Enhanced Antioxidant Activity of Nano-Selenium Produced Using a Bacterial Isolate Citrobacter sp.

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Abstract: Selenium is essential for human, plant, and animal growth and reproduction. Because of their biological activity, bioavailability, and low toxicity, selenium nanoparticles are regarded as a promising material for many applications in biomedicine and health. Selenium-resistant bacteria were isolated from garden soil and identified as Citrobacter amalonaticus strain ARB01. TEM, UV-visible spectrophotometer, FTIR, and EDAX were used to characterize biosynthesized SeNPs using the cell-free extract of ARB01. The antioxidant activity of biologically functionalized SeNPs was evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant strength (FRAP), and 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. The Dot-blot test was used to perform a qualitative study of the antioxidant activity of SeNPs using the TLC-DPPH technique. The TEM analysis revealed spherical SeNPs with diameters ranging from 50 nm to 80 nm. Because of the synergistic effect of biomolecules involved in nanoparticle synthesis, SeNPs showed higher antioxidant activity. This study reveals that the antioxidant activity of nanoparticles increased due to functionalization by biomolecules present in the cell-free extract of bacterial isolate.

Keywords: nanoparticles; selenium; Citrobacter amalonaticus; antioxidant

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1. Introduction

In recent years, nanotechnology has taken a significant role in scientific research fields like health, nutrition, medicine, and progress in physics, chemistry, and biology[1,2]. Nanoscale particles have distinct optoelectronic, magnetic, and mechanical properties that distinguish them from bulk materials [3–5]. Because of their small size and chemistry, nanoparticles have a wide range of applications [6,7]. Selenium (Se) is a naturally occurring metalloid element in the environment. Selenium resources are limited and non-renewable [8]. Selenium is a multifunctional material with unique physical properties such as high optical conductivity, anisotropy of thermal conductivity, and X-ray sensing responses. Nanoparticles have distinct properties due to their greater surface/volume ratio and higher surface energy [9]. Selenium nanoparticles have a broad range of applications in chemistry, physics, biology, biomedical, pharmaceutical, and material sciences [10]. The biological approach for the synthesis of nanoparticles has sparked the interest of researchers because it is both eco-friendly and cost-effective, as reported by many studies [11–13]. Ion-sputtering solvothermal
procedure, sol-gel technique, ultraviolet irradiation, laser ablation, Riley oxidation, and ultrasonic treatment are examples of physical and chemical methods. Microwave irradiation was used to synthesize hexagonal selenium nanoparticles using selenium tetrachloride as a precursor and hydrazine as a reducing agent [14]. A simple wet chemical method was used to synthesize spherical SeNPs with a 76-150 nm size range by reducing selenosulphate with an ionic liquid [15]. Nanoparticles of different sizes and shapes have been synthesized using biological reducing agents with varying bioactivity [16–18]. *Staphylococcus aureus*, a Gram +ve bacteria, was used to synthesize small-sized selenium nanoparticles [19]. *Lactobacillus acidophilus*, a probiotic bacteria, was used to synthesize non-toxic selenium nanoparticles [20]. Selenium is important for human health because it regulates many oxidation and reduction processes through seleno-enzymes and selenoproteins. During the metabolic process, free radicals and reactive oxygen species are formed, which cause oxidative stress and cellular damage. Antioxidants are molecules that act as reducing agents by donating electrons to free radicals to stabilize them. Antioxidants protect DNA, cell, and organ systems from free radical damage. Antioxidant substances like vitamins, enzymes, flavonoids, and polyphenols are abundantly found in plants, fruits, vegetables, and some naturally derived products [21]. Synthesized selenium nanoparticle shows antimicrobial [22,23], antioxidant [24], antidiabetic [25,26], and cytotoxic activity [27–29].

2. Materials and Methods

This study synthesized SeNPs using a cell-free extract of the strain ARB01, a biological reducing agent, followed by physical characterization and evaluating their qualitative and quantitative antioxidant activity. During the experiment, analytical grade chemicals obtained from Merck (India) were used.

2.1. Isolation of selenium resistant bacteria.

The soil sample was collected and dissolved in a 0.9 % saline solution. In a flask, 50 ml of nutrient broth media containing 500 µmol/L Na₂SeO₃ was taken and inoculated with 1 ml of the prepared soil solution. In brief, the approach was followed as per the previously published study[24]. Red colonies of selenium-resistant bacteria appeared on the culture plates after incubation (Figure 1).

![Figure 1. The growth of selenium-resistant bacteria onto nutrient agar plate supplemented with sodium selenite, reduction of selenite ions is evident by the presence of red bacterial colonies.](https://biointerfaceresearch.com/)
2.2. Genomic DNA isolation, agarose gel electrophoresis, PCR, and analysis of nucleotide sequences.

The methods employed for isolation of DNA, agarose gel electrophoresis, PCR, and sequence analysis were followed according to the earlier study [24].

2.3. Preparation of cell-free extract and synthesis of selenium nanoparticles.

The cell-free extract of *Citrobacter amalonaticus* strain ARB01 was prepared, and SeNPs were synthesized by following the experimental procedure published previously [24].

2.4. Physical characterization of synthesized selenium nanoparticles.

Physical characterization of SeNPs was done by using UV-visible spectroscopy, Transmission electron microscopy, Fourier-transform infrared (FTIR) spectroscopy, and Energy-dispersive X-ray spectroscopy (EDAX) [24].

2.5. Assay for evaluation of the antioxidant activity of SeNPs.

The antioxidant activity of synthesized selenium nanoparticles was evaluated using the TLC-DPPH assay (for qualitative analysis), DDPH, ABTS, and FRAP assay (for quantitative analysis).

2.5.1. DPPH assay.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was done according to the procedure described by Kumar *et al.*[24]. The qualitative analysis for the antioxidant activity of SeNPs was carried out by a Dot-blot test using the TLC-DPPH assay adapted from the study by Cieśla *et al.* [30] with some technical modification. Spraying was done on the TLC plate (silica plate) with a 0.2% methanolic DPPH solution instead of immersing the TLC plate. All the samples were prepared at a concentration of 2 µg/ml. The antioxidant activity of DPPH was calculated as % inhibition of DPPH by Eq.1.

\[
%\text{ inhibition of DPPH radicals} = \frac{(\text{Absorbance blank} - \text{Absorbance})}{\text{Absorbance blank}} \times 100 \quad \text{Equation 1}
\]

2.5.2. ABTS assay.

In brief, the experimental procedure for the 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was followed according to the method described [24]. The percent inhibition of ABTSº+ radicals was determined by the Eq. 2

\[
%\text{ inhibition of ABTSº + radicals} = \frac{(\text{Absorbance blank} - \text{Absorbance})}{\text{Absorbance blank}} \times 100 \quad \text{Equation 2}
\]

2.5.3. FRAP assay.

Ferric reducing antioxidant power (FRAP) assay is a widely used method. In this method, antioxidant materials are used for the reduction of colorless ferric tripyridyltriazine (Fe³⁺ TPTZ) complex into a colored ferrous tripyridyltriazine (Fe²⁺ TPTZ) complex at low pH [31]. The experimental work for the FRAP assay was carried out according to the procedure previously published [24].
3. Results and Discussion

3.1. Identification of selenium resistant bacterial isolate.

*Citrobacter amalonaticus* strain ARB01 was isolated as selenium-resistant bacteria from soil (Figure 2). The phylogenetic study of the bacterial isolate *Citrobacter amalonaticus* strain ARB01 revealed similar homology towards *Citrobacter amalonaticus* strain CECT883. It was found to be distantly related to *Citrobacter gillenii* strain CDC4693-86. A dendrogram was constructed to determine ARB01’s phylogenetic relationship to other *Citrobacter* species.

![Figure 2](https://biointerfaceresearch.com/)

**Figure 2.** The phylogenetic tree depicts the evolutionary relationships of *Citrobacter amalonaticus* ARB01 with other members of their family at the bootstrap value of 1000.

3.2. UV-visible spectroscopy.

The cell-free extract was used to synthesize selenium nanoparticles. The UV-visible absorption spectrum shows absorption maxima at 360 nm (Figure 3). The absorbance spectra of selenium nanoparticles synthesized from cell-free extract *Citrobacter amalonaticus* strain ARB01 were observed in the wavelength range of 250 nm to 700 nm (Figure 3).

![Figure 3](https://biointerfaceresearch.com/)

**Figure 3.** After 48 hours of incubation, the UV-Visible absorption spectrum shows absorption maxima at 360 nm, and a red-colored colloidal solution formed due to the formation of SeNPs.

There were no noticeable absorbance maxima in the zero-hour spectrums. After 48 hours, the absorbance increased significantly, with a maximum absorbance at 360 nm.
According to Ingole et al., the absorbance maxima of synthesized nanoparticles have been at 300 nm [32]. Prasad and Selvaraj recorded the absorption maxima of SeNPs at 390 nm as well [33]. As a result, particle size is responsible for forming absorption maxima at 360 nm, which is consistent with the previous studies, supporting the formation of selenium nanoparticles. The molecules involved in the synthesis of selenium nanoparticles cause differences in absorption maxima.

3.3. Transmission electron microscopy of SeNPs.

According to the TEM study, the synthesized nanomaterial is spherical with a diameter of 50-80 nm (Figure 4). Research reveals that selenium nanoparticles formed by the bacterium *Bacillus subtilis* ranged in size from 50 nm to 400 nm [34]. Similarly, in another study, the diameter of biosynthesized SeNPs was found to be in the 20–80 nm range [35]. In addition, biomimetic spherical SeNPs with diameters ranging from 30 to 150 nm have been reported [3]. The obtained results were consistent with earlier published studies. The size of the synthesized nanomaterial is determined by the concentration of the precursor material, the reducing agent, and the reaction time. The crystalline nature of the synthesized selenium nanoparticles was confirmed by the selected area electron diffraction (SAED) pattern.

![Figure 4. TEM image showing the spherical SeNPs of 50-80 nm size along with SAED pattern confirming the crystalline nature of selenium nanoparticle.](image)

3.4. Fourier transform infrared spectroscopy.

The Fourier transform infrared (FTIR) spectroscopy generates spectra based on the biological compounds' absorbance of infrared radiation (lipids, proteins, and polysaccharides). The intensity can be plotted at each wavenumber as a percentage of light transmittance or absorbance [36–38]. The FTIR spectra of SeNPs synthesized using cell-free extract revealed bands associated with various biological compounds such as proteins, polysaccharides, and lipids. Figures 5 (a) and (b) display a comparison of the FTIR spectra of the control sample (cell-free medium) and the test sample (Bio-SeNPs). The spectral picture represents the number of absorption bands induced by molecular vibrations of various functional groups. One peak between 3400 and 3200 cm⁻¹ can be defined as the absorption peak of the NH stretching vibration or the OH stretching vibration of the H-bonded biomolecules. Weak bands found between 3000 and 2800 cm⁻¹ can be attributed to CH stretching vibrations of aliphatic side
chains in organic molecules caused by symmetric and asymmetric stretching vibrations of methyl and methylene groups. A very weak band at around 2090 cm\(^{-1}\) can be attributed to the stretching vibration of the C≡C bond. The absorption bands at around 1654–1634 cm\(^{-1}\) can be attributed to amide I of proteins involved in the capping of selenite ions. The spectral band at 1548 cm\(^{-1}\) and 1539 cm\(^{-1}\) can be assigned to the amide II of the proteins. The weaker band around 1410-1380 cm\(^{-1}\) can be assigned to the carboxylic group. The band at 1462 in the test sample (Figure 5b) can be assigned to scissoring vibrations of the methylene group of aliphatic side chains of biomolecules involved in the formation of SeNPs. The spectral bands around 1200-1000 cm\(^{-1}\) can be attributed to the various C–O, C–C, C–O–H, or C–O–C vibrations in biomolecules (proteins and polysaccharides) [36,37]. The absorption peaks around 1000-700 cm\(^{-1}\) may be due to the C–H bending of aliphatic side chains in proteins and polysaccharides. The spectral band at 620 cm\(^{-1}\) can be assigned to S-S stretching vibration.

![FTIR spectra](https://doi.org/10.33263/BRIAC125.61246133)

**Figure 5.** FTIR spectrums of (a) control and (b) biological samples containing SeNPs show the functional groups involved in nanoparticle synthesis.

### 3.5. Energy-dispersive X-ray analysis (EDAX).

The qualitative and quantitative status of elements present with the synthesized selenium nanoparticles is determined by energy-dispersive X-ray analysis. The EDAX study indicated a higher amount of selenium, as well as oxygen, carbon, and sodium (Figure 6) [39,40].

![EDAX spectrum](https://doi.org/10.33263/BRIAC125.61246133)

**Figure 6.** EDAX spectrum of synthesized nanomaterial showing the quantity of selenium along with other elements.
The study showed a higher concentration of selenite (55%) in nanoparticles, followed by carbon (40%), oxygen (18%), and sodium (1.18%). These elements are present in biosynthesized nanomaterials because biomolecules are involved in the functionalization and stabilization of SeNPs.

3.6. Antioxidant activity.

The qualitative TLC-DPPH assay revealed that SeNPs have antioxidant activity due to the synergistic effect of the biomolecules involved in nanoparticle synthesis. The antioxidant activity of selenium nanoparticles was demonstrated by the DPPH, ABTS, and FRAP assays (quantitative) (Figure 7). The antioxidant activity of synthesized SeNPs was evaluated by employing DPPH, ABTS, and FRAP assay [41–43]. The assay showed that selenium nanoparticles synthesized using the cell-free extract of *Citrobacter amalonaticus* strain ARB01 have antioxidant activity, which is higher than the cell-free extract and sodium selenite due to the synergistic effect of capping and stabilizing molecules. Above mentioned assays were done to evaluate the antioxidant activity of selenium nanoparticles, sodium selenite, the cell-free extract of ARB01, and ascorbic acid. After performing DPPH and ABTS assay, antioxidant activity was calculated by % inhibition of DPPH and ABTS radicals.

The Dot-blot test was performed using the TLC-DPPH assay for the qualitative evaluation of the antioxidant activity. Decolourization occurred at the spot applied on the TLC plate after spraying the DPPH solution due to scavenging activity. The DPPH assay shows that the scavenging activity of SeNPs (65±4) is higher than sodium selenite (20±4). Similarly, the ABTS assay shows that the scavenging activity of SeNPs (70±3) is higher than sodium selenite (25±2). FRAP results were shown as FeSO$_4$ equivalents in mmol/L. The result was shown in the form of mean±SE of the mean (n=6) using statistical calculations at 5% of significance level (Figure 7). The FRAP assay showed higher antioxidant activity of selenium nanoparticles.
than sodium selenite (55±3 and 20±2), respectively. The antioxidant activity of the SeNPs is higher due to the synergistic effect of biomolecules involved in nanoparticle synthesis.

4. Conclusions

In this present study, a cell-free extract of bacterial isolate *Citrobacter amalonaticus* strain ARB01 was used to synthesize selenium nanoparticles. The cell-free extract contains biomolecules like proteins, lipids, and polysaccharides, which were involved in the formation of SeNPs. The TEM analysis showed spherical selenium nanoparticles with a size range of 50 nm to 80 nm diameters. For the first time, the antioxidant activity was evaluated qualitatively by using the TLC-DPPH assay and quantitatively by DPPH, ABTS, and FRAP assay. The DPPH, FRAP, and ABTS assay manifested that selenium nanoparticles have enhanced antioxidant activity than sodium selenite and cell-free extract. The increase in the antioxidant activity of SeNPs was due to the synergistic effect of biomolecules involved in the functionalization of the nanomaterial. Besides, extensive investigation of SeNPs is needed for biomedical and pharmaceutical applications due to cytotoxic, antimicrobial, and antidiabetic activity.

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Conflicts of Interest

The authors declare no conflict of interest.

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