Green synthesis of less toxic Selenium nanoparticles: Their antibacterial, antioxidant and catalytic activity

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ABSTRACT

Introduction and Aim: Biosynthesis of metal nanoparticles is an important branch of nanobiotechnology. In recent years, microbial biosynthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness.

Materials and Methods: In this study, Selenium nanoparticles (Se NPs) were synthesized by using the fungi *Fusarium sp.* isolated from agricultural soil. Mycosynthesized Se NPs were characterized by UV-Vis spectrometer and Dynamic Light Scattering (DLS) analysis. Further biosynthesized Se NPs screened for different biological activities such as antibacterial, antioxidant, hemolysis, cytotoxicity and catalytic activity.

Results: Synthesis Se NPs was preliminary observed by a color change from pale yellow to orange red color and confirmed by UV peak at 342 nm. The DLS result shows the particles size ranges from 19 to 43 nm.The obtained result showed that the synthesized Se NPs possess good antibacterial activity against human pathogens such as *E. coli, Staphylococcus aureus, Salmonella typhi* and *Klebsiella pneumonia*. Further biogenic Se NPs showed less cytotoxicity on yeast cells and slight hemolysis.

Conclusion: The synthesized Se NPs have good antioxidant property and antibacterial activity with less haemolytic property these properties lead to use of Se NPs in different biomedical applications. This Se NPs Also showed an efficient catalytic activity by degrading hazardous dyes such as methylene blue and bromothymol blue.

Keywords: Green synthesis; Selenium nanoparticles; mycosynthesis; DLS; antibacterial activity.

INTRODUCTION

Nanotechnology is one of the emerging and promising fields of research, which generating new avenues and applications in medicine. The nanotechnology used in medical application is commonly known as "nanomedicine"; attempt to provide a new set of tools, devices and therapies for the treatment of human disease (1). The synthesis of nanoparticle using biological agents is an environment friendly and cost-effective alternative to physical and chemical methods. Use of bacteria, fungi and plant extracts for the synthesis of nanoparticles is slightly different leading to green chemistry which provides more advantages over physical and chemical methods as it is cost-effective and environment friendly (2). Many organisms required Selenium (Se) is an essential trace element and an important co-factor of antioxidant enzymes such as glutathione peroxidases and thioredoxin reductases (3).

Biologically synthesized nanoparticles are more stable for a longer time, show low toxicity, eco-friendly and cost-effective. Because of protein coating over Se NPs which do not aggregate, so it is stable for longer time (4). Properties of NPs depending upon their size and shape and many studies showed that size and shape of NPs could be controlled by optimization of physical and chemical parameters. A large number of studies suggest that selenium has a significant role in antioxidant protection, enhanced immune surveillance and modulation of cell proliferation and also reported to possess good antibacterial as well as antiviral activities (5). In biological synthesis, bacteria, fungi and plants are reported to synthesize Se NPs. Synthesis of nanoparticles by using fungi as reducing and stabilizing agent is known as mycosynthesis. Fungi are commonly used for the synthesis of metal nanoparticles. Synthesis of monodispersed nanoparticles which have well defined size and shape can be obtained by using different fungal strains. The fungal mediated green chemistry approach towards the fabrication of NPs has many advantages. This includes easy and simple scale up method, economic viability, easy downstream processing and biomass handling and recovery of large surface area with optimum growth of mycelia.

Previously reported that actinomycete mediated synthesized of Se NPs and their photocatalytic degradation of bromothymol blue using the purified Se NPs revealed 62.3% of dye removal under UV illumination (15 W) after 60 mins incubation of dye solution (6). There are only a few reports have described the fungal mediated synthesis of Se NPs. Extracellular production of monodispersed spherical Se NPs has been reported in *Aspergillus terreus* isolated from soil and *Alternaria alternate* isolated from leaf spot on *Stevia rebaudiana* (7).

The fungi *Fusarium sp*. already reported for the synthesis of other metal nanoparticles such as silver and gold nanoparticles. According to authors knowledge there are no reports on Se NPs synthesis by using *Fusarium sp.* The aim of this study was to isolate different fungi from an agricultural soil sample and screening for extra-cellular synthesize of Se NPs. The active fungal isolate was characterised by morphological and microscopic studies as *Fusarium sp.* And synthesised Se NPs characterised by visual observation, UV-spectroscopy and DLS analysis. Further, the synthesized Se NPs were studied for their different biological and environmental applications.

MATERIALS AND METHODS

Isolation and identification of fungi

Soil samples were collected from the agricultural field under sterile condition. Collected soil samples were serially diluted and known concentrations were poured into petri plates containing Sabouraud Dextrose Agar (SDA) media and incubated at 27 ^{0}C for 5 days. Isolated fungi were identified by observing colony morphology and under microscope i.e., under 10X and 40X using lactophenol cotton blue stain.

Preparation of fungal biomass for nanoparticles synthesis

Isolated fungi were separately cultured in 500 ml flask containing SDA broth medium at 27 ^{0}C and 150 rpm for 96 hours. After 96 hours of incubation fungal biomass was separated by filtration and centrifuged to get cell free supernatant at 10,000 rpm for 10 minutes at 4 ^{0}C in a cooling centrifuge. A volume of 250 ml of that cell free supernatant was taken in a sterilized flask, which was further used for the reduction of the Sodium selenite aqueous solution.

Synthesis of Se nanoparticles

Synthesis of Se NPs was optimized by using different concentrations 10, 25, 50 and 100 mM of sodium selenite (Na_2SeO_3). Cell free supernatant and Sodium selenite solution was mixed and kept for incubation at room temperature for 24 hours. Simultaneously, negative control of only sodium selenite solution without fungal biomass was maintained the under same experimental conditions. After obtaining the optimum substrate concentration, for the mass production of Se NPs, 200 ml of fungal supernatant mixed with 50 mM substrate and incubated at room temperature for 24 hours. Color change was observed in flask indicated the formation of Se nanoparticles. The synthesized Se NPs were separated by centrifugation at 14,000 rpm for 15 minutes at 4^{0}C. After centrifugation metal nanoparticle were collected from the bottom of the centrifuge tube. The collected nanoparticles were washed with sterile distilled water (three times) followed by alcohol wash to remove biological molecules and used for characterization and to study different biological properties.

Characterization of nanoparticles

Visual observation by a color change

After incubation, the synthesis of Se NPs was preliminarily observed by color change from pale yellow to orange red. The change in the color was routinely monitored visually, which would signify the bio-reduction of selenium ions and formation of Se NPs.

UV-Spectrophotometer

The formation of Se NPs was monitored by UV-visible spectrophotometer in the wavelength range of 200-800 nm. The colloidal Se NPs solution was added into a quartz cuvette and followed by spectral measurements.
Particle size analysis by DLS

Distribution and particle size of synthesized Se NPs was measured by DLS analysis (Microtrac, FLEX 11.0.0.2) in the range between 0.1 nm and 10.0 mm.

Biological properties of Se NPs

Hemolysis assay

Haemolytic property of biologically synthesised Se NPs was performed according to the earlier report [8]. In the present study, chicken blood was used to carry out the haemolytic assay of the Se NPs. The experimental sample was prepared by mixing 1 ml of Se NPs to 9 ml of 0.85% of sodium saline (NaCl). Blood sample of chick was collected with EDTA. Collected 20 ml blood sample was centrifuged at 3000 rpm for 5 min at 4 ℃ and washed thrice with 0.85% sodium saline. Prepared 1 ml of Se NPs sample with saline and mixed with 1 ml of RBC and incubated at room temperature for 2-3 hours. Distilled water with RBC used as positive control and without Se NPs with saline used as negative control and incubated under the same experimental conditions. After incubation, the final solution was centrifuged at 14,000 rpm for 5

minutes and supernatant reading was taken at 540 nm in UV-Vis spectrophotometer.

Cytotoxicity

To test the cytotoxic effect of synthesized Se NPs, yeast inoculum was prepared by using *Saccharomyces cerevisiae* which was inoculated into a 100 ml sterilized YEPD broth and incubated at 35° C for 24 hours and maintained as seeded broth. Again, seed broth of 0.5 ml was added to 2.5 ml of freshly prepared YEPD broth and treated with 1 ml of Se NPs and incubated at 37^0C for different time intervals (0, 2, 4, 8, 12 and 24 hours (9). Control was prepared by without adding Se NPs. The viability of yeast cells after treating with or without nanoparticles were counted using hemocytometer after staining with trypan blue dye. The viable cells were observed transparent, unstained and were counted against the dead cells which had taken the dye and appeared blue colored. The cell viability counts were performed for each time interval of incubation.

Antioxidant assay

DPPH free radical scavenging assay

The antioxidant activity of synthesized Se NPs was tested using the previously reported protocol (10). DPPH stock solution was prepared by mixing 0.004% (w/v) of DPPH (HIMEDIA) in methanol and preserved in opaque container and stored at 4 ^{0}C to prevent degradation of DPPH. 01 ml of DPPH (in methanol) was added to different volumes (0, 20, 40, 60, 80, 100 µl) of methanolic extract of Se NPs. The reaction mixtures were shaken and incubated in the dark for 30 minutes. The UV-vis absorbance at 517 nm was checked against a blank (methanol). Ascorbic acid was used as the standard. Decreasing the absorbance of reaction mixture indicate a high percentage of free radical scavenging activity. Purple coloured DPPH radical changes into a yellow coloured stable compound in the presence of an antioxidant and the reaction depends on hydrogen donating ability.
The percentage of inhibition or scavenging of free radicals was calculated as follows:

DPPH radical scavenging ability (%) = $[(A_c-A_s)/A_c] \times 100$
Where, A_s is absorbance of the sample, A_c is absorbance of the control solution.

Antimicrobial activity

Agar well diffusion method

Antibacterial activity of synthesized Se NPs was studied by agar well diffusion method (11). Nutrient agar plates were prepared and swabbed with pathogenic bacteria such as *E. coli, Salmonella typhi, Klebsiella pneumonia* and *S. aureus*. Wells were made using a 6 mm cork borer to which the different concentrations (25, 50, 75 and 100 µl) of Se NPs were loaded and incubated at 37 °C for 24 hours. After incubation, the plates were observed for a clear zone of inhibition around the wells.

Catalytic activity

The catalytic property of the Se NPs was studied using the probe reaction (12). This property was determined by degrading hazardous dyes such as Rhodamine and methylene blue. 1 mg of each dye was added to 100 ml of distilled water and used as stock solution. For experiment 1 ml of synthesized Se NPs with a known amount of $NaBH_4$ was added to the 10 ml of each dyes and incubated for 1 hour under room temperature. Degradation of dyes was observed based on the color changes during the incubation. Control was maintained under the same experimental conditions without adding Se NPs.

RESULTS

Biosynthesis of nanoparticles

In the present study, totally four morphologically different fungal colonies were obtained. All the four isolates were pure cultured and screened for the synthesis of Se NPs. One isolate was showed a positive result for the synthesis of Se NPs (Fig 1).

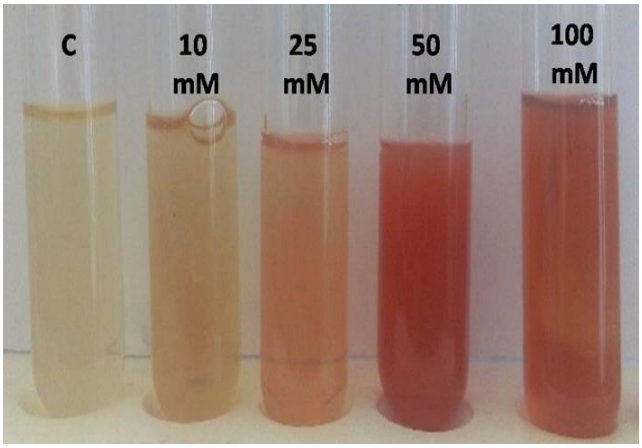


Fig. 1: Visual observation of biosynthesized Se NPs in different substrate concentrations

The active isolate was identified based on the morphological features and microscopic observation as *Fusarium sp*. It was further pure cultured and used for the synthesis of nanoparticles.

Characterization of nanoparticles

UV-Spectrophotometer

The formation of Se NPs was monitored on UV-spectrophotometer; absorption spectrum of selenium nanoparticle was showed around 342 nm (Fig. 2).

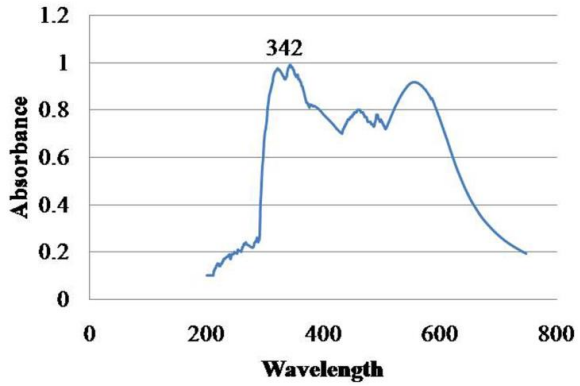


Fig. 2: UV-visible spectra of biogenic Se NPs shows peak at 342 nm indicated the formation of Se NPs.
This confirms the formation of Se NPs from the selected fungal extract.

Dynamic light scattering

The particles size and dispersity of synthesised Se NPs were studied by DLS analysis. Laser diffraction studies revealed that particle size obtained from the highly dispersed mixture was in the range of 19 to 43 nm and majority size of Se NPs was 25 nm (Fig 3).

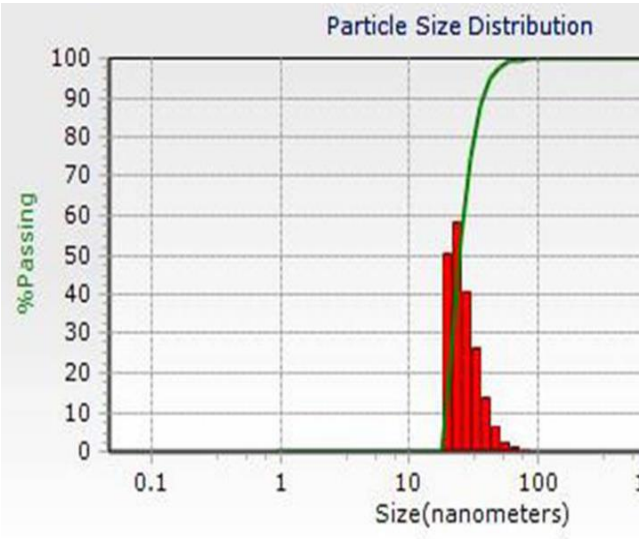
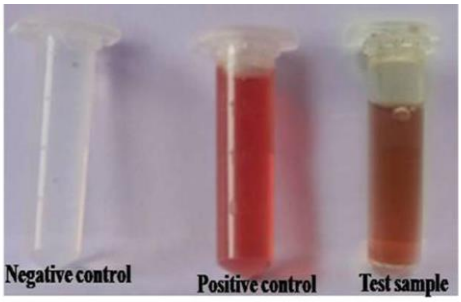


Fig. 3: DLS analysis for size distribution of biosynthesized Se NPs at optimized substrate concentration.

Biological Properties

Hemolysis: Future therapeutic applications of nanoparticles are based on oral or intravenous administration, experiments on their interaction with red blood components are of extreme importance. The *in-vitro* hemolytic assay is a screening tool for developing in vivo toxicity towards host cells. In the present study chick red blood cells checked for hemolysis by Se NPs (Fig. 4).



Sample	Content	O.D at 540 nm	% of Haemolysis
Negative control	Saline +RBC	00	00
Positive control	Distilled water +RBC	1.2	100
Test sample	Se NPs +RBC	0.21	17

Fig. 4: Haemolytic property of RBCs incubated with biogenic Se NPs.

Cytotoxic activity: Cytotoxic activity of Se NPs was done by using the yeast cells. The yeast cells in media and Se NPs was incubated in different time intervals 0, 2, 4, 8, 12 and 24 hours. After incubation of each time intervals the cells treated with trypan blue stain and observed for enumeration of dead and viable cells using hemocytometer. Fig. 5 and Table 1 show the percentage of cytotoxicity in different timeintervals.

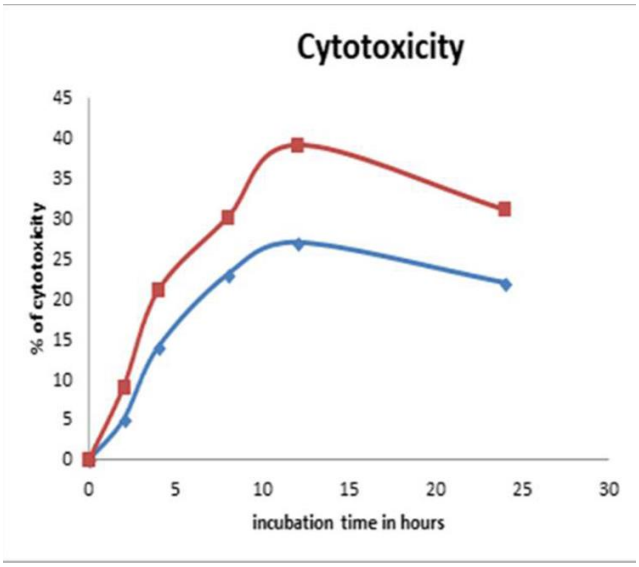
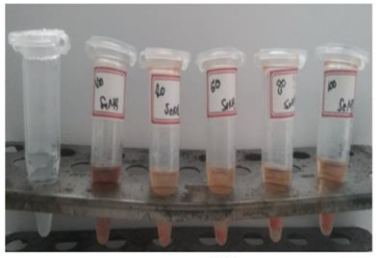


Fig. 5: Cytotoxic effect of Se NPs on yeast cells.

Table 1: Cytotoxic effect of biogenic Se NPs on yeast cells *Saccharomyces cerevisiae.*

Trial No	Incubation (Hrs)	Total cells in control		% of Toxicity	Total cells in Test sample		% of Toxicity
		Viable cells	Dead cells		Viable cells	Dead cells	
1	00	12	00	00	16	00	00
2	02	18	01	05	21	02	09
3	04	21	03	14	19	04	21
4	08	26	06	23	30	09	30
5	12	29	08	27	33	13	39
6	24	31	09	30	35	11	31

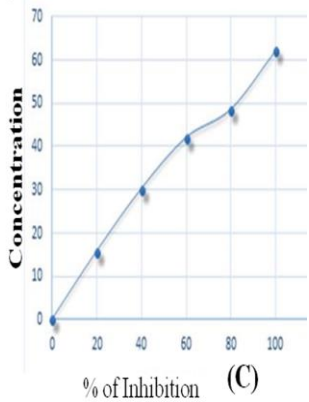
Antioxidant studies: The percentage inhibition of free radical scavenging activity of biogenic Se NPs was measured by DPPH radical scavenging assay. DPPH is a stable compound and showed a deep purple color with a maximum absorbance at 517 nm. It was reduced by accepting the hydrogen or electrons from antioxidant molecule coated on Se NPs. Percentage inhibitions of DPPH Free radical scavenging activity of Se NPs were presented in a graph (Fig. 6).



(A)

Concentrations	% of Inhibition
blank	00
20	15.78
40	30.20
60	42.00
80	48.50
100	62.00

(B)



(C)

Fig. 6: Antioxidant property of biogenic Se NPs (A) Visual observation (B) Percentage of free radical scavenging activity (C) Graphical representation of antioxidant property.

- **Antibacterial activity:** The synthesized nanoparticles were checked for their antimicrobial activity against selected bacterial pathogens shown in Fig 7. Biogenic Se NPs showed antibacterial activity against bacterial pathogens such as *Klebsiella pneumonia, E. coli, Salmonella typhi* and *S. aureus* (Fig. 7).

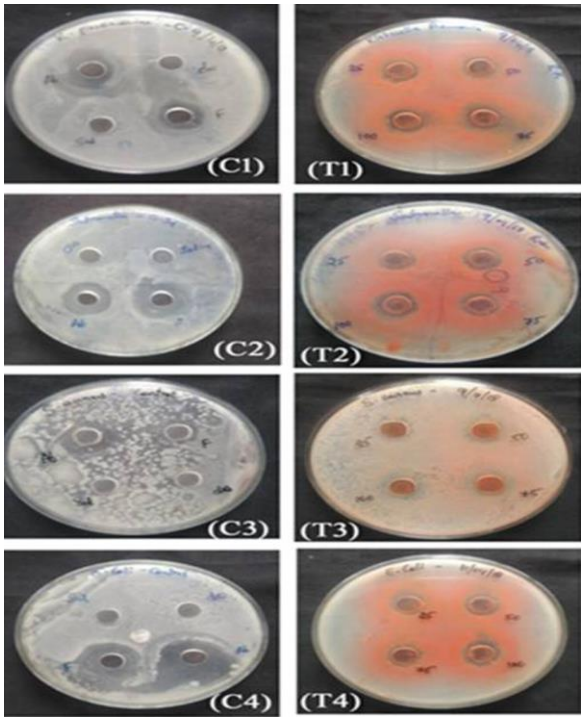


Fig. 7: Antibacterial activity of Se NPs on bacterial pathogens.

Catalytic activity: The two hazardous dyes namely Bromothymol blue (BB) and Methylene blue (MB) were selected in the present study. The degradation of dyes was checked by the color change. When Se NPs was added to Bromothymol blue dye it showed the color change after 1 hour of incubation and Methylene blue degradation was observed immediately after adding Se NPs to the reaction mixture as shown in Fig 8.

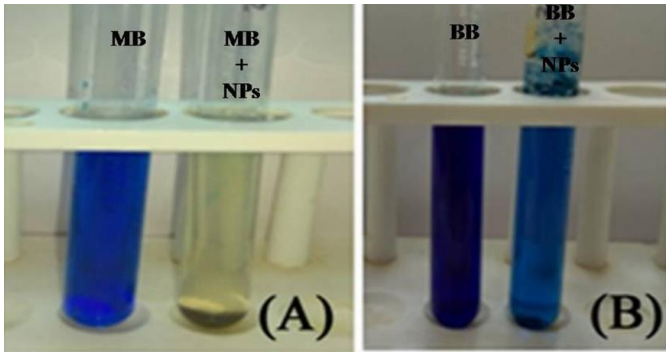


Fig. 8: Catalytic activity of Se NPs shows color change (A) Methylene blue and (B) Bromothymol blue.

DISCUSSION

All four fungal isolates were tested for the efficiency of Se NPs synthesis. One isolate was showed a positive result for Se NPs synthesis. Optimization studies for the synthesis of Se NPs based on the intensity of color formation by Se NPs, 50 mM of a substrate selected as an optimum concentration and further used for the mass production. The color change was observed in experimental tubes indicated the formation of Se nanoparticles (Fig.1).

After 24 hours of incubation, the high intense color change was observed in optimized substrate concentration. The color change was observed after incubation from pale yellow to orange red color which indicates the formation of Se NPs. In case of control without fungal biomolecules, no colour change was observed. In the Previous report, the formation of Se NPs was immediately visualized by a colour change from the colourless to orange red color (13).

Previous studies reported that, all the prepared nanoparticles were scanned from 200 nm- 600 nm. Strong UV-visible absorption peak between 320 nm to 550 nm was observed with a maximum at 390 nm. The intensity of the absorption spectra increased gradually indicating the amount of reduced Se nanoparticles increased in the solution of different concentrations (14).

Size distribution was analysed by DLS studies. Similar results were observed in the previous studies, Se NPs synthesised by *E. coli* were poly-dispersed, varied from 100 to 183 nm and the average size was about 155 nm (15).

The Se NPs showed a very low level of toxicity towards chicken red blood cells. The obtained result showed that 17% of hemolysis was observed at the tested concentrations, while positive control showed 100% Hemolysis. Previously reported the percentage of hemolysis by Se NPs was found around 18% (16).

To study the cytotoxicity of Se NPs appropriate volume of yeast culture containing a defined number of cells was incubated with nanoparticles for different time intervals and viable cells were counted against the dead cells that had taken the trypan blue dye. The obtained results showed that Se NPs shows less cytotoxicity towards yeast cells. The advantage of non-mammalian organisms particularly of yeast, as systems for anticancer drug screening, is considered a potential alternative to human models arose in the light of advances in genomic research. *Saccharomyces cerevisiae*, share similar signalling and growth regulatory pathways with humans. In this study, the yeast cells were treated with the nanoparticles.

Free radical scavenging activity was observed concentration dependent, as the concentrations of Se NPs increases the percentage of free radical scavenging activity was increased. The biogenic Se NPs scavenges reactive oxygen species (ROS), such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), this activity of nanoparticles is size dependent, where smaller Se NPs possess higher free radical scavenging potential (17).

In the antibacterial studies showed highest zone of inhibition against *Klebsiella pneumonia* and *Salmonella typhi* when compared to other pathogenic bacteria. In previous studies Se NPs displayed antibacterial effect against six food borne pathogens; *B. cereus, S. aureus, E. faecalis, E. coli, S. typhimurium, S. enteritidis* (18). Other studies show that Se NPs can inhibit both gram negative and gram-positive bacteria with equal efficacy. The Se NPs have the ability to disrupt microbial biofilms and confirms that the biogenic Se NPs as an alternative antibiofilm agent that can be used against food borne pathogens. Se NPs showed a considerable antibacterial activity against Gram positive bacteria, *Staphylococcus aureus* and *Staphylococcus epidermidis*, one of the main causative agents of orthopaedic infections (13). Se NPs have also been reported to possess antibacterial as well as antiviral activities (19). Additionally, a number of findings suggest that selenium plays an important role in a variety of physiological processes and selenium intake may be necessary for bone health.

Dye degradation result indicates that biogenic Se NPs having efficient catalytic activity towards the degradation of environmental hazardous dyes. Evaluation of the catalytic activity of biogenic Se NPs was reported by a few researchers in previous studies (20, 21).

CONCLUSION

Green synthesis of nanoparticles serves as an important alternative in the development of clean, nontoxic, economical and environmentally friendly procedures for the synthesis of Se NPs. In the present study, we have explored the synthesis of Se NPs by fungus *Fusarium* sp. as a reducing and stabilizing agent. The synthesized Se NPs have good antioxidant property and antibacterial activity with less haemolytic property these properties lead to use of Se NPs in different biomedical applications. Se NPs also showed an efficient catalytic property for the degradation of toxic dyes, these could be used in environmental applications.

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