



Synthesis of Selenium Nanoparticles Using Probiotic Bacteria *Lactobacillus acidophilus* and Their Enhanced Antimicrobial Activity Against Resistant Bacteria

Hammad Alam¹ · Nafeesa Khatoon¹ · Mohammad Aasif Khan² · Syed Akhtar Husain² · Muthupandian Saravanan³ · Meryam Sardar¹

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Abstract

In the present study, nontoxic selenium nanoparticles were synthesized extracellularly using probiotic bacteria *Lactobacillus acidophilus*. The synthesized *Lactobacillus acidophilus* mediated selenium nanoparticles (LA-SeNPs) show the surface plasmon resonance (SPR) at 385 nm. The hydrodynamic radius of LA-SeNPs was found to be 34.13 nm along with polydispersity index (0.28) and zeta potential (+ 37.86 mV). TEM shows that the average diameter of LA-SeNPs is 2–15 nm. FTIR suggest that extracellular proteins present in bacterial culture were responsible for reduction and stabilization of Se ions to LA-SeNPs. The antibacterial activity of synthesized nanoparticles was studied against five different sensitive and resistant bacterial strains. The MIC₉₀ for bacterial strains were in the range ± 1 to ± 10 $\mu\text{g/ml}$. The inhibition and degradation of bacterial biofilm were studied against all the tested strains. The synthesized nanoparticles were cyto-compatible against human HEK-293 normal cell lines shown by MTT assay.

Keywords *Lactobacillus acidophilus* · Selenium nanoparticles · Biocompatible · Biofilm · Antioxidant

Introduction

Resistant bacteria have become a major threat for human beings, so there is a need to develop a potent and cost-effective antibacterial drug. Since the decade's antibiotics are the preferred drugs for the treatment of bacterial infections due to their powerful outcomes and cost-effectiveness [1]. However, recent studies have proved that extensive use of antibiotics result in the emergence of resistance among bacteria. The antibiotics that are currently available inhibit bacterial growth either by (1) affecting bacterial DNA replication machinery,

(2) blocking cell wall synthesis and (3) inhibiting translational machinery [1]. Unfortunately, the bacteria have become resistance against all these three modes of action. The resistance mechanism includes modification of cellular components of bacteria, modification of drugs or expression of enzymes by bacteria which degrades and modifies the antibiotics [1–3]. So far, no bacterial resistance has been reported against nanomaterials; therefore, researchers are focusing on synthesizing new nanoparticles with efficient antibacterial properties. Among nanoparticles metal nanoparticles such as silver and gold are known to be promising antibacterial agents against both gram-positive and gram-negative bacteria, but the concerned have been raised on the toxicity profiling of these nanoparticles [4, 5]. Selenium nanoparticles are reported to have excellent antimicrobial properties and are less toxic to humans as compared to other metal nanoparticles [6, 7], therefore; research is required to synthesize selenium nanoparticles at large scale. Selenium nanoparticles can be synthesized by physical, chemical and biological methods [6]. Although physical and chemical methods lead to the synthesis of well-defined nanoparticles, biological methods are considered green and sustainable as

✉ Meryam Sardar
msardar@jmi.ac.in

¹ Enzyme Technology, Lab Department of Biosciences, Jamia Millia Islamia, New Delhi 110025, India

² Human Genetics Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi 110025, India

³ Department of Medical Microbiology and Immunology, Division of Biomedical Sciences, School of Medicine, College of Health Sciences, Mekelle University, 1871 Mekelle, Ethiopia

compared to the other two methods. Therefore, the need arises to identify the biomaterial, which is sustainable and can be employed for large scale production of selenium nanoparticles [6]. The microorganisms employ detoxification mechanism for reduction of selenites/selenates to nano selenium and are referred to as potential bio-factories for the synthesis of well-defined selenium nanoparticles [8]. However, the exact mechanism of synthesis of the nanoparticle by microbes is still not clearly understood, and the synthesis could be extracellular, intracellular or membrane-bound. Extracellular synthesis is advantageous over intracellular or membrane-bound as it is easier to extract the nanoparticles. Bao et al. uses nitrate-reducing bacterium *Bacillus oryzae* sp. for reduction of selenite into selenium nanoparticles intracellularly [9]. Tan et al. and Xu et al. reported the intracellular synthesis of selenium nanoparticles using aerobic bacteria *Comamonas testosteroni* S44, and it reduces Se (VI) using the enzymes of the sulfate-reducing pathway [10, 11]. *Vibrio natriegens* has been reported as a suitable bio-catalyst for bioremediation of selenite, electron microscopy, and X-ray studies demonstrated that *Vibrio natriegens* growing aerobically in selenite containing medium produced 100–400 nm size nanoparticles [12]. Recently, Xu et al. [13] investigated the synthesis of selenium nanoparticles by *Lactobacillus casei* ATCC393, they observed red selenium nanoparticles of size 50–80 nm accumulated in the bacterium intracellularly. So far, a number of microorganisms viz. *Pseudomonas putida* KT2440 [14], *Bacillus licheniformis* [15], *Enterococcus faecalis* [16] *Rhodococcus aetherivorans* BCP1 [17], *Bacillus subtilis* [18], *Bacillus cereus* [19], *Pseudomonas alcaliphila* [20] *Pantoea agglomerans* [21] and *Klebsiella pneumoniae* [22] etc. have been reported who helps in the reduction of selenium salt into nano selenium but most of them are not commonly available, and some are pathogenic like *Bacillus Subtilis*, *Bacillus cereus*, *Streptomyces minutiscleroticus*, *Pseudomonas alcaliphila*, *Pantoea agglomerans* and *Klebsiella pneumoniae*.

Hence, the present study focuses on the extracellular synthesis of selenium nanoparticles using a probiotic bacteria *Lactobacillus acidophilus*, a type of bacteria found in human intestines and have numerous health benefits. Further, these synthesized nanoparticles were elaborated for their antibacterial and antibiofilm activities against drug-resistant bacteria.

Materials and Methods

Materials

Lactobacillus acidophilus was isolated from curd purchased from mother dairy, Delhi and sodium selenite from

Alfa Aesar. The bacterial strains were obtained from the Microbial Type Culture Collection (IMTECH, Chandigarh), DMEM—Dulbecco's Modified Eagle Medium media and trypsin from Gibco. The cell culture media, MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) were obtained from Hi-media, India. All other chemicals used were of analytical grade.

Synthesis and Characterization of Selenium Nanoparticles

The *Lactobacillus acidophilus* was isolated from curd, using serial dilution method as described earlier [23]. The isolated bacteria were grown in 200 ml Luria broth at 37 °C, 180 rpm up to log phase. The grown culture was centrifuged at 6000 rpm for 5 min, the pellet was discarded, and the concentration of 15 mM was made by adding sodium selenite in the supernatant. The reaction mixture was incubated for 48 h at 37 °C. The sample was purified by centrifugation at 9000 rpm, followed by subsequent washing with distilled water. The purified selenium nanoparticles were characterized by using UV–Vis spectrophotometer, Dynamic light scattering (DLS) Transmission electron microscope (TEM), X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy was done.

UV–Vis Spectrophotometer

The reduction of Se ions by bacteria *Lactobacillus acidophilus* was monitored by UV–visible spectra of reaction mixture. The UV–visible spectroscopy was done using a Mecasys Optizen 3220UV spectrophotometer.

Dynamic Light Scattering (DLS)

DLS measurements for determining the average size and size distribution of the selenium nanoparticles (LA-SeNPs) were carried out using the spectroscatterer RiNA, GmbH class3B. The dried powder was dispersed in distilled water and all the analyses were done at 20 °C for ten cycles.

Transmission Electron Microscopy (TEM)

The TEM sample was prepared by placing a drop of sonicated powdered LA-SeNPs sample, in absolute ethanol for about 15 min on ultrasonicate (UP-500 Ultrasonic Processor), on a carbon-coated copper grid and dried in air for 1 h. TEM analysis was performed on a JEOL model JEM-2000FX instrument at an accelerating voltage of 200 kV.

X-ray Diffraction (XRD)

XRD analysis was performed by X'PertPro X-ray diffractometer (PANalytical B.V.) by using Cu-K α radiation and operating X-ray tube at 45 kV and 35 mA.

Fourier Transform Infrared (FTIR)

The spectrum of biosynthesized SeNPs and *Lactobacillus acidophilus* were done on a Perkin-Elmer FTIR spectroscopy using KBr pellets. Spectra of washed and purified selenium nanoparticles were recorded on a Perkin Elmer FTIR spectroscopy using KBr pellets. To obtain good signal-to-noise ratio, 32 scans of nano-selenium were taken in the range 500–4000 cm^{-1} and the resolution was kept as 4.0 cm^{-1} .

Antimicrobial Activity

Minimum Inhibitory Concentration (MIC)

The MIC value was determined on 96-well microdilution plates according to published protocols of NCCLS document M7-A3 [24].

Time-Kill Assay

Before the tests were performed, the bacterial cultures (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) were sub-cultured at least twice and grown for 24 h at 37 °C on Luria agar plates. The adjusted inoculum suspension of 5×10^6 CFU/ml was diluted to 1:10 ratio in media to give a final inoculum concentration of 5×10^5 CFU/ml. Each concentration of the LA-SeNPs was diluted to 1:10 ratio in media containing 5×10^5 CFU/ml. This procedure yielded an initial inoculum of 4.5×10^5 CFU/ml. The final concentrations of LA-SeNPs were $0.5 \times \text{MIC}$, $1 \times \text{MIC}$ and $2 \times \text{MIC}$ for each bacterial strain. Gentamycin (at MIC) was taken as positive control and bacterial cells without any treatment were taken as negative control. At different time intervals the aliquots of 100 μl were withdrawn, diluted and plated on Luria agar plates. The plates were incubated for 24 h at 37 °C and bacterial colonies formed were counted, the time-kill curve was plotted as log CFU/ml vs. time [25].

Effect of Selenium Nanoparticles on Preformed Biofilm

The biofilms of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were formed by inoculating 150 μl of cell

Table 1 Minimum inhibitory concentration for different bacterial strains in $\mu\text{g/ml}$

Strains	MIC ₉₀ ($\mu\text{g/ml}$)	
	LA-SeNPs	Gentamycin
<i>E. coli</i> (Gram negative)	9.4	2.0–3.0
<i>S. aureus</i> (Gram positive)	1.2	0.5–2.0
<i>B. subtilis</i> (Gram positive)	3.5	0.5–1.0
<i>K. pneumoniae</i> (Gram negative)	6.5	≥ 120
<i>P. aeruginosa</i> (Gram negative)	4	≥ 180

K. pneumoniae and *P. aeruginosa* are resistant against aminoglycosides, cephalosporins and carbapenems class of antibiotics

cultures (10^6 CFU/ml) in 96 well plates and incubated for 48 h (for mature biofilm) at 37 °C for each bacterial strain [26]. The media were removed, and formed biofilms were washed with PBS thrice for each case. Then the varied concentration of selenium nanoparticles (MIC/4, MIC/2,

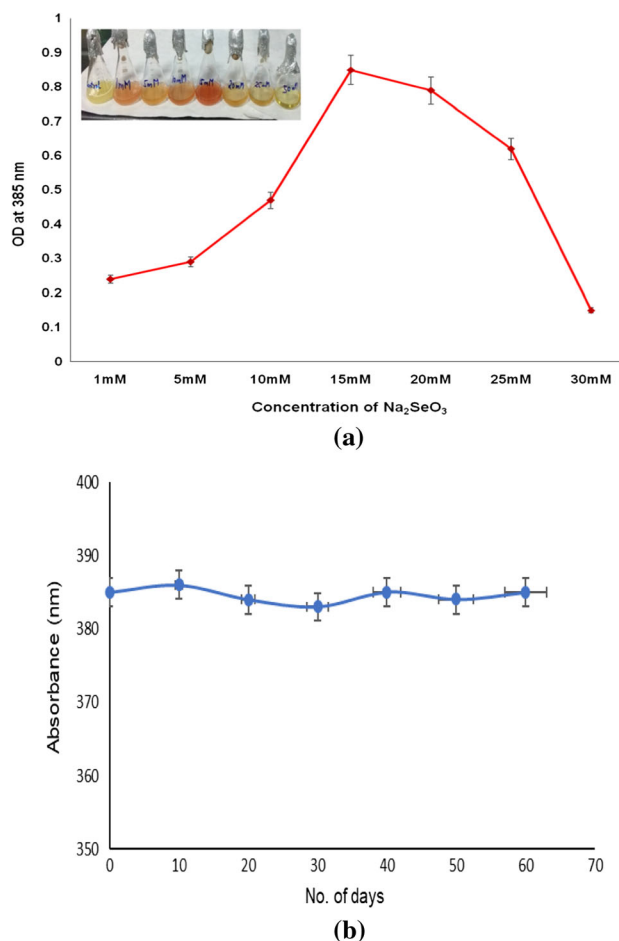


Fig. 1 **a** UV absorbance at 385 nm at different concentration of sodium selenite. Inset showing change in color during synthesis at different concentrations of sodium selenite. **b** Stability of LA-SeNPs by recording UV-Vis spectra at different time interval up-to 60 days at room temperature. The value represents the mean \pm SD of the experiment

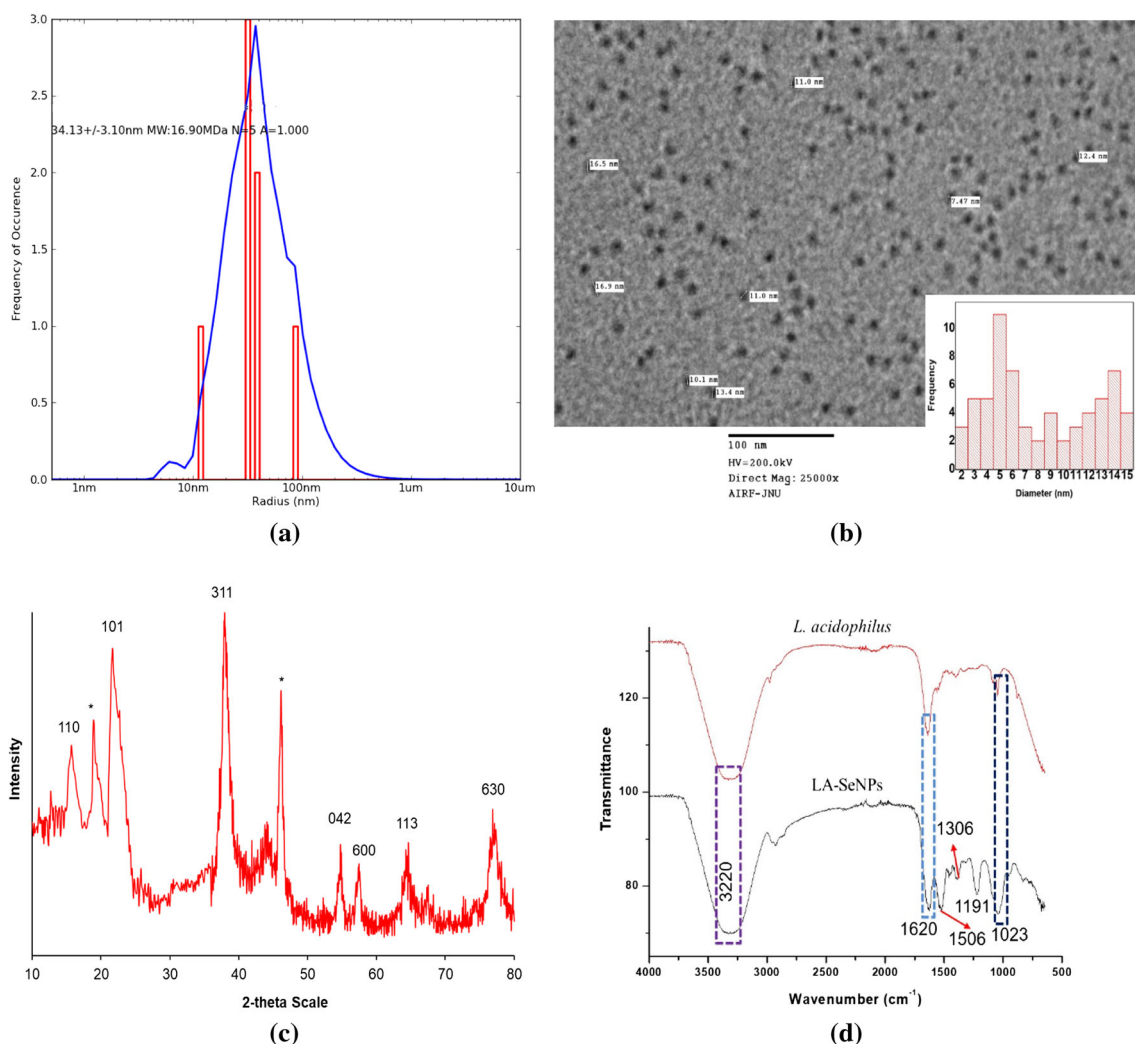


Fig. 2 **a** Dynamic light scattering of LA-SeNPs. **b** Transmission electron microscopy with particle size distribution, **c** X-ray diffraction pattern and **d** FTIR spectrum of LA-SeNPs and *L. acidophilus*

MIC, 2MIC and 4MIC) were added to each well, incubated at 37 °C. After 24 h, the wells were washed with PBS and 0.1% crystal violet solution was added to each well. After 5 min, crystal violet solution was removed, each well washed by PBS followed by the addition of 100 μ l of ethanol to solubilize the stain and absorbance were recorded at 595 nm [25].

The percentage of biofilm degradation was calculated as follows:

$$\text{Percentage biofilm degradation} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100.$$

Scanning Electron Microscopy of the Biofilm

For SEM, the bacterial cells (10^6 CFU/ml) were incubated in Luria broth in 6 well Petri plate having coverslips of

8 mm diameter at MIC concentration of LA-SeNPs for 24 h at 37 °C. The controls were grown without the presence of selenium nanoparticles. Biofilm formed over coverslips were washed with PBS, fixed and dehydrated after incubation. The samples were then air-dried, gold-coated, and viewed under a scanning electron microscope (Zeiss EVO 50, Carl Zeiss, Germany).

Reactive Oxygen Species Detection

The bacterial suspensions (selenium treated and control) were used to study two oxidative enzymes: superoxide dismutase (SOD) and catalase. The superoxide dismutase and catalase assays were performed according to the published protocol of Kuthan et al. and Visick and Clarke [27, 28], respectively.

Cytotoxicity Using MTT Assay

The cell lines HEK-293 (Human Embryonic Kidney) were seeded in triplicate in 96-well plate (150 μ l/well containing 2×10^4 cells in each well) and were kept in CO₂ incubator. After 24 h, cells were treated with various

concentrations of selenium nanoparticles (0–160 μ g suspended in nutrient media). The cytotoxicity was assessed by MTT assay as described previously [29]. Percentage cytotoxicity was calculated as a fraction of control (untreated) and the cytotoxicity was expressed in IC₅₀.

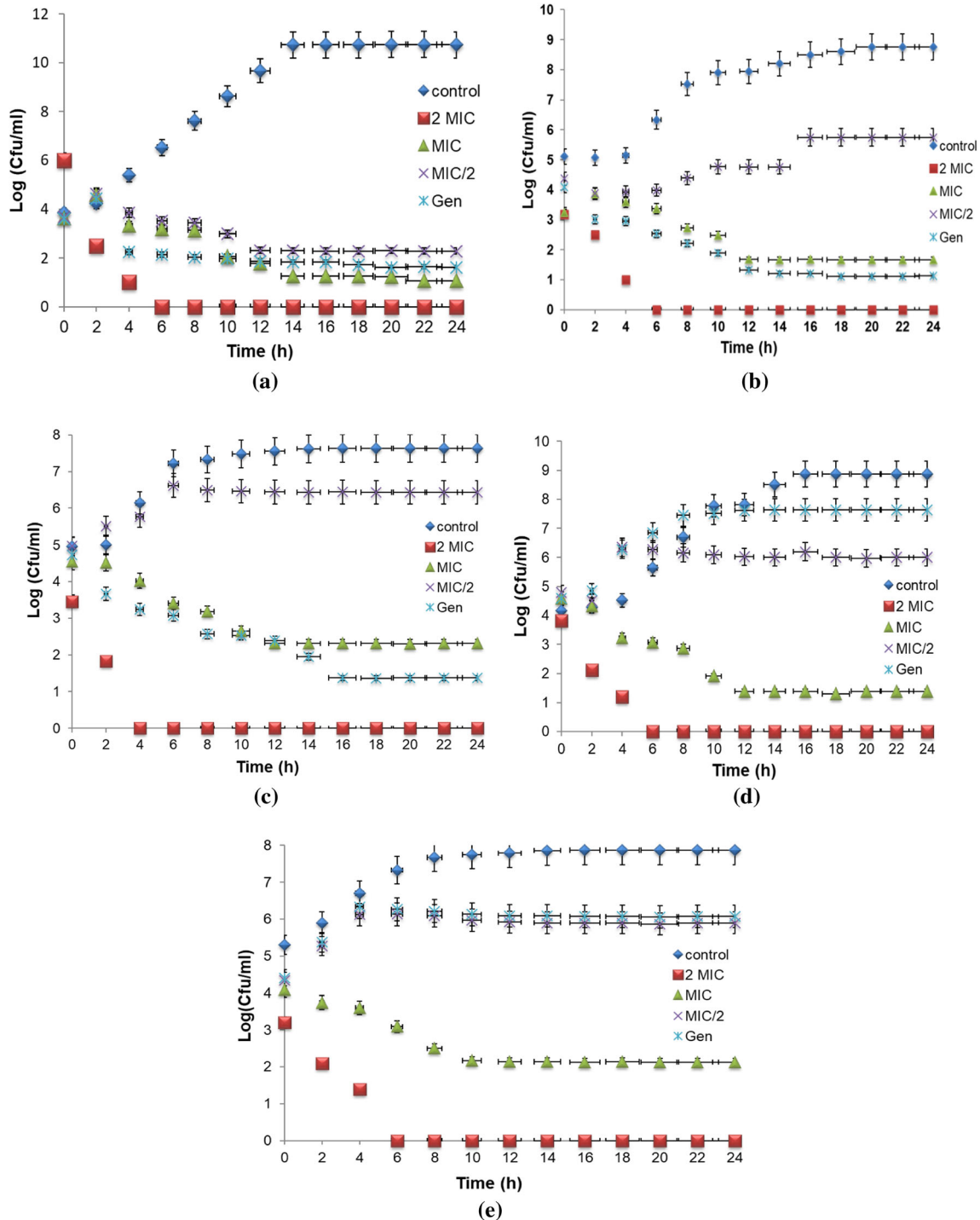


Fig. 3 Time kill assay performed against sensitive strains *E. coli* (a), *S. aureus* (b), *B. subtilis* (c), *P. aeruginosa* (d) and *K. pneumoniae* (e) cells treated with LA-SeNPs at concentrations of MIC/2, MIC and

at 2MIC. For all the strains gentamycin (at MIC) was used as a positive control. All the values are means of triplicate (n = 3) \pm SD. ANOVA significant at $P \leq 0.05$

Statistical Analysis

Analysis of variance (ANOVA) was performed, followed by Dunnett's test and values were represented as means of three replicates ($n = 3$) \pm SD. The significance level was maintained as P -value < 0.05 .

Results and Discussions

The tailoring and monitoring of selenium nanoparticles with low toxicity or biocompatible nature is acquiring pace. Microorganisms especially bacteria have been extensively explored for the synthesis of biocompatible nano-selenium [8, 30]. Still, research is required to identify the microorganism which can serve as the factory for the synthesis of nanomaterials of significance. Recently, Xu and his group describe the intracellular synthesis of nano-selenium using probiotic bacteria *Lactococcus lactis* NZ9000 and *lactobacillus casei* 393 [12, 30, 31]. They found that when these bacterial strains were subjected to sodium selenite salt stress, SeNPs of 50–80 nm size were accumulated in *lactobacillus casei* 393, whereas in case of *Lactococcus lactis* NZ9000 SeNPs of 38–152 nm were seen distributed within the bacterial cell [13, 31, 32]. Purification/extraction of nanoparticles synthesized intracellularly is a tedious process. Therefore, in the present study, selenium nanoparticles were synthesized extracellularly using culture extract of probiotic bacteria *Lactobacillus acidophilus*. The bacteria found in intestine confers various health benefits, reduces cholesterol, diarrhea and boost the immune system [33, 34]. When 200 ml of bacterial culture extract was incubated with sodium selenite (15 mM) for 48 h, the mixture turned reddish-brown having surface plasmon resonance at 385 nm as observed by UV visible spectroscopy. The synthesis was optimized at different growth phases of bacteria, that is lag phase of 6 h, log phase of 16 h, and the stationary phase. The maximum synthesis was achieved in the extracellular extract of log-phase, which indicates optimum concentrations of reducing agents were presents in log phase. Further concentration of sodium selenite was also optimized using UV-Vis spectroscopy, and the best synthesis was observed at the concentration of 15 mM shown in Fig. 1a. The results indicate that the concentration of salt is an important parameter and needs to be optimized; the results are in accordance of earlier published results [35]. The stability of LA-SeNPs was monitored by keeping them at room temperature for 2 months in solution phase, UV-Vis spectra were recorded, and no change in SPR was observed indicating that nanoparticles were stable (Fig. 1b). The hydrodynamic radius of LA-SeNPs was measured by using

dynamic light scattering (DLS) and was found to be 34.13 nm (Fig. 2a). The polydispersity index (PDI) was determined to know the particle size distribution of the selenium nanoparticles and PDI was found to be 0.28. The stability of the colloidal solution was determined by zeta potentials and was found to be + 37.86 mV, which indicate the long-term stability of the colloidal solutions. The morphology of LA-SeNPs was studied by using TEM; the TEM micrograph shows that the nanoparticles formed are spherical in shape having the average diameter in the range of 2–15 nm (Fig. 2b). The present study shows that the nanoparticles formed using culture extract of *Lactobacillus acidophilus* are small compared to the previous report of Xu and his group [13, 31, 32]. Size play an important role in governing the applications of nanoparticles, small size nanoparticles are considered better for biological activities. XRD was done to study the crystalline nature of synthesized nanoparticles, which is accordance with JCPDS#891617 (Fig. 2c).

To study the biomolecules responsible for the reductions and stabilization of selenium ions into nanoparticles FTIR spectroscopy was performed. The FTIR spectrum of selenium nanoparticles (Fig. 2d) shows the prominent peaks at 3220, 1620, 1506, 1306, 1191 and 1023 cm^{-1} whereas *L. acidophilus* shows a peak at 3220, 1620 and 1023 cm^{-1} . The peak 3220 corresponds to –OH bond due to the presence of phenolic compounds, 1620 and 1506 bands are attributed to amide I and amide II bond rotation respectively. The peak 1306 is due to amide III band of proteins, 1191 and 1023 may be due to C–C bond rotation [36]. The FTIR results indicate that the proteins present in the bacterial extract are mainly responsible for the synthesis of LA-SeNPs. Xu et al. [11] performs the proteomics of selenium nanoparticles synthesized using *Comamonas testosterone* S44 and found that proteins enriched with essential amino acids are significant factors that govern the synthesis.

Selenium nanoparticles are widely explored for their anticancerous properties but at present, very few reports are available on antibacterial and antibiofilm properties of biologically synthesized selenium nanoparticles against

Table 2 Reactive oxygen species (ROS) detection of superoxide dismutase (SOD) and catalase

Strains	SOD ($\mu\text{U/ml}$)		CATALASE ($\mu\text{U/ml}$)	
	Control	Treated	Control	Treated
<i>E. coli</i>	2.2 ± 0.1	53 ± 0.2	1.9 ± 0.2	41.5 ± 0.3
<i>S. aureus</i>	3.3 ± 0.2	38.9 ± 0.2	3.2 ± 0.2	42.1 ± 0.2
<i>B. subtilis</i>	3.6 ± 0.2	42.7 ± 0.3	3.8 ± 0.1	53.3 ± 0.2
<i>P. aeruginosa</i>	1.8 ± 0.1	64 ± 0.2	4.8 ± 0.3	62.4 ± 0.1
<i>K. pneumonia</i>	2.6 ± 0.5	44 ± 0.2	2.6 ± 0.2	58.4 ± 0.4

drug-resistant bacteria [37]. Huang et al. reported the synergistic effect of chemically synthesized selenium nanoparticles with quercetin against superbugs having MIC value of 32, 24, 8 and 24 µg/ml for *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumonia* respectively [38]. In the present study the MIC of LA-SeNPs against drug-sensitive strains were in the range of 1–10 µg/ml whereas in the case

of drug-resistant bacteria MIC was found to be 6.5 µg/ml for *K. pneumonia* and 4 µg/ml *P. aeruginosa* respectively (Table 1). Thus, MIC of LA-SeNPs alone without any drug observed in this study is approximately 4–5 times lesser than reported by Huang et al. [38]. The *K. pneumonia* and *P. aeruginosa* are drug-resistant strains that are resistant to the different classes of antibiotics,

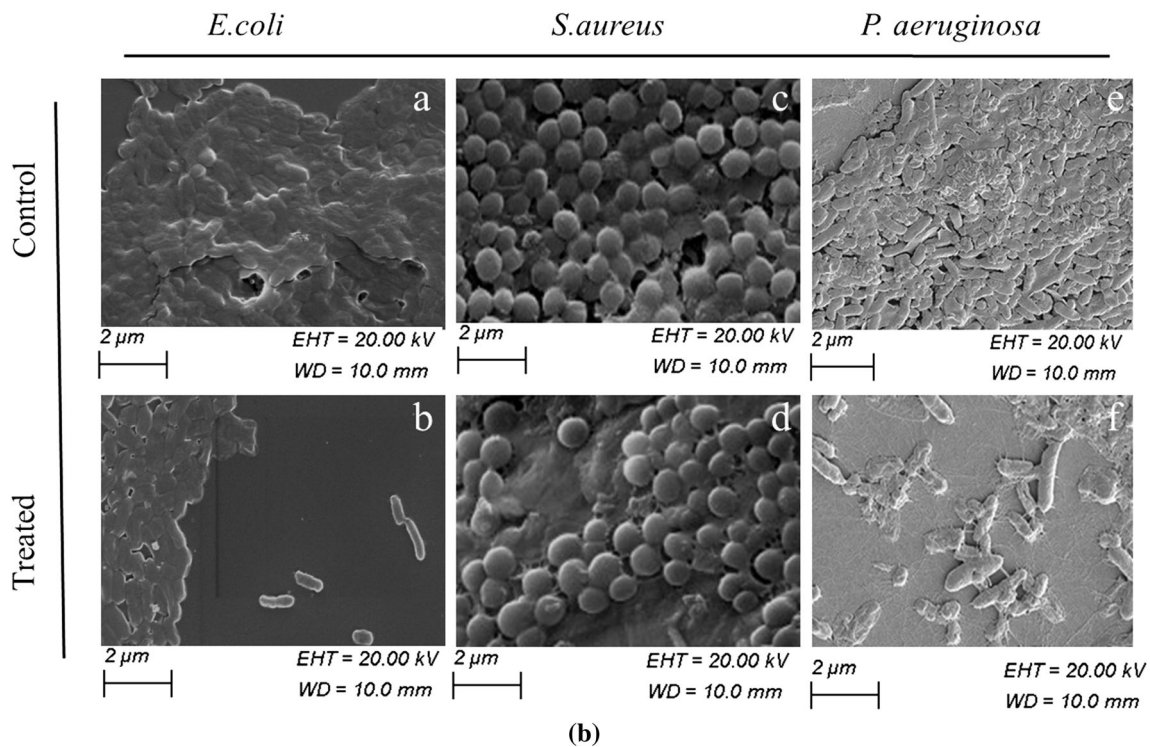
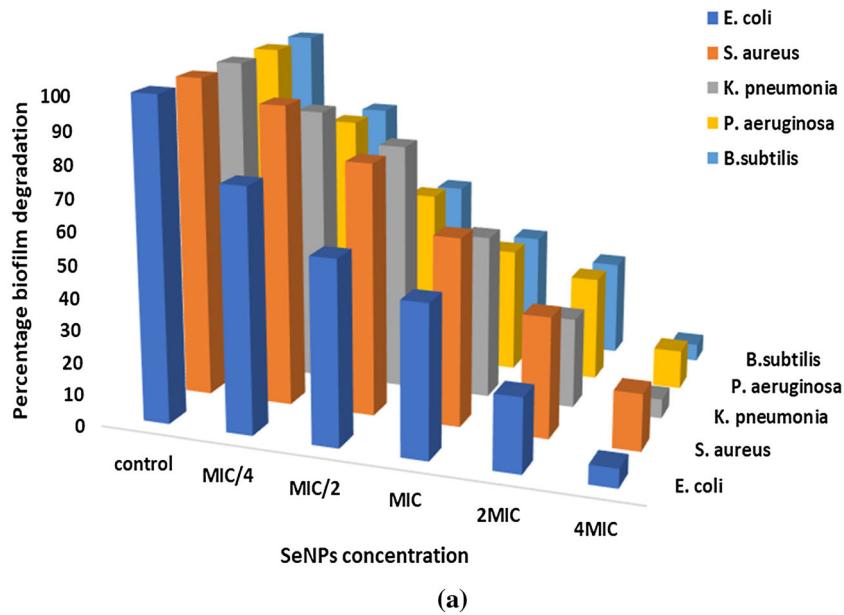


Fig. 4 a Biofilm degradation against different bacterial strains treated with LA-SeNPs at MIC/4, MIC/2, MIC, 2MIC and 4MIC concentrations. **b** SEM images of *E. coli*, *S. aureus* and *P. aeruginosa* with LA-

SeNPs at MIC concentration. All the values are means of triplicate (n = 3) ± SD. ANOVA significant at P ≤ 0.05

cephalosporins, aminoglycosides and carbapenems. The difference in MIC value for each strain may be due to the difference in composition of the bacterial cell wall. One can generalize that a good antibacterial agent is the one which interact strongly with the bacterial cell wall. The electrostatic interactions play important role in adhesion of nanoparticles to bacterial cell wall i.e. higher surface interactions led to more cell death. The different cell surface features of different types of bacteria led to different kinds of binding behaviors with the nanoparticles hence different MIC values.

The time-kill assay of selenium nanoparticles was performed at 2MIC, MIC and sub MIC concentrations (Fig. 3). The results show that complete inhibition of growth after 4–6 h at MIC concentration in all the tested strains, whereas complete killing was observed after 6 h at 2MIC concentration. At a concentration of MIC, the killing or inhibition activity for the sensitive strains was almost comparable to the conventional drug gentamycin (Fig. 3a–c), but in the resistance strains, LA-SeNPs was more effective as compared to gentamycin. Figure 3d, e clearly indicate the log CFU values of gentamycin at MIC in *K. pneumoniae* and *P. aeruginosa* was near about control.

The exact mechanism for the antibacterial activity of selenium nanoparticles still needs to be evaluated, but the previous studies suggested that the oxidative stress may be one of the reasons responsible for the control of bacterial growth [28]. The oxidative enzymes are a marker of a generation of reactive oxygen species (ROS) even under the mild stress conditions, superoxide dismutase (SOD) and catalase enzyme activity in treated and control bacterial cultures were determined. The results clearly indicate the presence of nanoparticles increases both the enzyme activities in all bacterial strains selected for study, which suggested the generation of ROS and thus leading to antibacterial action (Table 2). Increase in oxidative enzymes after treatment with selenium nanoparticles has been observed by several investigators [39, 40].

Further, degradation of preformed biofilms by selenium nanoparticles was studied. Figure 4a shows the UV spectrophotometric analysis of biofilm degradation in the presence of selenium nanoparticles at different concentrations of MIC/4, MIC/2, MIC, 2MIC and 4MIC. As the concentration of selenium nanoparticles increases degradation of biofilm increases. These nanoparticles are more effective against *E. coli*, *S. aureus* and *P. aeruginosa* as compared to *B. subtilis* and *K. pneumoniae*. Future work needs to be done to study their mode of action or mechanism of biofilm degradation. The biofilm degradation of three strains was further analyzed using electron microscopy (Fig. 4b), the SEM micrograph clearly shows the reduction of bacterial cells with degraded biofilm as compared to control which shows a higher number of bacteria

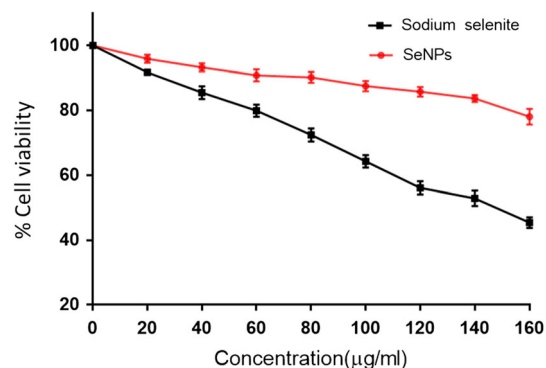


Fig. 5 a Growth inhibition of LA-SeNPs on cancer cell lines (HEK-293). All the values represent the mean \pm SD of the experiment, performed in triplicate

with dense biofilms. Toxicity is the major concern when using nanoparticles for biomedical applications. For this MTT assay was performed to confirm the toxicity of LA-SeNPs against normal human embryonic kidney cell lines (HEK-293) (Fig. 5). The result indicates LA-SeNPs are biocompatible up to two times of MIC value. The above study suggested LA-SeNPs can be used as a potent antibacterial agent. They can be used as coating agents on biomedical equipment like catheters as they inhibit biofilm formation.

Conclusions

The selenium nanoparticles synthesized using a “friendly” bacteria *Lactobacillus acidophilus* has potent antibacterial properties as they are effective against drug-resistant bacteria. The extracellular proteins present in the bacterial culture extract are mainly responsible for the synthesis of selenium nanoparticles. The bacteria can be found in fermented foods and access to culture. Therefore, the synthesis protocol of selenium nanoparticles can be easily scaled up. The nanoparticles formed were stable, biocompatible and had applications in medicine. The synthesized nanoparticles can significantly degrade the preformed bacterial biofilm that enhances the application of these selenium nanoparticles in various fields.

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