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Bio-manufacturing of selenium nanoparticles by *Bacillus subtilis* isolated from Qarun Lake and evaluation their activity for water remediation

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ABSTRACT

Selenium nanoparticles (Se-NPS) have gently stimulated more extensive interest due to their vital properties. During this intensive investigation, we have to tend to restrict aquatic bacteria isolated from Qarun Lake for the inexperienced green synthesis of nanoselenium and its potential applications as antimicrobial for water purification. Twenty-four distinctive bacterial isolates were purified and screened for selenite resistance, and ten were positive. Out of them, isolate of Bats2 had been excellently tolerant of sodium selenite with minimal inhibitory concentrations (MIC) 18 g/l. They have continually been the best isolate in the manufacture of Se-NPS with maximal coloration and absorbance at 420 nm, in 3 days growth at pH 7 and 35 °C with 1g/l sodium selenite. The strongest extracellular biomanufacturing of Se-NPS has been properly identified as *Bacillus subtilis*. The facile fabricated Se-NPS by the acceptable and most powerful isolate *Bacillus subtilis* have been consistently characterized using UV-visible spectroscopy, XRD, SEM and TEM techniques. Biosynthesized Nanoparticles had diameter of 31-193 nm using and the typical XRD patterns sufficiently established the imprecise nature of the fabricated nanoparticles. The significant impact of Se-NPs was fastidiously observed on the potential growth of pathogenic Gram-positive and Gram-negative bacteria. Our desired outcomes absolutely confirmed the antimicrobial activity of Se-NPs only against *Staphylococcus aureus*. Thus, bacterial Se-NPs composite might realize appropriate application as a bioremediation tool of pathogens in required water.

Keywords: *Biosynthesis; Se-NPs; polyurethane; Bacillus subtilis; bioremediation.*

1. INTRODUCTION

Selenium is a trace element commonly found in materials of the earth's crust. Selenium exists in the environment in a variety of redox states as well as VI, IV, 0, -II and organic selenium species like methylated compounds, seleno amino acids and seleno proteins are common in biosphere [1,2]. Nano-selenium is widely used because of its distinctive optical, spectral and different properties [3,4]. In biological and medical fields, nano-selenium has great potential for practical applications. As an example, nano-selenium showed excellent antibacterial activity against *Staphylococcus aureus* compared with commercially available drug Ampicillin [5,6].

Most methods that are utilized in industry for the synthesis of selenium nanomaterials need complex mechanisms with very high temperatures and pressure that these processes are related to risks to the environment [7]. Recently, there are several reports concerning the synthesis of metal nanoparticles via "green chemistry" methodology (e.g., in bioorganic polymers or/and biological systems) through the event of a spread of size and morphology of nanoparticles [8, 9]. Some microorganisms like bacteria, fungi, and yeasts are ready to survive and grow within the designated concentrations of metal ions and reduce toxic ions into well-defined nanoscale particles [10, 11]. As an example, Natarajan *et al*. [12] have described the formation of selenium nanoparticles using *Escherichia coli*. Zare *et al*. [13] have reported an approach to synthesize spherical selenium particles using the fungus *Aspergillus terreus* in just throughout 60 min of the beginning of the reaction. Fesharaki *et al*. [14] have described a technique to recover Se-NPs made by *Klebsiella pneumonia*. Studies show that there are important variations in terms of the toxicity of Se-NPs and selenium oxyanions like selenite [15,16].

Selenite was able to stop growth and even cause liver toxicity, despite its antioxidant properties [17]. Therefore, Se-NPs with effective antioxidant properties and very low toxicity are effective to maintain the correct functioning of the body and health.

Resistance to antimicrobial drugs has become more widespread over the last decades resulting in a significant threat to public health. Infections caused by antibiotic-resistant bacteria need higher doses of drugs, additional toxic treatments and extended hospital stays, and ultimately result in increased mortality [18,19]. Despite the need for new antibiotics, only limited resources have been allocated by the pharmaceutical industry to support the discovery of new antibacterial agents, largely because the financial returns are likely to be small. To prevent or overcome antimicrobial resistance, non-antibiotic therapies will be necessary to treat bacterial infections and alternative strategies that show promise for the management of resistant infections are already under investigation [20,21]. Recent developments in nanotechnology allow the production of tailored metal/metalloid nanoparticles with physicochemical properties that can inhibit microorganisms. These nanoparticles have been shown to overcome existing drug

resistance mechanisms, including slow drug uptake and accelerated efflux and intracellular bacterial parasitism [22,23]. Thus, the objectives of this study were the inexperienced green synthesis of Se-NPs by aquatic bacteria isolated from Qarun Lake and the development of a bio nanocomposite sponge for the hope of instant removal of aqueous mercury.

2. MATERIALS AND METHODS

2.1. Isolation of nano-selenium fabricating bacteria.

Water samples had been collected from 12 different locations at Qarun Lake, Fayoum Governorate, Egypt. The samples were taken in sterile plastic bottles and transported to the laboratory in an icebox for bacteriological analysis [24]. These samples were taken the codes from Q1 to Q10, El-Batts and El-Wadi Drains according to their sites of collection from Qarun Lake. Isolation of bacterial strains resistant to selenite and has the capacity to convert it to nano-form from the water samples was carried out using enrichment isolation procedure. Mineral salt medium [25] and nutrient broth media were supplemented with sodium selenite (Na_2SeO_3) by a concentration of 0.1 %. Ten ml of water samples was added to 90 ml media and incubated at 30 °C for 48 h. The cultures had been added to plates of nutrient agar (NA) pH 7.0 supplemented with 0.5 g\l Na_2SeO_3 (Sigma, USA) as selenium supply. The selenite solution was added to the medium as a filter out-sterile solution after autoclaving the medium. Incubation was performed aerobically at 30 °C for 3 days. After that, the plates located microbial growth with red or orange zones were selected to the isolation step. Colonies rounded by cleared zone on agar plates were segregated on fresh selenite containing nutrient agar plate. Pure colonies, red or orange in color were selected for further study [2, 26].

2.2. Screening for extracellular biosynthesis of selenium nanoparticles (SeNPs).

In order to screen the efficiency of bacterial isolates for the synthesis of SeNPs, bacterial isolates have been inoculated into 250 ml Erlenmeyer flask contained 100 ml nutrient broth medium supplemented with 0.05 g/l selenite salt as inducer after which incubated at 30 °C for 48 h under shaking conditions (120 rpm). After incubation, cultures supernatants were obtained by centrifugation at 8000 rpm for 10 min followed by filtration with 0.24 µm disks. The harvested supernatants were transferred to clean and sterile flasks and mixed with an equal volume of sodium selenite solution (0.5 g/l) and again incubated at 30 °C under constant agitation at 150 rpm for 72 h. The flasks contained red or orange color had been cited as the positive reaction and uncolored flasks considered negative reaction. The bio-reduction of selenite was monitored with the aid of visual color changing and detection by using UV/VIS spectrophotometer (Genway 6800) [27]. Another way for screening based at the time of reduction, the efficient isolates were used for the manufacturing of nano-selenium at different incubation times (1:7 day) [28].

2.3. Identification of the most efficient isolates.

Based on the previous screening, the most powerful isolates in nano-selenium fabrication have been subjected to primary identification according to Bergey's Manual of Systematic Bacteriology [29]. These bacteria had been identified on the basis of morphological and biochemical characters. The results of morphological and biochemical identification of selected isolates were confirmed through molecular and phylogenetic methods [30]. The genomic DNA was extracted from the most potent producing isolates and the 16S rRNA gene from each become amplified by means of PCR using a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) as formerly described. The PCR products had been purified using a QIAquick PCR purification Kit (Qiagen, USA). The purified 16S rRNA fragments were analyzed via agarose gel electrophoresis and visualized using UV-transilluminator [8, 31].

The sequencing of the amplified 16S rRNA fragments was performed through a BigDyeR Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an Applied Biosystems 3730xl DNA Analyzer. Similarities of the bacterial nucleotide sequences with other recognized sequences were tested with the aid of comparisons with the National Center for Biotechnology Information (NCBI) database for reference and type strains using the BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree based on partial 16S rRNA sequences was constructed using the neighbor-joining method contained within the Clustal X program and MEGA6 software. The received sequences had been submitted to GenBank [32, 33].

2.4. Optimization of SeNPs bio-manufacturing using *Bacillus subtilis.*

Microbial synthesis of SeNPs via the most efficient isolated bacterial strain, *Bacillus subtilis*, had been investigated under different conditions. The bio reduction of selenium ions was evaluated at initial and final pH values (5 to 9) using 0.2M NaOH and 0.1M HCl. For initial pH value, the cultivation medium was prepared at different pH values (5 to 9) and inoculated by the examined strain, and the cell-free supernatant (after harvesting) was applied as a reducing source. In the case of final pH, the examined strain was cultivated into the medium at the best initial pH, and the supernatant was collected after growth. The cell-free supernatant was divided into samples and pH adjusted to 5-9. The bio reduction process was started by adding equal volume from treatment to 2 g/l selenite solution. Also, incubation temperature (20 - 45 °C) was applied twice. The first time is the bacterial growth temperature, another one as the bio reduction incubation temperature and the pH was 7.0 with incubation period for 72 h.

2.5. Determination of minimal inhibitory concentrations (MICs).

In order to determine MICs of the two strains, they had been grown in a nutrient agar medium supplemented with sodium selenite by gradually increasing concentrations till the strains did not develop on the plates. The bacterial strain growing on the first concentration was transferred to the higher one (2, 4, 6, 8, 10, 12, 14, 16, 18, 20 g/l) by streaking on the plates. MIC was noted when the isolates did not grow on plates, even after 48 h of incubation [34].

2.6. Production and purification of SeNPs by *Bacillus subtilis.*

In order to discover the best cultivation method for SeNPs production, the bacterial strain was cultivated into a nutrient broth medium as rapidly elaborated. One group of flasks contained

nutrient broth solely with no additives, another group contained nutrient broth supplemented by 0.5 g/l sodium selenite solution.

The inoculant was 1 ml of fresh bacterial suspension (0.5 McFarland turbidity) and additionally, the un-inoculated media were used as control. All flasks have been incubated at 30 °C for 24 h. The bacterial suspensions were centrifuged (15 min at 6000 rpm), the harvested supernatant was mixed with an equal volume of sodium selenite solution (1g/l) and incubated for an additional 24 h at 30 °C. Orange to red color was monitored within the experiments as an indicator of selenium nanoparticle production. In order to purify SeNPs, after centrifugation of red color solutions (10 min at 5000 rpm), the precipitate was washed with 0.9 % NaCl solution and so centrifuged at 5000 rpm for 10 min. Three consequent freezes and thaw (-20 and 40°C) were done. The final precipitate was dissolved in 5 ml distilled water and sonicated. Following centrifugation (5 min at 5000 rpm), the precipitate was washed three times with 1.5 M Tris-HCl (pH 8.3) containing 0.5 % sodium dodecyl sulfate (SDS) and re-centrifuged for 10 min at 8000 rpm. So as to get rid of the remaining SDS, the precipitate contained SeNPs was washed 3 times by distilled water and centrifuged at 8000 rpm for10 min. The precipitate was re-suspended in distilled water [35]. Four ml of prepared suspension was vigorously mixed with 2 ml of octanol and incubated for 24 h at 4 °C for dissociation of two phases. During this manner, nanoparticles had been collected in the organic phase and impurities have remained in the upper phase. The organic and aqueous phases have been slowly separated and discarded and additionally the remained nanoparticles were washed consequently with chloroform, absolute ethanol (Merck, Germany) and distilled water. These steps were once more to realize pure nanoparticles. The SeNPs were dried and preserved as lyophilized powdered form until used [10, 14].

2.7. Characterization the biosynthesised SeNPs by X-ray diffraction analysis (XRD).

The powdered, purified and red-colored SeNPs had been characterized for its structural properties by X-ray diffractometer (XRD), model Brukur advanced D8 Kristalloflex (Ni-filtered Cu K radiation; 1.5406 Å) according to the method elaborated by Abdelhameed et al. [36].

2.8. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX).

The morphology and topography of the synthesized SeNPs have been studied using Scanning Electron Microscope at the National Research Center (SEM, Quanta FEG 250, FEI, Republic of Czech). Also, the elemental composition of tested SeNPs was analyzed by Energy Dispersive X-Ray Spectrometer (EDS) "Phoenix" detector for integrative analysis.

2.9. Transmission Electron Microscopy (TEM).

The dimension and form of the synthesized SeNPs have been analyzed using transmission electron microscopy (JEOL, JEM 1400) at an accelerating voltage of 80 kV. Samples for TEM analysis have been prepared by drop-coating SeNPs solution onto carbon-coated copper grids. The films on the TEM grids have been allowed to stand for 2 min. The extra solution was removed employing a blotting paper and additionally the grid dried before measurement [37].

2.10. Evaluation of the antibacterial activity of the produced SeNPs.

The antibacterial effect of SeNPs was evaluated using well diffusion method according to the widespread techniques [6,38,39]. It utilized against common Gram-positive bacteria i.e., *Staphylococcus aureus* ATCC-47077 and *Bacillus cereus* ATCC-12228 and Gram-negative bacteria i.e., *E. Coli* ATCC-25922 and *Pseudomonas aeruginosa* PTCC-1074. Overnight culture of bacterial suspension was adjusted to 0.5 McFarland equivalents (1.5×10^8 CFU/ml). The dried surface of Muller–Hinton agar (MHA) was inoculated with the examined bacterial suspension by the usage of streaking using a swab and then 6 mm well was made and impregnated with 70 µl of SeNPs solution. The wells contained distilled water has been regarded as negative controls.

All plates had been incubated at 37℃ for 24 h. Erythromycin and Vancomycin have been performed as standard antibacterial agents against all pathogens. The zone of inhibition was measured. Experiments have been performed in triplicate and numbers reported on average [40].

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of SeNPs producing bacteria.

Selenium nanoparticles (SeNPs) are gaining significant in the field of medication owing to their antibacterial and anticancer properties. SeNPs are biocompatible and non-toxic in comparison to their counterparts, selenite (SeO_3^{-2}) and selenite (SeO_4^{-2}). Biogenic SeNPs are stable and do not aggregate owing to the natural coating of the biomolecules. Numerous hypotheses have been proposed to explain the mechanism of microbial synthesis of SeNPs. It is primarily a two-step reduction process from SeO_4^{-2} to SeO_3^{-2} to insoluble elemental selenium (SeO) catalyzed by selenate and selenite reductases. In the current research, the SeNPs have been manufactured by aquatic bacterial isolates. A total of 24 morphologically distinct isolates were isolated from the collected water samples. The isolates were selected based on the formation of red or orange halo zones on agar plates supplemented with selenite **Fig. 1**. Those isolates have been re-streaked on new agar plates supplemented with selenite for purification. The purified isolates have been screened for their potential for the extracellular formation of nano-selenium. The supernatant of ten bacterial isolates became had the ability to produce red color of Se-NPs. The codes of the positive isolates were Q3, Q5, Q7, Q8, Q9, Q10, Bats1, Bats2, Bats3 and Bats4.

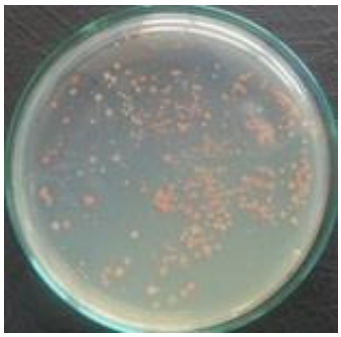

Figure 1. Red and orange color of various bacterial colonies on nutrient agar medium supplemented with sodium selenite.

The culture supernatants of bacterial strains after mixed with Na_2SeO_3 solution, the color of the solution becomes changed gradually from mild yellow to red by extraordinary bacterial

isolates **Fig. 2**. The appearance of the red color indicated the prevalence of Se-NPs formation in solution [28]. The feature of red color in the reaction solution was due to the excitation of the surface plasmon vibrations of the Se-NPs and provided a handle spectroscopic signature of their formation [41]. The Color changing in the reaction solution of various isolates was monitored each by means of visual inspection and spectrophotometric scanning using double beam UV–Vis spectrophotometer. The maximum absorbance of formed color after 72 h of incubation was recorded.

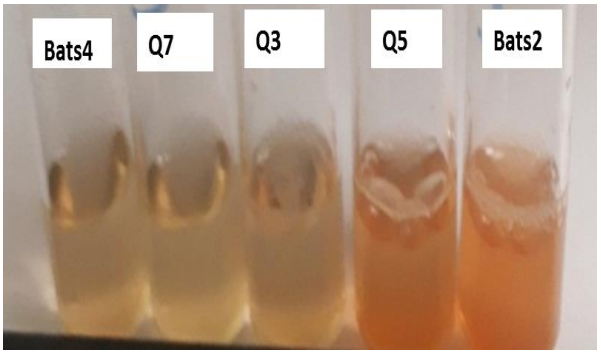

Figure 2. Red and orange color produced by various bacterial isolates supernatants after three days of incubation with sodium selenite solution.

The synthesis of SeNPs in solution was monitored through measuring the time dependent changes within the onset absorbance at an interval of 24, 48, 72, 96 and 120 h. The onset absorption was adjusted at 420 nm [42]. A time-dependent increase was seen in the absorption peaks of Se-NPs. No absorption peak similar to the control (supernatant without Na_2SeO_3) or Se ion solution. The absorbance of color formed by distinctive bacterial isolates at 420 nm with different incubation periods was illustrated in **Table 1**. Additionally, the symmetric plasmon band implies that the solution doesn't contain much of aggregated particles. From the data in **Table 1** it is proven that Bats 2 and Q5 isolates have been the best isolates in the manufacturing of nano selenium with higher absorbance 1.921 and 2.760 at 420 nm of wavelength, respectively. The spectrum of most potent isolate, Bats 2 was illustrated in **Figure 3**.

Table 1. Development of the color formation by special bacterial isolates measured at 420 nm.

Sample	Incubation period (days)				
	1	2	3	4	5
Q3	0.1029	0.1671	0.2218	0.2995	0.3928
Q5	0.7375	1.5682	2.0345	2.6144	2.7597
Q7	0.0843	0.3098	0.4133	1.1328	1.2026
Q8	0.0230	0.0647	0.1746	0.2525	0.3439
Q9	0.0864	0.1069	0.2793	0.5618	0.9621
Q10	0.0445	0.4176	0.4216	0.7441	1.1006
Bats 1	0.1373	0.4049	0.4957	0.7349	0.8354
Bats 2	0.1571	0.3497	0.4109	0.848	1.921
Bats 3	0.1420	0.3212	0.5165	0.6307	0.6652
Bats 4	0.0071	0.1515	0.2794	0.2794	0.5398

3.2. Identification of the most efficient isolate.

Many bacterial genera had been recorded for reduction of selenium ions to SeNPs like *Bacillus*, *Pseudomonas, Enterobacter, Microbacterium* and *Lactobacillus* [43]. In the present study, the bacterial isolate of Bats2 had been the most efficient bio-manufacture of SeNPs.

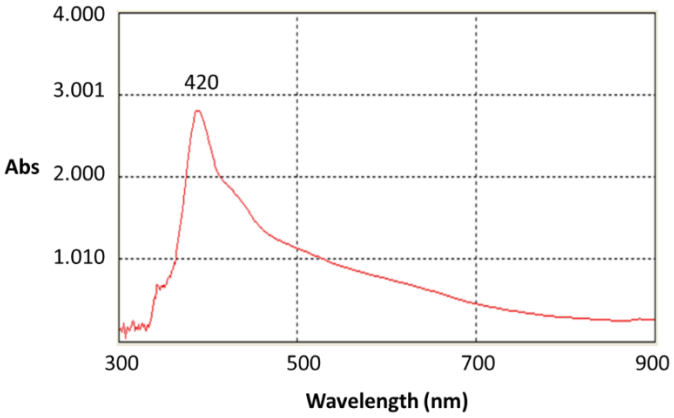

Figure 3. Spectrophotometric scanning of SeNPs produced by the Bats 2 bacterial isolate.

These isolates had been identified based on morphological and biochemical characteristics, as well as molecular finger prints. In the case of classical identifications, Bats 2 was rod in shape, motile, Gram positive, oxidase positive, catalase positive, citrate positive and positive of gelatin hydrolysis. For molecular identification of the chosen bacterial isolate, the genetic materials, DNA of this isolate was isolated, purified and the sequences data of amplified 16s rRNA have been analyzed and the bacterium was identified as *Bacillus subtilis*. The phylogenetic trees of two strains have been displayed in **Figure 4**.

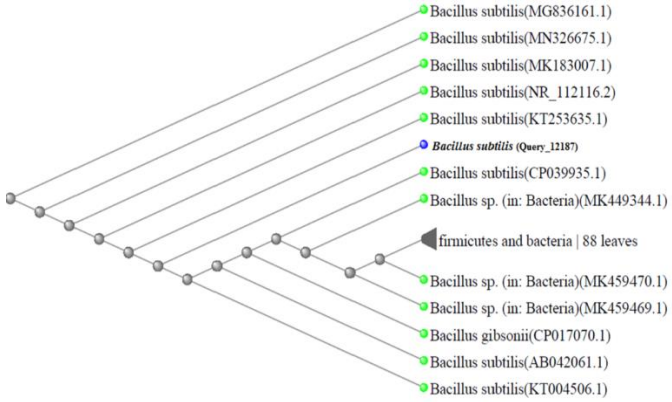

Figure 4. Phylogenetic tree based on the 16S rDNA genes of *Bacillus subtilis* (Bats 2).

3.3. Optimization of selenite concentration for SeNPs bio-production by *Bacillus subtilis.*

Five concentrations of Na_2SeO_3 were applied with the cell free supernatant separately. The purpose of this test was to determine the impact of quite a number of concentrations of selenite on the capacity of the tested strain for producing selenium nanoparticles. **Figure 5** symbolizes absorbance of produced nanoparticles by *Bacillus subtilis* at distinctive concentrations of Na_2SeO_3 after five days at 420 nm. Selenium nanoparticles levels produced by bacteria showed the highest absorption peak in the optimum time with 1g/l sodium selenite concentration, while the lowest peak was acquired at a concentration of 2 and 2.5g/l. These results may additionally reflect the fact that many bacteria can continue to exist in low concentrations of Na_2SeO_3 and accordingly will produce a larger amount of elemental selenium [44]. If compounds such as selenite produce hydrogen selenide (H_2Se) at high concentrations, H_2Se can react with oxygen to produce reactive oxygen species (ROS) toxic to cells [42]. Glutathione peroxidase is an enzyme that protects tissues from oxidative stress

and for that reason can block the action of destructive free radicals [45]. Increasing concentrations of sodium selenite enter a lot of injury to the genetic material of bacteria. Thus, large numbers of bacteria die due to the stress brought about through the presence of toxic inorganic compound in the environment and as a result decrease production of selenium nanoparticles [46]. In this regard, Ramoutar and Brumaghim [42] additionally showed that selenium dioxide (SeO_2) as Na_2SeO_3 can cause harmful results on bacterial plasmid DNA under H_2O_2 stress condition, however with greater power, while other compounds containing selenium such as Na_2SeO_4 and Na_2Se have any inhibitory effects on *Escherichia coli* plasmid DNA.

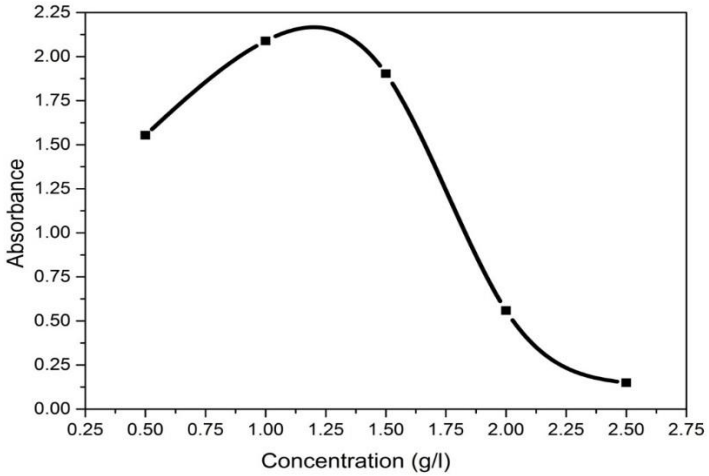

Figure 5. Absorbance of produced nanoparticles by *Bacillus subtilis* at different concentrations of Na_2SeO_3 after five days.

Table 2. Absorbance of the produced nanoparticles by *Bacillus subtilis* at different temperatures after three days at 420 nm.

Temperature	Absorbance
20	0.5014
25	0.6887
30	0.8343
35	1.3661
40	1.0872
45	0.825

Table 3. Absorbance of the produced nanoparticles by *Bacillus subtilis* at different pH after three days at 420 nm

pH	Absorbance
5	1.1538
6	1.0108
7	1.1554
8	0.9965
9	0.8382

3.4. Effect of different pH values and temperatures on production of SeNPs by *Bacillus subtilis.*

Five different pH values and temperatures have been experimentally tested for the production of nano selenium. It was found that the most manufacturing conditions of SeNPs based on coloration and absorbance at 420 was at pH 7 and 35 °C. These experiments had been repeated several times. **Table 2** represents the absorbance of produced nanoparticles by *Bacillus subtilis* at different temperatures after three days at 420 nm, while **Table 3** represents the absorbance of the produced nanoparticles by *Bacillus subtilis* at different pH after three days at 420 nm.

3.5. Determination of minimal inhibitory concentrations (MICs)

The minimum inhibitory concentration (MIC) is the minimal concentration of a metallic or metalloid in a given culture medium at an optimum condition below which bacterial growth is not inhibited. The MIC of the potent isolate Bats2 against sodium selenite was determined in solid media and ranged from 8-18 g/l. Bacterial isolate confirmed a very high degree of resistance to selenite with MIC value 18 g/l. It is interesting to observe that this strain could tolerate high concentrations of selenite. Although the MICs determined with the traditional media could not be related to the actual metal concentrations in the habitat from which bacteria had been isolated, this technique is nevertheless considered as a valid approach to evaluate the impact of heavy metals on microbial activity in polluted habitats [46].

3.6. Production and purification of SeNPs by *Bacillus subtilis.*

Bacillus subtilis when grown on nutrient agar or nutrient broth supplemented with sodium selenite for 24 h, the color of medium turned from yellow to red indication for production of nano selenium in the medium (**Fig. 6**). Also, the culture supernatant of *Bacillus subtilis* when challenged with Na_2SeO_3 solution, exhibited a change in color of the solution from light yellow to red. The appearance of the red color indicated the occurrence of the reduction reaction ensuring in the formation of elemental Se^0 in solution. The attribute red color of the reaction solution was due to excitation of the surface plasmon vibrations of the Se-NPs and provided a convenient spectroscopic signature of their formation [47].

Figure 6. Growth of *Bacillus subtilis* on nutrient broth media for 48 hr., (A) in the presence and (B) in absence of selenite. Liquid cultures confirmed that reduction to red elemental selenium occurred solely in the presence of selenite.

In order to purify selenium nanoparticles from cell debris, we used octanol, washed consequently with chloroform, absolute ethanol, and distilled water. These steps have been repeated again in order to gain pure nanoparticles. The final suspension was dried and stored at 4°C until used. Among the various forms of selenium, selenite reduction by microbes has been greater attention because of its high toxicity [9] and that is a serious environmental pollutant [48]. Red cell suspension formed by *E. faecalis* indicates that this bacterium is able to bioreduce toxic and colorless selenite to nontoxic and red metallic Se-NPs. Application of biological technology in latest years due to the fact of their simplicity, low cost and fast process have been made large development in the production of Se-NPs [49].

3.7. Characterization of the produced SeNPs by X-ray diffraction analysis (XRD).

The crystal structure of the produced nanoparticles used to be determined by using XRD **Fig.7**. The XRD pattern was noisy and broader with no sharp Bragg reflections. Thus, the data suggests the amorphous nature of the synthesized selenium nanoparticles, which is red in color. These results are in agreement with previous research carried on biological synthesis of selenium nanoparticles using bacterial strains *Bacillus* sp., *Pseudomonas alcaliphila* and *Capsicum annuum* extract [50,51].

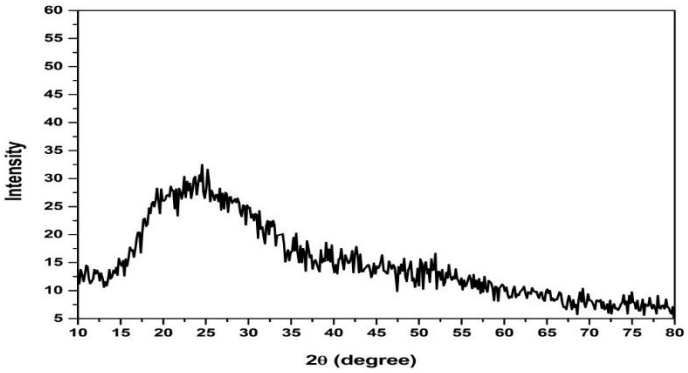

Figure 7. X-ray diffraction pattern of Se-NPs produced by *Bacillus subtilis.*

3.8. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX)

The formation of selenium nanoparticles by *Bacillus subtilis* was proven with SEM (**Fig. 8**). As shown in the electron micrograph, many spherical formed nanoparticles are surely seen.

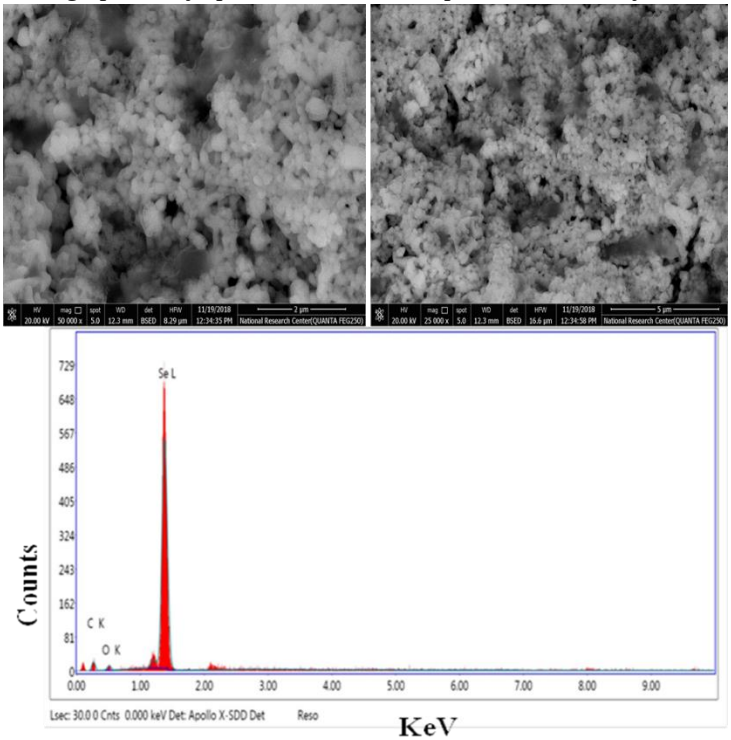

Figure 8. Electron microscope image and EDX spectra of Se-NPs produced by *Bacillus subtilis.*

Also, the presence of selenium in the nanoparticles was additionally established from the EDX spectrum of the bacteria. The samples show off strong absorption peaks at 1.37, 11.22 and 12.49 keV, corresponding to the characteristic absorption of SeL, SeK, and SeK, respectively (**Fig. 8**). Noted extra peaks of carbon and oxygen can be attributed to the protein molecules which are probably involved in the capping of the produced nanoparticles. These outcomes are in concurrence with the earlier characterization research carried out on selenium nanoparticles synthesized with *B. cereus*, *B. megaterium*, *B. mycoides* and *St. minutiscleroticus* [52,53].

3.9. Transmission Electron Microscopic (TEM).

The TEM images of selenium nanoparticles synthesized by *Bacillus subtilis* after 72 h of incubation are proven in **Fig. 9**. The produced nanoparticles were polydisperse, spherical in shape; measurement of the particles ranged from 31 to 193 nm and the average particle size obtained from the corresponding diameter distribution was about 95.9 ± 35.9 nm. These effects in concurrence with biologically produced selenium nanoparticles using bacterial strains *Shewanella* sp., *E. coli* and *Pantoea agglomerans* [53]. Selenium nanoparticles have been reported to be amorphous, a characteristic feature of elemental selenium produced by biological reduction [54]. Earlier studies carried out on bacterial mediated synthesis of selenium nanoparticles, using *Rhodopseudomonas palustris*, *Shewanella* sp., *Sulfurospirillum barnesii*, *B. selenitireducens*, *Selenihalanaerobacter shriftii* anaerobic conditions are required for the production of nanoparticles of 80-200, 100-250 and 200-400 nm, respectively.

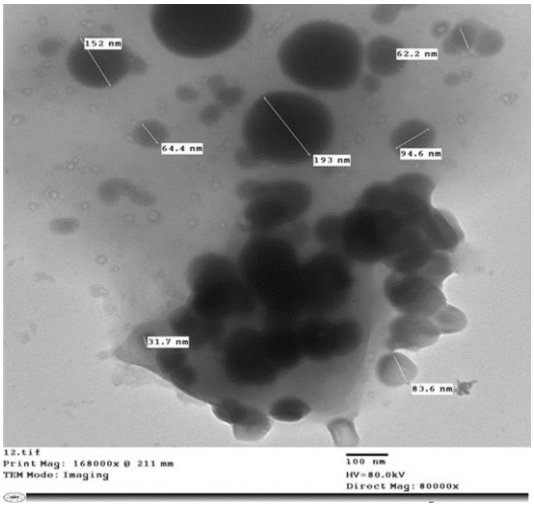

Figure 9. TEM image of Se-NPs produced by *Bacillus subtilis.*

Table 4. Average inhibition zone for Se-NPs

Bacteria	Inhibition zone (mm)			
	Test	Negative control	Positive control	
	Se-NPs	Water	VA	E
S. aureus	10	NA	14	16
B. cereus	NA	NA	15	19
E. coli	NA	NA	7	NA
P. aeruginosa	NA	NA	19	NA

NA = not appearing, VA = Vancomycin, E= Erythromycin.

3.10. Evaluation of antibacterial activity of the biosynthesised SeNPs.

The antibacterial activity of the Se-NPs against Gram-positive bacteria *Staphylococcus aureus* ATCC-47077, *Bacillus cereus* ATCC- 12228 and Gram-negative bacteria *Escherichia coli* ATCC- 25922, *Pseudomonas aeruginosa* PTCC-1074 was tested by well diffusion test. Antimicrobial activity of SeNPs was determined by measuring the growth inhibition zones around the well in mm according to [55]. A negative control disc was applied using distilled water only. Inhibition zone values represented as

averages in Table 4 and Fig. 10. However, effects showed the antimicrobial activity of Se-NPs solely towards *St. aureus*. This end result on the antibacterial impact of selenium on Staphylococcus confirms previous researches [56] and suggests that selenium may prevent bacteria from forming biofilms. In addition, Se-NPs have demonstrated a cytotoxic impact on *S. aureus* in live assays, so it was stated that Se-NPs prevented ventilator-associated pneumonia in rat dermal fibroblast cell lines [57]. However, the influence of Se-NPs on microorganisms, which include bacteria, is unknown.

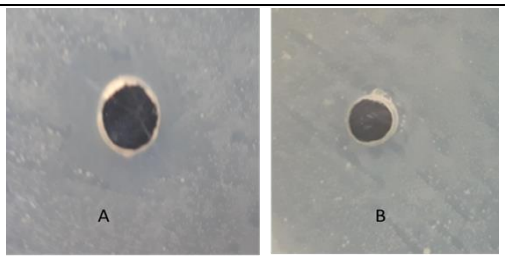

Figure 10. Inhibitory effect of Se-NPs on *Staphylococcus aureus*, A) test, B) control

4. CONCLUSIONS

Based on the obtained results, a novel *Bacillus subtilis* was isolated from Qarun Lake, Egypt. The isolated bacteria exhibited extraordinary ability to produce a reduction system as metabolities for Se-NPS manufacturing. In addition, the biosynthesized nanoparticles had diameter of 31-193 nm with spherical shape. The anti-microbial activity of the produced agents against several serious pathogens was reported. Thus, bacterial Se-NPs composite might realize appropriate application as a bioremediation tool of pathogens in required water.

5. REFERENCES

1. Huang, B.; Zhang, J.; Hou, J.; Chen**,** C. Free radical scavenging efficiency of Nano-Se in vitro. *Free Radical and Biological Medicine* **2003**, *35*, 805-813, https://doi.org/10.1016/s0891-5849(03)00428-3.

2. Hussein, H.A.; Darwesh, O.M.; Mekki, B.B.; El-Hallouty, S.M. Evaluation of cytotoxicity, biochemical profile and yield components of groundnut plants treated with nano-selenium. *Biotechnology Reports* **2019**, *24*, e00377, https://doi.org/10.1016/j.btre.2019.e00377.

3. Cui, Y.H.; Li, L.L.; Qing, Z. N.; Liu, J.H.; Huang, Q., Wang, H.J.; Jie Tian, C.; Yu, H.Q. In vivo synthesis of nano-selenium by *Tetrahymena thermophila* SB210. *Enzyme and Microbial Technology* **2016,** http://dx.doi.org/10.1016/j.enzmictec.2016.08.017.

4. Hussein, H.A.; Darwesh, O.M.; Mekki, B.B. Environmentally friendly nano-selenium to improve antioxidant system and growth of groundnut cultivars under sandy soil conditions. *Biocatalysis and Agricultural Biotechnology* **2019**, *18*, 1https://doi.org/10.1016/j.bcab.2019.101080.

5. Srivastava, N.; Mukhopadhyay M. Green synthesis and structural characterization of selenium nanoparticles and assessment of their antimicrobial property. *Bioprocess Biosystem Engineering* **2015,** *38*, 1723–1730, https://doi.org/10.1007/s00449-015-1413-8.

6. Darwesh, O.M.; Barakat, K.M.; Mattar, M.Z.; Sabae, S.Z.; Hassan, S.H. Production of antimicrobial blue green pigment Pyocyanin by marine Pseudomonas aeruginosa. *Biointerface Research in Applied Chemistry* **2019**, *9*, 4334 – 4339, https://doi.org/10.33263/BRIAC95.334339.

7. Elshahawy, I.; Abouelnasr, H.M.; Lashin, S.M.; Darwesh, O.M. First report of *Pythium aphanidermatum* infecting tomato in Egypt and its control using biogenic silver nanoparticles. *Journal of Plant Protection Research* **2018**, *15*, 137–151, https://doi.org/10.24425/122929.

8. Kheiralla, Z.H.; Hewedy, M.A.; Mohammed, H.R.; Darwesh, O.M. Isolation of Pigment Producing Actinomycetes from Rhizosphere Soil and Application It in Textiles Dyeing. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2016**, *7*, 2128-2136.

9. Marrez, D.A.; Abdelhamid, A.E.; Darwesh, O.M. Eco-friendly cellulose acetate green synthesized silver nano-composite as antibacterial packaging system for food safety. *Food Packaging and Shelf Life* **2019**, *20*, 1-8, https://doi.org/10.1016/j.fpsl.2019.100302.

10. Dhanjal, S.; Cameotra, S.S. Aerobic biogenesis of selenium nanospheres by *Bacillus cereus* isolated from coalmine soil. *Microbial Cell Factory* **2010,** *52*, 1–11, https://dx.doi.org/10.1186%2F1475-2859-9-52.

11. Darwesh, O.M.; Matter, I.A.; Eida, M.F.; Moawad, H.; Oh, Y. Influence of Nitrogen Source and Growth Phase on Extracellular Biosynthesis of Silver Nanoparticles Using Cultural Filtrates of *Scenedesmus obliquus*. *Applied Sciences* **2019**, *9*, 1-13, https://doi.org/10.3390/app9071465.

12. Natarajan, K.; Selvaraj, S.; Murty, V.R**.** Microbial production of silver nanoparticles. *Digital Journal of Nanomaterial and Biostructure* **2010,** *5*,135–140, https://doi.org/10.4172/2155-952X.S13-007.

13. Zare, B.; Babaie, S., Setayesh, N.; Shahverdi, A.R. Isolation and characterization of a fungus for extracellular synthesis of small selenium nanoparticles. *International Journal of Nanomedicine* **2013**, *1*, 14–20.

14. Fesharaki, J.P.; Nazari, M.; Shakibaie, S.; Rezaie, M.; Abdollahi, A.H.; Shahverd A.M. Biosynthesis of selenium nanoparticles using Klebsiella pneumoniae and their recovery by simple strerilization process`. *Brazilian Journal of Microbiology* **2010,** *2*, 461-466, https://doi.org/10.1590/S1517-83822010000200028.

15. Hasanin, M.S.; Mostafa, A.M.; Mwafy, E.A.; Darwesh, O.M. Eco-friendly cellulose nano fibers via first reported Egyptian *Humicola fuscoatra* Egyptia X4: Isolation and characterization. *Environmental Nanotechnology, Monitoring & Management* **2018,** *10*, 409–418, https://doi.org/10.1016/j.enmm.2018.10.004.

16. Ali, S.I.; Mohamed, A.A.; Sameeh, M.Y.; Darwesh, O.M.; Abd El-Razik, T.M. Gamma-Irradiation Affects Volatile Oil Constituents, Fatty Acid Composition and Antimicrobial Activity of Fennel (*Foeniculum vulgare*) Seeds Extract. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2016**, *7*, 524-532.

17. Eszenyi, P.; Sztrik, A.; Babka, B.; Prokisch, J. Elemental, nano-sized (100–500 nm) selenium production by probiotic lactic acid bacteria. *International Journal of Bioscience, Biochemical and Bioinformatics* **2011,** *1*, 148–153.

18. Darwesh, O. M.; Elsehemy, I. A.; El-Sayed, M. H.; El-Ghamry, A. A.; El-Hawary, A. S. *Thermoflavimicrobium dichotomicum* as a novel thermoalkaliphile for production of environmental and industrial enzymes. *Biointerface Research in Applied Chemistry* **2020,** *10*, 4811–4820. https://doi.org/10.33263/BRIAC101.811820.

19. Hatfild, D.L.; Berry, M.J.; Gladyshev, V.N. *Selenium: Its Molecular Biology and Role in Human Health*. Springer Science & Business Media, USA 2011; https://doi.org/10.1007/978-1-4614-1025-6.
20. Mohamed, A.A.; Ali, S.I.; Darwesh, O.M.; El-Hallouty, S.M.; Sameeh, M.Y. Chemical compositions, potential cytotoxic and antimicrobial activities of Nitraria retusa methanolic extract sub-fractions. *International Journal of Toxicological and Pharmacological Research* **2015**, *7*, 204-212.
21. Barakat, K.M.; Mattar, M.Z.; Sabae, S.Z.; Darwesh, O.M.; Hassan, S.H. Production and Characterization of Bioactive Pyocyanin Pigment by Marine *Pseudomonas aeruginosa* OSh1. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2015**, *6*, 933-943.
22. Abdelhameed, R.M.; Darwesh, O.M.; Rocha, J.; Silva, A.M. IRMOF-3 Biological Activity Enhancement by Post-Synthetic Modification. *European Journal of Inorganic Chemistry* **2019**, 1243-1249, https://doi.org/10.1002/ejic.201801442.
23. Sultan, Y.Y.; Ali, M.A.; Darwesh, O.M.; Embaby, M.A.; Marrez, D.A. Influence of Nitrogen Source in Culture Media on Antimicrobial Activity of *Microcoleus lacustris* and *Oscillatoria rubescens*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2016**, *7*, 1444-1452.
24. APHA (American Public Health Association) *Standard methods for examination of water and wastewater*. Washington DC, USA, 20th Ed, 1998.
25. Darwesh, O.M.; Moawad, H.; Barakat, O.S.; Abd El-Rahim, W.M. Bioremediation of Textile Reactive Blue Azo Dye Residues using Nanobiotechnology Approaches. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2015,** *6*, 1202-1211.
26. Yaghoobizadeh, F.; Ardakani, M.R.; Zolgharnein, H. Isolation, screening and identifiation of the best selenium reducing bacteria and study on the sorption mechanism. *Environmental and Experimental Biology* **2017,** *15*, 161-171, https://doi.org/10.22364/eeb.15.15.
27. Musarrat, J.; Dwivedi, S.; Singh, B.R.; Al-Khedhairy, A.A.; Azam, A.; Naqvi, A. Production of antimicrobial silver nanoparticles in water extracts of the fungus *Amylomyces rouxii* strain KSU-09. *Bioresource Technology* **2010**, *101*, 8772–8776, https://doi.org/10.1016/j.biortech.2010.06.065.
28. Kora, A.J.; Rastogi, L. Biomimetic synthesis of selenium nanoparticles by *Pseudomonas aeruginosa* ATCC 27853: An approach for conversion of selenite. *Journal of Environmental Management* **2016,** *181*, 231-236, https://doi.org/10.1016/j.jenvman.2016.06.029.
29. Holt, J.G.; Krieg, N.R.; Sneath, P.H.; Staley, J.S.; Williams, S.T. *Bergey's Manual of Determinative Bacteriology*. 9th Ed., Williams and Wilkins, 1994; pp. 787.
30. Darwesh; O.M.; Eida, M.F.; Matter, I.A. Isolation, screening and optimization of L-asparaginase producing bacterial strains inhabiting agricultural soils. *Bioscience Research* **2018**, *15*, 2802-2812.
31. Barakat, K.M.; Hassan, S.W.M.; Darwesh, O.M. Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea, Egypt. *Egyptian Journal of Aquatic Research* **2017,** *43*, 205–211, http://dx.doi.org/10.1016/j.ejar.2017.09.001.
32. Eida, M.F.; Darwesh, O.M.; Matter, I.A. Cultivation of oleaginous microalgae *Scenedesmus obliquus* on secondary treated municipal wastewater as growth medium for biodiesel production. *Journal of Ecological Engineering* **2018**, *19*, 38–51, https://doi.org/10.12911/22998993/91274.
33. Hasanin, M.S.; Darwesh, O.M.; Matter, I.A.; El-Saied, H. Isolation and characterization of non-cellulolytic *Aspergillus flavus* EGYPTA5 exhibiting selective ligninolytic potential. *Biocatalysis and Agricultural Biotechnology* **2019**, *17*, 160–167, https://doi.org/10.1016/j.bcab.2018.11.012.
34. Ghosh, A.; Mohod, A.M.; Paknikar, K.M.; Jain, R.K. Isolation and characterization of selenite- and selenate-tolerant microorganisms from selenium-contaminated sites. *World Journal of Microbiology and Biotechnology* **2008**, *24*, 1607–1611, https://doi.org/10.1007/s11274-007-9624-z.
35. Pouri, S.; Motamedi, H.; Honary, S.; Kazeminezhad, I. Biological Synthesis of Selenium Nanoparticles and Evaluation of their Bioavailability. *Brazilian Archives of Biology and Technology* **2017,** *60*, 1-12, https://doi.org/10.1590/1678-4324-2017160452.
36. Abdelhameed, R.M.; El-Sayed, H.A.; El-Shahat, M.; El-Sayed, A.A.; Darwesh, O.M. Novel Triazolothiadiazole and Triazolothiadiazine Derivatives Containing Pyridine Moiety: Design, Synthesis, Bactericidal and Fungicidal Activities. *Current Bioactive Compounds* **2018**, *14*, 169-179, https://doi.org/10.2174/1573407213666170127095158.
37. Darwesh, O.M.; Matter, I.A.; Eida, M.F. Development of peroxidase enzyme immobilized magnetic nanoparticles for bioremediation of textile wastewater dye. *Journal of Environmental Chemical Engineering* **2019**, *7*, https://doi.org/10.1016/j.jece.2018.11.049.
38. El-Baz, F.K.; Mahmoud, K.; El-Senousy, W.M.; Darwesh, O.M.; El Gohary, A.E. Antiviral – Antimicrobial and Schistosomicidal Activities of *Eucalyptus camaldulensis* Essential Oils. *International Journal of Pharmaceutical Sciences Review and Research* **2015**, *31*, 262-268.
39. Abdel-Rahman, M.A.; Sadek, Z.I.; Azab, M.S.; Darwesh, O.M.; Hassan, M.S. Incorporation of microencapsulated *Lactobacillus rhamnosus* into infant foods inhibit proliferation of toxicogenic *Bacillus cereus* strains. *Biocatalysis and Agricultural Biotechnology* **2019**, *18*, https://doi.org/10.1016/j.bcab.2019.01.051.
40. Shoeibi, S.; Mashreghi, M. Biosynthesis of selenium nanoparticles using *Enterococcus faecalis* and evaluation of their antibacterial activities. *Journal of Trace Elements in Medicine and Biology* **2017,** *39*, 135–139, https://doi.org/10.1016/j.jtemb.2016.09.003.
41. Oremland, R.S.; Herbel, M.J.; Blum, J.S.; Langley, S.; Beveridge, T.J., Ajayan, P.M.; Sutto, T.; Ellis, A.V.; Curran, S. Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. *Applied and Environmental Microbiology* **2004**, *70*, 52-60, https://dx.doi.org/10.1128%2FAEM.70.1.52-60.2004.
42. Ramoutar R.R.; Brumaghim J.L. Effects of inorganic selenium compounds on oxidative DNA damage. *Inorganic Biochemistry* **2007**, 101, 1028–1035, https://doi.org/10.1016/j.jinorgbio.2007.03.016
43. Yamada, A.; Miyashita, M.; Inoue K.; Matsunaga T. Extracellular reduction of selenite by a novel marine photosynthetic bacterium. *Applied Microbiology and Biotechnology* **1997,** *48*, 367–372, https://doi.org/10.1007/s002530051064.
44. Debieuxa, C.M.; Dridgea, E.J.; Muellera, C.M.; Splatt, P.; Paszkiewicz, K.; Knight, I.; Florance, H.; Love, J.; Titball, R.W.; Lewis, R.J.; Richardson, D.J.; Butler, C.S. A bacterial process for selenium nanosphere assembly. *PNAS* **2011,** *108*, 1–6, https://doi.org/10.1073/pnas.1105959108.
45. Shakibaie, M.; Khorramizadeh, M.R.; Faramarzi, M.A.; Sabzevari, O.; Shahverdi, A.R. Biosynthesis and recovery of selenium nanoparticles and the effects on matrix metalloproteinase-2 expression. *Biotechnology and Applied Biochemistry* **2010,** *56*, 7-15, https://doi.org/10.1042/BA20100042.

46. Shikuo, L.; Yuhua, S.; Anjian, X.; Xuerong, Y.; Xiuzhen, Z., Liangbao, Y.; Chuanhao, L. Rapid, room-temperature synthesis of amorphous selenium/protein composites using *Capsicum annuum* L extract. *Nanotechnology* **2007,** *18*.
47. Mishra, R.R.; Prajapati, S.; Das, J.; Dangar, T.K.; Das, N.; Thatoi, H. Reduction of selenite to red elemental selenium by moderately halotolerant *Bacillus megaterium* strains isolated from *Bhitarkanika* mangrove soil and characterization of reduced product. *Chemosphere* **2011**, *84*, 1231-1237, https://doi.org/10.1016/j.chemosphere.2011.05.025.
48. Lampis, S.; Zonaro, E.; Bertolini, C.; Bernardi, P.; Butler, C.S.; Vallini, G. Delayed formation of zero-valent selenium nanoparticles by *Bacillus mycoides* SeITE01 as a consequence of selenite reduction under aerobic conditions. *Microbial Cell Factory* **2014**, 13- 35, https://doi.org/10.1186/1475-2859-13-35.
49. Ramya, S.; Shanmugasundaram, T.; Balagurunathan, R. Biomedical potential of actinobacterially synthesized selenium nanoparticles with special reference to anti-biofilm, anti-oxidant, wound healing, cytotoxic and anti-viral activities. *Journal Trace Elemental and Medical Biology* **2015**, *32*, 30-39, https://doi.org/10.1016/j.jtemb.2015.05.005.
50. Dobias, J.; Suvorova, E.I.; Bernier-Latmani, R. Role of proteins in controlling selenium nanoparticle size. *Nanotechnology* **2011,** *22*.
51. Torres, S.K.; Campos, V.L.; Leon, C.G.; Rodríguez-Llamazares, S.M.; Rojas, S.M.; Gonzalez, S.M.; Smith, C.; Mondaca, M.A. Biosynthesis of selenium nanoparticles by *Pantoea agglomerans* and their antioxidant activity. *Journal of Nanoparticle Research* **2012,** *14*, 1-9, https://doi.org/10.1007/s11051-012-1236-3.
52. Li, B.; Liu, N.; Li, Y.; Jing, W.; Fan, J.; Li, D.; Zhang, L.; Zhang, X.; Zhang, Z.; Wang, L. Reduction of selenite to red elemental selenium by *Rhodopseudomonas palustris* strain N. *PLoS One* **2014,** *9*, e95955, https://doi.org/10.1371/journal.pone.0095955.
53. Lee, J.H.; Han, J.; Choi, H.; Hur, H.G. Effects of temperature and dissolved oxygen on Se (IV) removal and Se(0) precipitation by *Shewanella* sp. HN-41. *Chemosphere* **2007,** *68*, 1898-1905, https://doi.org/10.1016/j.chemosphere.2007.02.062.
54. Darwesh, O.M.; Sultan, Y.Y.; Seif, M.M.; Marrez, D.A. Bio-evaluation of crustacean and fungal nano-chitosan for applying as food ingredient. *Toxicology Reports* **2018**, *5*, 348-356, https://doi.org/10.1016/j.toxrep.2018.03.002.
55. Sonkusre, M.K.; Motamedi, H.; Akbarzadeh, K.A.; Mehrabi, M.R. Gold Nanoparticle Biosynthesis by *E. coli* and Conjugation with Streptomycin and Evaluation its Antibacterial Effect. *Current Nanotechnology* **2014**, *4*, 553- 561, https://doi.org/10.2174/15734137096661312032313 44.
56. Tran, P.A.; Webster, T.J. Selenium nanoparticles inhibit *Staphylococcus aureus* growth. *International Journal Nanomedicine* **2011,** *6*, 1553–1558, https://doi.org/10.2147/IJN.S21729.
57. Ramos J.F.; Webster, T.J. Cytotoxicity of selenium nanoparticles in rat dermal fibroblasts. *International Journal Nanomedicine* **2012**, *7*, 3907–3914, https://dx.doi.org/10.2147%2FIJN.S33767.

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