin 5 µg /mL	13	12	18	13	-
	Control supernatant	-	-	-	-	-
BLH 5-22	Se 3 mM	13	32	-	13	-
	Streptomycin 5 µg /mL	14	10	9	14	-
	Control supernatant	-	-	-	-	-
BLH 12-1	Se 3 mM	12	33	10	10	10
	Streptomycin 5 µg /mL	14	11	18	15	-
	Control supernatant	-	-	-	-	-

Note: *Bs*: *Bacillus subtilis*, *Sa*: *Staphylococcus aureus*, *Ml*: *Micrococcus luteus*, *Ec*: *Escherichia coli*, *Ca*: *Candida albicans*

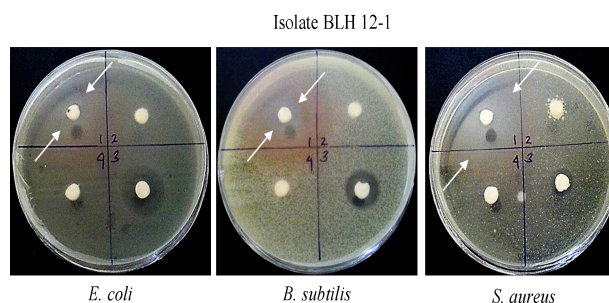


Figure 8. Antibacterial activity of Selenium nanoparticles synthesized by isolate BLH 12-1 against test bacteria: *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*.

Among metal nanoparticles, SeNPs are known for their valuable biological activities

like antibacterial and antifungal properties (Beheshti *et al.*, 2013; Soflaei *et al.*, 2014). Different biological resources such as bacterial, fungal, and plant extracts for the biosynthesis of SeNPs have gained significant interest in two decades (Soflaei *et al.*, 2014; Fesharaki *et al.*, 2010; Forootanfara *et al.*, 2014). The antimicrobial activity was reportedly due to the penetration of SeNPs into the bacteria, damaging the cell membrane and releasing cell contents. Another possibility suggested was the release of Se (⁰) ions from the nanoparticles, which may contribute to the bactericidal properties of SeNPs.

Determination of the Minimum Inhibitory Concentration for BLH 12-1 SeNPs against Test Microorganisms

The effect of biogenic SeNPs as antimicrobial agents evaluated by measuring the determination of minimum inhibitory concentration (MIC) values was first carried out against the test microorganisms *B. subtilis*, *E. coli*, *S. aureus*, *M. luteus*, and *C. albicans* (Figure 9). SeNPs on test microorganism's cell viability were studied by performing a Resazurin assay. The test microorganisms were cultured in various concentrations of the SeNPs.

We found that the SeNPs from BLH 12-1 can inhibit and reduce the viability of *B. subtilis* and *C. albicans* on all tested concentrations up to 128 dilutions. All wells inside the microplate are blue. This blue color indicates that the salt of resazurin does not turn into resofurin (pink color) (Sarker *et al.*, 2007) in the absence of live bacteria. The growth of *E. coli* can be inhibited by SeNPs BLH 12-1 to 16× dilution, whereas *S. aureus* and *M. luteus* growth is inhibited until a dilution of 32× SeNPs.

These results indicate the observed antimicrobial activity due to the presence of nanoparticles.

Several studies have analyzed the antimicrobial activity of SeNPs that are biologically synthesized against different pathogenic bacterial strains. The results of Shakibae *et al.* (2017) showed selenium biogenic SeNPs having antifungal activity against *Aspergillus fumigatus* and *C. albicans*. Selenium is an effective antimicrobial agent with inherent activity (Ramos & Webster, 2012). The actinomycetes *Micromonospora tulbaghia* strain BLH 12-1 appear to be promising organisms for antimicrobial applications such as antibiotics and medical devices.



Figure 9. MIC Plate after 24 hours incubation using resazurin assay method. The pink color indicates the growth of test microorganisms. The blue color indicates inhibition of the development of the test microorganism, i.e.: (1) Positive control streptomycin in serial dilution 5-0.039 µg/mL of streptomycin, *B. subtilis* (2-3), *E. coli* (4-5), *S. aureus* (6-7), *M. luteus* (8-9), *C. albicans* (9-10) and medium control without addition of SeO₂.

Conclusion

Biosynthetic methods, developed for producing metallic nanoparticles using microorganisms such as actinomycetes, are considered more promising due to their non-toxic, environmentally friendly, and energy-efficient characteristics, in contrast to physically and chemically based methods for nanoparticle synthesis. Actinomycetes can produce Selenium nanoparticles with antimicrobial activity against pathogenic bacteria and fungi such as *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *E. coli*, and *Candida albicans*. We have demonstrated that Selenium nanoparticles synthesized by *Micromonospora tulbaghia* strain BLH 12-1 possess potent antimicrobial activities. Further investigation needs to be done to optimize the production of nanoparticles.

Author contributions

All authors contributed equally to this work and reviewed the manuscript.

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