Research Article

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Biosynthesis, characterization, and investigation of cytotoxic activities of selenium nanoparticles utilizing *Limosilactobacillus fermentum*

Abstract: The biosynthesis of selenium nanoparticles (SeNPs) is one of the methods used alongside other conventional methods for SeNP synthesis. In this research, we used the cell-free culture (CFC) of *Limosilactobacillus fermentum* for SeNP synthesis. We investigated the biosynthesis of SeNPs under various levels of temperature, pH, and Se⁴⁺ concentration and characterized the biosynthesized SeNPs using FE-SEM, energy-dispersive X-ray, Fourier transform infrared spectroscopy, X-ray diffraction, ultraviolet–visible spectroscopy (UV–Vis), and dynamic light scattering–zeta potential analyses to find nanoparticles with desirable properties. Also, the cellular toxicity of SeNPs against the MCF-7 cell line was analyzed. The scavenging activity of free radicals in CFC before and after SeNP synthesis was examined using the DPPH method. The selected SeNP has an average hydrodynamic radius of 92.52 nm and a polydispersity index of 0.134. This nanoparticle also has a mostly spherical shape, amorphous nature, and zeta potential of −32.2 mV. The toxicity of nanoparticles for MCF-7 was much lower than sodium selenite salt. It was also confirmed that during nanoparticle synthesis, the reducing ability of CFC significantly decreases. This research aimed to design a safe, cheap, and eco-friendly protocol for the biosynthesis of SeNPs using the CFC of *Limosilactobacillus fermentum*. As a result, SeNPs possess great potential for further exploration in the realm of biomedicine.

Keywords: biosynthesis, *Limosilactobacillus fermentum*, selenium nanoparticle, characterization, cytotoxic

1 Introduction

Nanotechnology is thought to be the next industrial revolution and is also expected to have a significant impact on society, the economy, and every aspect of life [1–3]. Nanoparticles can offer new ways to solve unresolved issues in industry, the environment, and medicine. Therefore, nanoparticles have been extensively studied and investigated in recent years [4]. The characterization of nanoparticles is of great importance to understanding their properties and applications. Size, dispersion, geometric shape, optical properties, crystallinity, and cellular toxicity are among the most critical specifications that help us choose different nanoparticles for specific applications [5].

The need for environmentally friendly and sustainable methods for nanoparticle production is crucial. Conventional chemical and physical synthesis methods often use hazardous chemicals, require high energy consumption, and can generate toxic byproducts [6,7]. Green nanotechnology, which utilizes biological resources for nanoparticle synthesis, offers a more sustainable and eco-friendly alternative [8,9]. Green synthesized nanoparticles often exhibit good biocompatibility and high stability, do not require various biological and chemical stabilizers, and require minimal post-processing steps [7,10]. Also, it is undeniable that some conventional methods of nanoparticle synthesis can harm the environment [6]. On the other hand, physical synthesis of nanoparticles sometimes has fewer risks than chemical methods; still, the complexity of the tools and the high cost of this method will limit the application of synthesized nanoparticles [11].

Selenium nanoparticles (SeNPs) have gained significant interest in recent years due to their unique properties and potential applications in biomedicine and other fields [12,13]. Selenium is an essential element for various enzymes and proteins, including selenoproteins, which play a crucial role in DNA production and protection against cellular damage and infection [14]. However, some selenium supplements, especially mineral forms, can be toxic at high doses [15]. SeNPs have been shown to be less toxic and exhibit...
improved biological activities compared to their bulk counterparts [16,17]. They possess significant anticancer and antioxidant properties, making them promising candidates for various biomedical applications such as anti-inflammatory agents, drug carriers, cancer therapy, diabetes treatment, and immune stimulators [18–21]. The biological synthesis of SeNPs offers several advantages over conventional methods. Biologically synthesized nanoparticles are typically less toxic, more stable, and require fewer purification steps [22]. Also, the ecologically sustainable method of producing nanoparticles offers the chance to use them safely in different sectors [23,24].

Biological SeNPs have been produced using a variety of techniques and are derived from a variety of biological sources, such as plant extract, bacteria, fungi, etc. [25–28]. The novelty of this research lies in our exploration of the potential of the Limosilactobacillus fermentum strain ATCC 14931 for the green synthesis of SeNPs. While various biological sources have been employed for SeNP synthesis, Limosilactobacillus fermentum offers distinct advantages. Furthermore, limited research has explored the ability of cell-free culture (CFC) from Limosilactobacillus fermentum for SeNP synthesis. We explore the influence of various factors, such as pH, temperature, and sodium selenite concentration, on the production and properties of the synthesized nanoparticles. We chose Limosilactobacillus fermentum due to its Generally Recognized As Safe status, implying its safety for large-scale production processes. Additionally, L. fermentum was chosen due to its well-documented reducing power, a key factor for SeNP biosynthesis [20]. Uniformity in size and shape is crucial for consistent nanoparticle properties and functionalities [29]; therefore, the production of uniform NPs is a primary focus of this research. To assess the role of reducing power in the NP synthesis process, we further evaluated the pre- and post-synthesis reducing power of the resulting CFC. This evaluation employed the DPPH assay to determine if the reducing potential of the CFC had been diminished during the NP synthesis process.

2 Material and methods

2.1 Chemical compounds used in the synthesis of NPs

SeNPs were synthesized using sodium selenite pentahydrate (Na2SeO3·5H2O) obtained from Merck, Germany. MRS broth medium (Q-LAB, Germany) was used for bacterial cultivation.

2.2 Bacterial strain and growth conditions

The Limosilactobacillus fermentum strain ATCC 14931 was obtained from the Iranian biological resource center. The bacteria were grown in an MRS broth medium at 37°C under aerobic conditions for 24 h.

2.3 Preparation of CFC

Preparation of CFC began by thawing a frozen aliquot of the bacterial stock culture, which had been previously stored at −80°C. This thawing process should be rapid (around 30–37°C using a water bath) to minimize the risk of damaging the frozen bacterial cells; 25 μL of thawed bacterial cells were then aseptically inoculated into 10 mL of autoclaved MRS broth medium. Aseptic techniques are crucial at all steps of CFC preparation and synthesis to prevent contamination with unwanted microorganisms. The flask containing pre-culture was incubated at 37°C, which is the optimal growth temperature for L. fermentum. Constant shaking at 180 rpm ensures proper aeration and the homogeneous distribution of nutrients and bacterial cells throughout the culture medium. The pre-culture was incubated for 16 h. This incubation period allows the bacteria to adapt from their frozen state, revive, and begin multiplying. After the pre-culture incubation, 10 mL of the pre-culture was aseptically transferred into a larger flask containing 100 mL of fresh, autoclaved MRS broth medium. The main culture was then incubated under similar conditions as the pre-culture (37°C, 180 rpm shaking) for an extended period of 24 h. This extended incubation allows for exponential growth of the bacterial population, maximizing the production of metabolites and enzymes. Aerobic conditions were maintained throughout the incubation using flasks with breathable caps. Following incubation, the bacterial culture was centrifuged at 5,000 rpm for 20 min using a refrigerated centrifuge. Using a refrigerated centrifuge helps maintain low temperatures during centrifugation. The resulting supernatant, referred to as the CFC, was carefully collected under sterile conditions. The sterile collection of the CFC ensures minimal contamination that could interfere with the nanoparticle biosynthesis process.

2.4 Biosynthesis of SeNPs

After preparing CFC, the pH was adjusted to the desired values (pH at values of 5.4, 7.4, and 9.4), and the final concentration of sodium selenite salt in the synthesis solution was also adjusted (0.375, 0.75, 1.5, and 3 mM). All were done
under sterile conditions. After adding the salt, the synthesis solution was immediately placed at the desired temperatures (37°C, 25°C, and 4°C) and incubated for 3 days under the conditions. MRS broth was used as a control. After completing the synthesis reaction, samples were centrifuged at 9,000 rpm for 20 min at 4°C using a centrifuge. Separated SeNPs were washed in distilled water to eliminate the unreacted salts and other components. Throughout this study, the selected SeNPs were synthesized several times. Then, the SeNPs were resuspended in deionized water and kept at 4°C. If it was needed, SeNPs dried in an oven at 37°C, and their powder was stored.

2.5 Ultraviolet–visible (UV–Vis) spectroscopy

The UV–visible spectrum of NPs was investigated using a UV–visible spectrophotometer (PerkinElmer Inc., USA) operated at a resolution of 1 nm.

2.6 DLS and zeta potential

The Zetasizer Zeta–DLS instrument (Malvern, UK) was used to assess the samples’ size, polydispersity index (PDI), and zeta potential at 25°C.

2.7 FESEM-EDX analysis

Images of SeNPs were acquired using an FE-SEM microscope (Sigma VP, ZEISS Germany). A smear of samples on glass was used, and the material was gold-coated. Also, energy-dispersive X-ray (EDX) analysis was used to establish the presence of elemental selenium. At a magnification of 10 kV, images were captured.

2.8 Fourier transform infrared spectroscopy (FTIR)

SeNPs were added to KBr pellets for this analysis, and the results were analyzed using a Nicolet IR100 FTIR spectrometer (Thermo Scientific). Against a potassium bromide background, 400–4,000 cm⁻¹ wave numbers were used to get the FTIR spectra. The acquired peaks were represented as wave number (cm⁻¹) on the Y-axis and transmittance percentage on the X-axis.

2.9 X-ray diffraction (XRD)

Using a Panalytical X-Pert Pro XRD with Cu Ka 1.5406 Å radiation, the XRD patterns of sodium selenite and the selected SeNP sample were obtained. The measurement was recorded over 10–70 (2θ).

2.10 MTT assay

The cytotoxicity activity of SeNP was determined by MTT assay using the protocol performed by Riss et al. [30] with some modifications. MCF-7 cell lines were purchased from the Pasteur Institute of Iran. The cell lines were raised in 10% fetal bovine serum (DMEM). The cultured cells were incubated in a biological incubator at 5% CO₂ at 37°C. After that, 1 × 10⁴ cells were planted in each well in a 96-well cell culture dish and incubated for 48 h. Then, MCF-7 cells were treated with different concentrations (200, 100, 50, 25, and 12.5 μg·mL⁻¹) of biosynthesized SeNPs. Also, cells were treated with a solution of the same concentration of sodium selenite. Five wells without treatment were considered as the negative control (without treatment). Then, the treated cells and the control sample were incubated for another 24 h under the previously mentioned conditions. The entire experimental process was carried out in an aseptic environment. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test was then performed to assess the vitality of the cells. Using a spectrophotometer, the cell viability was measured at 570 nm. Each experiment was carried out in triplicate. The cell viability percentage (%) was computed using this formula:

\[
\text{Cell viability} \% = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where “A” is the optical density in 570 nm.

2.11 DPPH assay

In this study, the radical scavenging activity of CFC was assessed both before and after the synthesis of SeNPs using DPPH (1,1-diphenyl-2-picrylhydrazyl). The CFC before and after the synthesis of NPs was studied. In order to investigate the effect of a longer synthesis process on the final reducing power of the CFC, the synthesis of NPs proceeded for 3 and 5 days. Also, to demonstrate how the MRS culture medium’s reducing power changes as bacterial growth proceeds, the MRS medium was examined. To perform the assay, 100 μL of 0.2 mM DPPH and 100 μL of each sample were mixed in a well of a 96-well microplate, and
the microplates were placed in darkness at 25°C for 30 min; 100 μL DPPH solution and 100 μL ethanol 50% were used as controls, and the blank was 100 μL of sample and 100 μL ethanol 96%. With the use of a microplate reader, the absorbance was determined at 517 nm. The following formula is used to calculate the percentage of radical scavenging activity:

$$\text{Radical scavenging activity (\%)} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100$$

where $A_{\text{control}}$ is equal to the absorbance of the control sample, $A_{\text{sample}}$ is the absorbance of each of the investigated samples, and $A_{\text{blank}}$ is the absorbance of samples without DPPH. All absorbances were investigated at 517 nm. The test was done in triplicate.

3 Results and discussion

3.1 Biosynthesis of SeNPs

The initial step in the shaping of nanoparticles is visual coloration [31,32]. The formation of SeNPs can be detected by a change of color in the synthesis colloid (Figure 1) and confirmed by additional tests [33]. Previous studies have reported that during the synthesis of SeNPs in an aqueous environment, the color of the synthesis solution changes to red and then to dark red [34], and this is an indication of the reduction of ionic Se to elemental Se and probably the formation of SeNPs. As can be seen in Figure 1, since the MRS and therefore CFC have a darkish red color, it is not easy to detect the color change of the MRS-containing NPs. Therefore, we verified the color change in the NP synthesis solution by observing it after the separation of the MRS using a centrifuge. To validate the formation of SeNPs, we further investigate the samples with FESEM, XRD, FTIR, etc.

3.2 Selection of advantageous condition

We conducted the synthesis process at different temperature levels (37°C, 25°C, and 4°C), pH levels (5.4, 7.4, and 9.4), and sodium selenite concentrations (0.375, 0.75, 1.5, and 3 mM) to find conditions that are necessary to achieve nanoparticles with desirable physicochemical properties. Out of the 36 tested conditions, we only detected a color change in 12 of them. In the rest of the cases, no considerable color change was seen. Additional experiments were needed to confirm the synthesis of nanoparticles in those 12 samples since a change in the color of the synthetic solution is a required but insufficient criterion to validate the synthesis of nanoparticles. However, we handled these 12 samples, as nanoparticles so that we could narrow down the sample pool in order to discover the optimal condition. It should also be noted that the synthesis of SeNPs in all experiments occurred only in CFC, and color change in the synthesized solution never occurred in the control (MRS + sodium selenite). This can indicate that the synthesis of SeNPs requires the presence of compounds that exist due to the growth of bacteria in the MRS medium. The different synthesis conditions of these 12 samples are presented in Table 1. Due to the

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>pH</th>
<th>The concentration of sodium selenite (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
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<td>7.4</td>
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<td>7.4</td>
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<td>9.4</td>
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<tr>
<td>11</td>
<td>37</td>
<td>9.4</td>
</tr>
<tr>
<td>12</td>
<td>37</td>
<td>9.4</td>
</tr>
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</table>
lack of considerable color change in other conditions, they were not mentioned.

An analysis of the combined data from Table 1 suggests that the temperature of 37°C yielded a significantly higher concentration of SeNPs compared to other temperatures tested. For instance, little to no SeNP formation was observed at 4°C, while 25°C resulted in an unfavorable synthesis rate. In terms of pH, most of the neutral and some of the alkaline conditions (pH 7.4 and 9.4) facilitated the formation of SeNPs. Acidic synthesis conditions were unable to do so. The concentration of sodium selenite ions exerted a significant influence. While SeNP synthesis was observed across various sodium selenite (Se⁴⁺) concentrations, our data suggest a critical dependence on Se⁴⁺ availability for optimal nanoparticle characteristics. Both excessive and insufficient Se⁴⁺ concentrations deviated from the desired outcome. High Se⁴⁺ levels resulted in the formation of larger nanoparticles, potentially due to uncontrolled particle aggregation. Conversely, limited Se⁴⁺ availability hindered proper nanoparticle formation, leading to either a complete absence of SeNPs or the production of inadequately sized or shaped nanoparticles. It is necessary to mention the crucial role of reaction time. Shorter than 3-day durations could lead to fewer and smaller SeNPs, whereas extended reaction times could lead to a significant increase in particle size and dispersity [35].

Long-term stability is a crucial factor for SeNP applications. In the next step, we selected the most stable SeNP. To achieve this, colloidal samples that underwent sedimentation or color change within 3 months after the synthesis were discarded due to potential changes in their properties. Sedimentation suggests a loss of stability and aggregation, which can alter the size, surface properties, and ultimately, the functionality of the SeNPs. Color change can be another indicator of morphological or structural transformations within the nanoparticles, potentially affecting their long-term performance [36].

We also consider that SeNPs with relatively sharp absorption peaks might be advantageous (Figure 2). This is because a strong and sharp absorption peak can indicate the creation of nanoparticles with uniform shape and size (low PDI) and low dispersion. Four of the 12 samples met the criteria we considered. The name and synthesis conditions of these nanoparticles are mentioned in Table 2.

These four samples were stable for more than 3 months and were more stable than other nanoparticles produced. Within 3 months of their production, the stability of these nanoparticles was evaluated based on changes in the colloid’s color and appearance as well as the UV–Vis spectrum. Among the four nanoparticles exhibiting 3-month stability, SeNP-1 displayed superior long-term stability, as evidenced by the absence of color change or sedimentation for an extended period. The UV–Vis spectra of these four selected nanoparticles and one example of not-stable SeNPs are shown in Figure 2. SeNP-1 nanoparticles should have a lower PDI and a more uniform distribution of size and shape, according to the result. This can be concluded from the higher and stronger peaks of these nanoparticles compared to other samples.

### Table 2: The synthesis conditions of the four selected samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>The concentration of selenite (mM)</th>
</tr>
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<tbody>
<tr>
<td>SeNP-1</td>
<td>37</td>
<td>7.4</td>
<td>0.75</td>
</tr>
<tr>
<td>SeNP-2</td>
<td>37</td>
<td>7.4</td>
<td>1.5</td>
</tr>
<tr>
<td>SeNP-3</td>
<td>37</td>
<td>9.4</td>
<td>0.75</td>
</tr>
<tr>
<td>SeNP-4</td>
<td>37</td>
<td>9.4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

### 3.3 Characterization of selected nanoparticles

Various tests were performed to confirm the formation of SeNPs and also identify the properties of four stable nanoparticles to achieve a SeNP with desirable properties (low PDI, high zeta potential, and consequently more stability). These tests include FE-SEM, EDX, and dynamic light scattering–zeta potential (DLS–ZETA).

#### 3.3.1 FE-SEM imaging and EDX elemental analysis

The four chosen nanoparticles were individually observed using FE-SEM microscopy. We can validate the creation of nanoparticles and see their geometric shape using the pictures captured by this microscope. The images of these nanoparticles are shown in Figure 3.
As shown in Figure 3, SeNP-1 is mostly spherical. SeNP-2 is mostly cubic or spherical. SeNP-3 seems to have irregular and spherical shapes, and SeNP-4 is irregular and different in shape. Thus, SeNP-1, among these four samples, has the most uniform shape and is generally spherical. Additionally, to confirm the presence of selenium in the image taken by FESEM and to ensure that observed nanoparticles are selenium we used EDX, in which the presence of selenium was confirmed by the existence of a peak at 1.347 keV (data not shown).

### 3.3.2 DLS–ZETA test

DLS is a typical method for determining a particle’s hydrodynamic size. The frequency of light scattered by colloidal particles is inversely related to the particle diameter per unit of time. The scattered light frequency by SeNP-1 nanoparticles shows that their particle size is in the nanometer range (Figure 4(a)).

The result shows that SeNP-1 nanoparticles have an average hydrodynamic radius below 100 nm (92.52 nm). Additionally, the PDI index has the lowest value (0.134) compared to other samples, indicating the monodispersity of these nanoparticles. SeNP-2 and 3 had particle radii of 122.9 and 126.2 nm, respectively, and greater hydrodynamic sizes. Additionally, both samples have much higher PDI values (0.350 and 0.372, respectively). SeNP-4 has a smaller hydrodynamic size than other samples. The PDI index in this sample was equal to 0.201. Therefore, SeNP-1 can be considered to have an advantageous PDI and size among SeNPs.
The high zeta potential of particles in colloids avoids aggregation and agglomeration. In general, nanoparticles with a zeta potential above $+30 \text{ mV}$ or less than $-30 \text{ mV}$ are regarded as stable colloidal systems [37]. Among the synthesized nanoparticles, SeNP-1 has a zeta potential equal to $-32.2 \text{ mV}$ (Figure 4(b) and Table 3). As can be seen in Table 3, SeNPs-2, 3, and 4 have a less than $30 \text{ mV}$ zeta potential value ($-11.5$, $-14.5$, and $-14.4 \text{ mV}$, respectively). It is expected that SeNP-1 colloids have more stability compared to other synthesized NPs. As mentioned before, to confirm the stability of the synthesized NPs, we kept them for a long time (3 months or more) under ambient conditions. SeNP-1 was more stable than the other tested NPs, and it did not change color or aggregate over time. Therefore, based on the results obtained, SeNP-1 was selected for further characterization steps as having high stability, low polydispersity, and an almost uniform shape.

To obtain a sufficient quantity of SeNP-1 for comprehensive characterization and establish the reproducibility of the SeNP-1 synthesis method, our biosynthesized nanoparticles, in contrast to some other research, exhibit a more ideal PDI and greater zeta potential, without any surface modification or extra chemical component addition [38–40]. The results of the DLS–ZETA test for the four stable NPs are shown in Table 3.

### 3.3.3 FTIR test

To confirm the synthesis of SeNP-1 and to identify the functional groups present on the NP’s surface, an FTIR test was carried out, and the result is shown in Figure 5(a).

Identification of functional groups on the surface of NPs is made possible by FTIR [41]. FTIR analysis of the SeNP-1 sample exhibits a significant peak at $3,450 \text{ cm}^{-1}$. This peak is connected to the stretching bonds of $\text{O–H}$, potentially arising from hydroxyl groups in alcohols, phenols, or carboxylic acids [42]. The peak at $1,633 \text{ cm}^{-1}$ could be attributed to either $\text{C=C}$ stretching in unsaturated carboxylic acids or $\text{C=O}$ stretching in amides. Additionally, the $1,392 \text{ cm}^{-1}$ band might be indicative of $\text{C–N}$ stretching vibrations in amines or nitro compounds [43]. While the FTIR analysis provided valuable insights into the presence of functional groups on the SeNPs, the definitive assignment of these groups requires further investigation. Even with our current understanding, the identified functional groups likely play a significant role in the biomedical applications of these SeNPs. For instance, the presence of hydroxyl groups could contribute to improved water dispersibility, facilitating their delivery within physiological environments. Additionally, these functional groups could serve as anchoring sites for biomolecules like drugs or targeting ligands, enhancing their potential for targeted drug delivery or specific cellular interactions. The possibility of protein adsorption onto the nanoparticle surfaces can further influence their stability in biological fluids and impact their interaction with eukaryotic cells. Depending on

<table>
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<th>Hydrodynamic radius (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
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<tbody>
<tr>
<td>SeNP-1</td>
<td>92.52</td>
<td>$-32.2$</td>
</tr>
<tr>
<td>SeNP-2</td>
<td>122.9</td>
<td>$-11.5$</td>
</tr>
<tr>
<td>SeNP-3</td>
<td>126.2</td>
<td>$-14.5$</td>
</tr>
<tr>
<td>SeNP-4</td>
<td>67.80</td>
<td>$-14.4$</td>
</tr>
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the adsorbed protein corona, the SeNPs could exhibit enhanced biocompatibility or altered targeting specificities.

Furthermore, the FTIR spectrum of sodium selenite, employed as a reference material, exhibits distinct and intense bands at 735 and 786 cm\(^{-1}\), corresponding to symmetric and asymmetric Se–O stretching vibrations, respectively [44]. Conversely, the SeNP-1 spectrum lacks these two distinct peaks. Instead, a broad and significantly weaker

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**Figure 5:** (a) FTIR spectrum of SeNP-1 and Na\(_2\)SeO\(_3\)·5H\(_2\)O salt, (b) and (c) XRD spectrum of SeNP-1(b) and Na\(_2\)SeO\(_3\)·5H\(_2\)O salt (c), (d) the percentage of viability of MCF-7 cells with different concentrations of SeNP-1 and sodium selenite salt treatment, and (e) radical scavenging activity of MRS and CFC (before and after NP synthesis).
band was observed at 614 cm\(^{-1}\) (400–875 cm\(^{-1}\) region). This stark difference suggests a substantially lower abundance of Se–O bonds in SeNP-1 compared to pure sodium selenite [15,45,46]. The FTIR analysis provides evidence supporting a reduction in Se–O bonds during the transformation of precursor materials to elemental SeNPs (Se\(^0\)) within the SeNP-1 sample.

### 3.3.4 XRD

The XRD analysis was employed to identify the kind and type of crystallinity of nanoparticles. In amorphous materials, the lattice planes are small but many. The amorphous nature of the nanoparticles is demonstrated by the continuous, wide, and jagged peaks. There is no discernible pattern in the XRD spectra of SeNP-1. The outcome of the XRD analysis of sodium selenite and SeNP-1 was in agreement with results from some other studies in which sodium selenite crystals were transformed into polymorph nanoparticles in the process of biosynthesis [15,36,39,47,48]. According to the result, the sodium selenite salt’s crystalline form was converted into amorphous SeNPs during the biosynthesis process (Figure 5(b and c)).

### 3.3.5 MTT assay

The MTT assay was conducted to investigate the toxicity of SeNPs compared to sodium selenite salt. As shown in Figure 5(d), cell viability increased as salt, and SeNP-1 concentrations were lowered. Cell viability was significantly higher following exposure to SeNP-1 compared to the salt at equivalent concentrations. The calculated IC\(_{50}\) values corroborated this observation, with sodium selenite salt exhibiting a lower IC\(_{50}\) (67 \(\mu\)g·mL\(^{-1}\)) compared to various other biologically synthesized SeNPs. The IC\(_{50}\) values for pomegranate peel extract SeNPs (69.8 \(\mu\)g·mL\(^{-1}\)), SeNPs fabricated by endophytic fungal strain Penicillium verhagenii (283.8 \(\mu\)g·mL\(^{-1}\)), and biosynthesized SeNPs Utilizing Lactobacillus casei (>100 \(\mu\)g·mL\(^{-1}\)) were all higher. This suggests a potentially greater anticancer efficacy of SeNP-1. However, a chemically synthesized starch-stabilized SeNP displayed a significantly lower IC\(_{50}\) of 11.3 \(\mu\)g·mL\(^{-1}\). This value might be attributed to the inherent cytotoxicity associated with chemically synthesized nanoparticles, warranting further investigation to distinguish between true anticancer properties and general cytotoxicity.

Based on these results, the synthesized nanoparticles hold promise as candidates for both cancer treatment and drug delivery applications. Furthermore, their reduced toxicity compared to sodium selenite salt suggests their

<table>
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<th>Nanoparticle type</th>
<th>IC(_{50}) ((\mu)g·mL(^{-1})) against MCF-7</th>
<th>Synthesis method</th>
<th>Refs.</th>
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<tr>
<td>Pomegranate peel extract SeNPs</td>
<td>69.8</td>
<td>green synthesis</td>
<td>[53]</td>
</tr>
<tr>
<td>SeNPs fabricated by endophytic fungal strain Penicillium verhagenii</td>
<td>283.8</td>
<td>green synthesis</td>
<td>[54]</td>
</tr>
<tr>
<td>Starch-Stabilized SeNPs</td>
<td>11.3</td>
<td>chemically synthesis</td>
<td>[55]</td>
</tr>
<tr>
<td>Biosynthesized SeNPs utilizing Lactobacillus casei</td>
<td>higher than 100 (20% inhibition by 100 (\mu)g·mL(^{-1})) SeNPs)</td>
<td>green synthesis</td>
<td>[56]</td>
</tr>
<tr>
<td>Biosynthesized SeNPs by Limosilactobacillus fermentum</td>
<td>67</td>
<td>Green synthesis</td>
<td>This study</td>
</tr>
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</table>
proposed, including the involvement of specific enzymes, regenerative compounds, or cellular processes, the exact role of these factors is unclear [57]. In this work, we hypothesize that the primary driver of SeNP biosynthesis is the presence of reducing compounds released by the bacteria during growth in the culture medium. We propose that these reducing compounds within the CFC contribute to the formation of nanoparticles, and other protein components secreted into the culture medium may contribute to the stability of the synthesized nanoparticles. To investigate this hypothesis that the presence of reducing compounds in the synthesis solution has the main role in the biogenic production of NPs, the DPPH assay was performed [58]. As shown in Figure 5(e), the radical scavenging activity percentage of MRS and CFC was significantly higher than that of samples after synthesis. It is also evident that with increasing synthesis time, the radical scavenging activity of CFC decreases, indicating higher consumption of reducing compounds during the longer biogenic synthesis process. The results showed that the antioxidant ability and reducing power of the MRS culture medium before the growth of bacteria are outstanding. However, NP does not synthesize in a culture medium. Therefore, it is likely that the substances in CFC that originate from the bacterium Limosilactobacillus fermentum and contribute to SeNP production are more than just reducing substances. As shown in Figure 5(e), with the start and completion of the synthesis, the antioxidant activity of the CFC decreases significantly. Based on this information, it can be claimed that the presence of reducing compounds in MRS is necessary for nanoparticle synthesis but not sufficient. Also, a significant amount of reducing compounds is consumed during the synthesis process. Therefore, it can be justified that the synthesis process can depend on a specific type of reducing compound produced by bacteria during their growth process, or more likely on proteins and enzymes released by bacteria into CFC that can consume reducing compounds and facilitate electron transfer between reducing compounds and selenium ions. It is also important to note that all of the samples were analyzed both on the day they were prepared and on the last day of analyses (5 days after the beginning of NP synthesis), and there was no discernible change in the outcomes throughout this time. Further research is needed to confirm the exact mechanism, protein, and reducing compound that plays a role in the biosynthesis process.

4 Conclusion

In conclusion, this study successfully demonstrated a safe and environmentally friendly method for producing stable, low-toxicity SeNPs using the CFC of Limosilactobacillus fermentum bacteria. This method offers a promising alternative to traditional nanoparticle synthesis techniques that can be hazardous or generate toxic byproducts. The optimal conditions for SeNP synthesis were identified as 37°C, pH 7.4, and a sodium selenite concentration of 0.75 mM. Furthermore, the nanoparticles exhibited desirable physical–chemical properties, including uniform size and shape, high stability at neutral pH, and lower cellular toxicity compared to sodium selenite salt. Additionally, the study suggests a correlation between reaction temperature and nanoparticle formation rate. Notably, we achieved the desired size distribution and shape without the use of external size-limiting agents or complex protocols. A precise examination of various synthesis parameters and their precise control during nanoparticle fabrication resulted in the selection of a uniform size, shape, and stability of SeNP.

Our investigation further suggests the utilization of reducing agents present in the CFC during the synthesis process, potentially indicating an enzymatic mechanism. However, a comprehensive understanding of this biogenic synthesis necessitates the identification of specific reducing agents, proteins, enzymes, and other bacterial secretions present in the CFC. The promising properties of SeNPs, particularly their reduced cytotoxicity relative to sodium selenite salt, make them promising candidates for additional biomedical research and investigation, with potential for a range of biomedical uses. In general, SeNPs have shown potential in several diseases like rheumatoid arthritis, inflammatory bowel disease, asthma, liver diseases, and various autoimmune disorders like psoriasis, cancer, and diabetes. They can also act as drug carriers and supplements in animal and poultry feed. Understanding the exact mechanism of synthesis will enable us to optimize the production process and tailor SeNP properties for specific biomedical applications. Therefore, due to the promising characteristics of our biogenic nanoparticles, further research is warranted to explore the exact mechanism and compounds that play a role in synthesizing SeNPs and their potential in various medical fields.
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