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Antibacterial Activity of Biosynthesized Nano-Selenium and ItsEnvironmental Applications

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ABSTRACT

Because the fast development of microbial resistance system, the finding of effective, ecofriendly and environmental compatible agent is difficult and will be more difficult in the future, it is difficult but not impossible. For that, the current research focused on production of nano-selenium by bacteria as effective and environmental compatible antibacterial agent. Forty nine samples (soil, water, wastewater and milk cracker) were collected from different locations in Delta region, Egypt. Hundred isolates of bacteria were obtained from the collected samples on MSM medium supplemented with sodium selenite and screened for selenium reduction; about 74 positive bacterial isolates were selected. The screening technology was developed to obtain the superior bacterial isolates for nano-selenium manufacturing. Three bacteria were finished these procedures as the best producers. The culture and environmental conditions were optimized for the 3 strains to obtain active and high yield of nano-selenium. The produced amount of nano-selenium was characterized using TEM, XRD, EDAX and UV/VIS spectrophometer techniques. The characterization technology confirmed producing selenium with nano-properties (15-30 nm with spherical shape). The antibacterial activity of the produced nano-selenium was evaluated against several bacterial pathogens and the MIC was calculated. The produced nano-selenium has high activity and low MIC with the most tested pathogens. For that, it can be applied as antibacterial agent for controlling the pathogens in hospital, agricultural fields and municipal wastewater treatment units.

Keywords: Nano-selenium, Bacterial Synthesis, Pathogens, Environmental Applications.

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INTRODUCTION

Nanotechnology is considered the synthesis of functional materials and systems through control of matter on the nanometer length scale and exploring of great phenomena and properties which arise because of the nanometer length scale. It includes the synthesis, characterization and utilization of nanostructured materials. Scientists are very interested in nanostructured materials for scientific reasons and great practical applications. (**Pathak et al., 2013; Cao et al., 2013**) A nanometer (nm) is an International System of Units (Système international d'unités, SI) unit that represents 10^{-9} meter in length.

Selenium is very important trace element which is required up to 40–300 μg for human body every day. Only small amount is required for maintaining the function, and a large amount of selenium may be harmful to the human body (**Srivastava et al., 2015a**). It is useful in regulating the function of the human body. It is useful in protecting cardiovascular health, regulating thyroid hormones and immune response, and preventing progression of cancer (**Gautam et al. 2017**). Selenium is less toxic and high active of selenium nanoparticles are used in many medical applications such as antitumor (**Huang et al. 2013; Ramamurthy et al. 2013**), antimicrobial (**Bartůněk et al. 2015**), drug delivery. Selenium has codons in mRNA which formseleno-cysteine by entering as selenoprotein (**Srivastava et al., 2015b**). The average size of the selenium nanoparticle is 80 nm and the property and activity of the selenium nanoparticles rely on the size, shape, and the biomolecule's nature (**Husen et al., 2014**). High surface area and the low particle size of selenium nanoparticles strength the biological activity. Selenium nanoparticles actively work against the antibiotic- resistant bacteria which has been increased recently (**Bartůněk et al. 2015**)

Micro-organisms play a key role in selenium transformations that involve the conversion of the highly soluble and biologically available selenite $(SeO_3)^{-2}$ to insoluble and relatively unavailable Se^0. (**Bao et al., 2013**) The objectives of this study were to find isolates capable of reducing the selenite ion to elemental selenium and to evaluate the effect of various parameters on the reduction rate. In the field of Se detoxification, reduction of selenium oxyanions by microbial cells plays an important role in selenium transformation in the biosphere. Utilizing this mechanism, some bacteria can use oxidized form of Se (selenite) as respiratory substrates and produce elemental selenium. The elemental selenium readily precipitates and is mostly unavailable for biological systems. Therefore the potential toxicity of selenium oxyanions is reduced. (**Yaghoobizadeh et al., 2017**)

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 (**Gadakh & Van Aerschot., 2015**). 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. 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 (**Pelgrift &Friedman., 2013**). In this study, selenium nanoparticles (SeNPs) possess antibacterial properties, suggesting they could be suitable as therapeutic candidates to combat infectious diseases. In particular, nanostructured particles can be synthesized using bacterial cells as biological catalysts, providing a non-toxic and environmentally beneficial approach for the production of nanoparticles, including SeNPs (**Xiangqian et al., 2011**). Several microbial strains can reduce the toxic selenite oxyanion to the less toxic elemental selenium through the formation of either intra-cellular or extracellular SeNPs, with a typical spherical shape and a diameter of 50–400 nm (**Cremonini et al., 2016**). In this trend, this study was designed to isolate and identify nanoselenium synthesizing bacteria from Egyptian environments. As well as optimize their production efficiency and environmental application of the produced nanoselenium.

MATERIALS AND METHODS

Isolation and purification of bacteria has reducing activity toward selenium

Soil slurry was prepared by suspending 5 g soil from the top layer in 100 ml tap water. Twenty ml portions of slurry were incubated in 100 ml Erlenmeyer flasks under gentle shaking overnight at 28 ± 2°C. From this slurry a mixed bacterial culture capable of reducing selenite was enriched by multiple transfers of 2.5 % of the initial soil suspension to enrichment medium (EM), described by Ghosh et al. (2008). To this medium 0.5g/l of $Na_2SeO_3{\cdot}5H_2O$ (= 50 mg/l Se (IV)) and 1 g/l glucose monohydrate as the main carbon source were added. Incubate the flasks in the shaking incubator at 30°C and 100 rpm for week. Bacterial activity was monitored visually (red color formation of elemental Se in EM). Make Se re-enrichment if there is no color change in the first week of incubation. Select individual bacterial colonies from any of the plates. More colonies can be selected if there is particular interest in the soil. Use a high dilution plate, as it tends to have pure colonies thatare separated well. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other. Taking a fresh modified MSM plate, make a streak a few centimeters long on one side. Sterilize and cool again, then make a streak that crosses the initial streak only on the first pass. Repeat this process twice more in the same manner. This streaking "dilution" results in cells on the loop being separated from one another. Place the plate in a dark area to incubate at 30°C for two weeks. Pure cultures of bacteria were maintained on modified MSM agar slants. Slants were kept in a refrigerator at 4°C and sub-cultured every 2 months. To ensure that the isolated colonies are bacteria, we made gram stain for them.

Differentiation between intracellular and extracellular reduction of selenium

Differentiation were done by preparing MSM broth medium, then divide it in 100ml flasks each one contain 50ml media, then sterilize it in autoclave at 121 Cfor 20 minutes. After sterilization inoculate each flask with one bacterial isolate under aseptic conditions, then label each flask and finally incubate them at 28C in shaking incubator for 48 h. During the incubation period prepare sodium selenite solution with concentration 0.5g/l and divide it into 74 test tubes, and then sterilized. After the incubation period make centrifugation to each isolate at 7000rpm for 15 minutes to precipitate bacteria and obtain bacterial metabolites. After that inculate each tube contains the sterilized selenium solution with one bacterial metabolite under aseptic conditions and the inoculation is done by passing the bacterial metabolite through bacterial filter to ensure the absence of bacterial cells. Incubate the tubes at 28C in shaking incubator for 7 days and take 2ml daily from each tube and measure it at spectrophotometer to differentiate among isolates by the spectral differences.

Screening the most reducing isolates at high concentrations of sodium selenite

A 100 ml each of nutrient broth medium were prepared and sterilized. To each 100 ml add specific concentration of sodium selenite using bacterial filter and make 4 groups the first one contains 0 g/l of sodium selenite, the second contains 1 g/l of sodium selenite ,the third contains 5 g/l of sodium selenite, the forth contains 7 g/l of sodium selenite. To each group add bacterial metabolites using bacterial filter then the cultures were maintained at 30 C until the nanoparticles were formed on a rotary shaker at 90 rpm. With the reaction, the color of solution changed from light yellow to bright red, indicating the presence of selenium nanoparticles in the solution.

Synthesis of Se nanoparticles using the most reducing isolates at high concentrations of sodium selenite

A 200 ml each of nutrient broth medium were prepared and sterilized. After sterilization inoculate each flask with one bacterial isolate under aseptic conditions, then label each flask and finally incubate them at 28C in shaking incubator for48 hours. During the incubation period prepare 1.5l of sodium selenite solution with concentration 7g/l and divide it into 3 flasks. After the incubation period make centrifugation to each isolate at 7000rpm for 15 minutes to precipitate bacteria and obtain bacterial metabolites. After that inoculate each flask contains the sterilized selenium solution with one bacterial metabolite under aseptic conditions and the inoculation is done by passing the bacterial metabolite through bacterial filter to ensure the absence of bacterial cells. Incubate the flasks at 28C in shaking incubator for 7 days. After that make centrifugation to each flask at 7000rpm for 15 minutes to precipitate selenium nanoparticles and wash them with distilled water and diluted alcohol then dry them in oven at 40 C.

Optimization of different factors affecting selenium reduction by bacteriaEffect of different media on selenium reduction

Different media were prepared such as Nutrient broth media, lauria broth media, Bennet medium, Glucose- asparagine broth media, Glycerol -asparagine broth media, Minimal salt media. After preparing the medium divide them in flasks 150ml in each one then autoclave them, by exposure to steam at 121 C for 20 minutes. After autoclaving label them and inoculate each one with bacterial isolate under aseptic conditions then incubate them at 30 C in shaking incubator at 50 rpm for 2 days. After incubation period, make centrifugation for each culture at 7000rpm for 15 minutes. After centrifugation, make filtration for each one by bacterial filter to ensure the absence of bacterial cells. Prepare 18 flasks each one contain 25ml distilled water and autoclave them at 121C at 1.5 atm for 20 minutes, then under aseptic conditions 0.25 g sodium selenite.

Prepare sodium selenite solution in 18 Erlenmeyer flask 25 ml in each one and label them, then add 25 ml metabolites in each flask under aseptic conditions and take in your consideration that the final concentration of salt is 5g/l, then incubate the flasks at 28C for 6 days with agitation 90 rpm and observe the color change daily and measure the change at spectrophotometer daily for 6 days at 300 nm.

Phytochemical studies

Qualitative and quantitative analysis of some secondary metabolites were carried out on the studied
isolates.

Preparations of bacterial metabolites

Isolated bacteria, grown earlier in Erlenmeyer containing sterile liquid medium each isolate grow on different six medium (nutrient broth, Lauria broth media, MSM, Bennet medium, glucose asparagine media, glycerol asparagine media),then were incubated in shaking incubator at 28C for 2 days with agitation of 50 rpm. This culture was inserted in a sterile tube and then centrifuged at 7,000 rpm for 15 min to separate the pellets and supernatant. The supernatant was filtered through bacterial filter to ensure that it contains metabolites without bacteria.

Preliminary phytochemical screening (qualitative analysis)

Qualitative chemical tests were carried with using standard procedures to identify some phytochemical constituents of the studied isolates such as flavonoids, tannins, cardiac glycosides and saponins.

Test for flavonoids

Five ml of dilute ammonia solution 10% was added to a portion of the filtrate (10ml) , followed by addition of few drops of concentrated H_2SO_4 . A yellow coloration indicates the presence of flavonoids. The yellow color may disappear after few minutes on standing.

Test for tannins

Few drops of 0.1% $FeCl_3$ were added and the mixture was observed for brownish green or blue-black coloration, which is an indication for the presence of tannins.

Test for cardiac glycosides (Keller- Killani test)

Two ml of glacial acetic acid containing 1ml of ferric chloride solution (10%) were added to 5ml of each filtrate. 1ml of concentrated sulphuric acid was added carefully down the wall of test tube. On standing, a brown ring appeared at the interface (due to aglycon) while, in the acetic acid layer, a greenish ring mayappear gradually (due to a deoxy sugar) in the presence of cardiac glycosides.

Test for saponins

A 10 ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable

persistent forth; it was allowed to stand for 10-15 minutes.

Estimation of primary metabolites Determination of total carbohydrates

A 0.1 ml of previously prepared filtrate was pipetted into a test tube and the volume was completed to 1ml with distilled water. The tubes were immersed in an ice bath. 4ml of cold anthrone reagent was added, tubes were put in a boiling water bath for 8 min, and then cooled rapidly and the produced green color was measured at 630 nm on spectrophotometer.

Determination of total protein

One ml of filtrate was mixed in a tube with 0.9 ml reagent A (2g potassium sodium tartarate+100 g sodium carbonate were dissolved in 1000 ml of 0.1N sodium hydroxide) and incubated in a water bath at 50C for 10 min. After cooling to room temperature, 0.1 ml of reagent B (2g potassium sodium tartarate+ 1g copper sulphate ($CuSO_4.5H_2O$) were dissolved in 100 ml of 0.1N sodium hydroxide) was added and mixed well, the tubes were allowed to stand for 10 min. 3ml of reagent C (1 volume of folin-ciocalteu reagent diluted to 10 volumes with distilled water) were added rapidly with mixing using a vortex mixer. The tubes were incubated in a water bath at 50C for 10 min. After cooling, the color was measured at 650 nm.

Estimation of secondary metabolites

Estimation of total phenolic compounds

One ml from the combined extract was mixed with 1ml folin ciocalteau reagent and 1ml sodium carbonate solution (20%w/v), then the mixture was completed up to a known volume with distilled water and all tubes were incubated in dark for color development. The absorbance was measured at 650 nm on spectrophotometer after exactly 30 min.

Estimation of reduction power on different carbon sources

Prepare 7 l of Bennt media without glucose and divide it in 27 flask, 250 ml in each one and labelthem then divide them into three groups, each group 9 flasks for each isolate .Calculate the amount of carbon source needed for each one and add it to each flask. The carbon sources that used were glucose, dextrose, fructose as monosaccharaides, maltose, lactose, sucrose as disaccharides and cellulose, starch, asparagine as polysaccharides. After adding carbon sources to flasks, sterilize media in autoclave, then inoculate each group with bacterial isolate, finally incubate them in shaking incubator at 28 C, 50 rpm for three days. After incubation period make centrifugation to all flasks to obtain metabolites for each one. Prepare 81 flasks each one contain 25 ml of sodium selenite solution with concentration 5g/l to make 3 replicates for the experiment. Add 25 ml metabolites for each flask and label them, then incubate them in shaking incubator at 28˚C, 90 rpm for 7 days and observe color change and measure daily on spectrophotometer at wavelength 300nm.

RESULTS AND DISSCUSSION

Isolation of bacterial isolates

The main objective of this study is to obtain some bacterial isolates capable of reducing selenium. To achieve this goal, forty nine samples (soil, wastewater, water and milk cracker) were collected from different locations. The collected samples were transferred to lab under septic conditions and used the source of selenium reducing bacteria. The details of these samples were noted in **Table (1)**. Seventy four isolates of bacteria were obtained from collected samples on agar MSM medium supplemented with sodium selenite salt. These isolates were purified on new agar MSM medium plates supplemented with sodium selenite.

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Table 1. Details of samples for selenium reducing bacteria

Sample code	Type of sample	The city (place of sample)	Type of plant
1	Milk cracker	Menof city	-------
2	Salted water	Wadi El Natroon	-------
3	Soil	Tala	Rocca
4	Soil	Ashmoon city	Lettuce
5	Soil	Berket El Sabaa	Grape
6	Soy bean	Shebeen El Koum	Bean
7	Soil	Kwesna	Cabbage
8	Soil	Ganzooor	Lemon
9	Soil	Ashmoon city	Spinach
10	Soil	Menouf city	Cauliflower
11	Soil	Menouf city	Cabbage
12	Soil	Menouf city	Garlic
13	Soil	El Hamool	Taro
14	Wastewater	Manshiet Sultan	------
15	Soil	Manshiet Sultan	Rocca
16	Soil	Manshiet Sultan	Onion
17	Soil	Manshiet Sultan	Rocca
18	Soil	Tala	Spinach
19	Soil	Kafr El Sokareya	Lettuce
20	Soil	Tala	Cabbage
21	Soil	Teta	Onion
22	Soil	Ashmoon city	Garlic
23	Soil	Meleeg	Lettuce
24	Soil	Shebeen El Koum	Cauliflower
25	Soil	Manshiet Esam	Mint
26	Soil	Manshiet Esam	Lemon
27	Soil	Manshiet Esam	Cauliflower
28	Soil	Tala	Spinach
29	Soil	Nader	Garlic
30	Soil	Nader	Lettuce
31	Soil	Shebeen El Koum	Grape
32	Pool water	Tala	--------
33	Pool water	Kafr Tanbdy	---------
34	Pool water	Ashmoon city	--------
35	Pool water	Kafr Tanbdy	--------
36	Soil	El Shohadaa	Grape
37	Soil	El Shohadaa	Mint
38	Soil	Shebeen El Koum	Cabbage
39	Soil	Tala	Grape
40	Soil	Ashmoon city	-------
41	Wastewater	Berket El Sabaa	--------
42	Pool water	Berket El Sabaa	---------
43	Wastewater	Berket El Sabaa	---------
44	Wastewater	Shebeen El Koum	------
45	Air	Shebeen El Koum	---------
46	Air	Shebeen El Koum	--------
47	Air	Shebeen El Koum	------
48	Air	Shebeen El Koum	--------
49	Air	Shebeen El Koum	------

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Isolation and screening of the more potent bacterial isolates

After 48 hours of incubation, we obtain pure plates from all isolates reducing selenim. Hundred isolates of bacteria were isolated from collected soil samples on MSM medium and screened for selenium reduction. About 74positive bacterial isolates were obtained from a total of 100 isolates. Bacteria reducing selenium were around 70% from all isolates. Repurification on media without selenium showed that the original color of each isolate is not red, so, the red colonies appeared on media containing selenium indicate that all these isolates reduce selenium. Gram staining showed that all isolates vary from gram positive to gram negative bacteria.

After purification, the 74 isolates obtained were reducing selenium salt (sodium selenite) from different places were tested for reduing selenium inside or outside the bacterial cell. There are 6 isolates reduced selenium from this city five of them reduced selenium intercellular and one of them reduced it extracellular.

Table 2. Differentiation between intracellular and extracellular reduction of selenium for Menouf cityisolates.

no of isolate	55	56	57	60	61	65
Day 0	0.366±0.001	0.353±0.002	0.304±0.001	0.327±0.001	0.371±0.001	0.306±0.001
Day 1	0.366±0.002	0.415±0.002	0.305±0.001	0.327±0.002	0.371±0.001	0.306±0.001
Day 2	0.368±0.003	0.525±0.001	0.306±0.002	0.328±0.002	0.372±0.002	0.306±0.001
Day 3	0.369±0.001	0.627±0.001	0.306±0.003	0.328±0.003	0.372±0.003	0.307±0.001
Day 4	0.369±0.001	0.733±0.002	0.306±0.003	0.328±0.003	0.373±0.003	0.307±0.001
Day 5	0.370±0.001	0.854±0.002	0.306±0.003	0.329±0.004	0.373±0.004	0.307±0.001
Day 6	0.371±0.001	0.919±0.002	0.306±0.003	0.329±0.004	0.373±0.004	0.307±0.001
LSD*	0.105	0	0.932	0.982	0.927	0.155

There are 15 isolates reduced selenium from this city twelve of them reduced selenium intercellular and three of them reduced it extracellular. There are 15 isolates reduced selenium from this city twelve of them reduced selenium intercellular and three of them reduced it extracellular.

Table 3. Differentiation between intracellular and extracellular reduction of selenium for Tala city isolates

no of isolate	8	19	21	22	23	24	25
Day 0	0.322±0.002	0.426±0.005	0.351±0.001	0.353±0.002	0.351±0.001	0.342±0.001	0.345±0.003
Day 1	0.323±0.001	0.690±0.005	0.353±0.001	0.354±0.002	0.353±0.001	0.345±0.003	0.352±0.004
Day 2	0.323±0.001	0.721±0.003	0.355±0.001	0.355±0.002	0.355±0.001	0.348±0.004	0.353±0.004
Day 3	0.325±0.002	0.810±0.006	0.356±0.001	0.356±0.002	0.356±0.001	0.352±0.003	0.353±0.008
Day 4	0.326±0.003	0.877±0.004	0.358±0.001	0.356±0.002	0.358±0.001	0.356±0.001	0.359±0.004

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Day 5	0.327±0.004	1.009±0.003	0.360±0.002	0.357±0.002	0.360±0.002	0.359±0.001	0.361±0.004
Day 6	0.329±0.006	1.288±0.004	0.361±0.002	0.357±0.002	0.361±0.003	0.364±0.001	0.363±0.003
LSD*	0.260	0.000	0.000	0.192	0.000	0.000	0.004

Screening the most reducing isolates at high concentrations of sodium selenite

After incubation in shaking incubator for seven days at 30C at 90 rpm with different concentrations of sodium selenite, the results were as the following according to the spectrophotometer readings.

Isolate number 5, According to the spectrophotometer readings. This isolate can reduce selenium salt at concentration 1g/l and the red color began to appear after 24 hours from incubation but it cannot reduce it at high concentrations.

Table 4 . Reduction of selenium by isolate number 5 at different concentrations of sodium selenite.

Se conc	**0**	**1**	**5**	**7**	**9**
Day 0	**0.733±0.002**	**0.748±0.004**	**0.759±0.002**	**0.763±0.003**	**0.781±0.002**
Day 1	**0.734±0.002**	**0.805±0.002**	**0.761±0.002**	**0.764±0.003**	**0.782±0.001**
Day 2	**0.735±0.002**	**0.826±0.003**	**0.762±0.002**	**0.765±0.002**	**0.784±0.002**
Day 3	**0.736±0.002**	**0.838±0.002**	**0.763±0.002**	**0.765±0.002**	**0.785±0.002**
Day 4	**0.737±0.001**	**0.848±0.003**	**0.764±0.002**	**0.766±0.001**	**0.785±0.002**
Day 5	**0.738±0.001**	**0.919±0.002**	**0.765±0.001**	**0.767±0.002**	**0.785±0.002**
Day 6	**0.738±0.001**	**0.943±0.002**	**0.766±0.001**	**0.768±0.001**	**0.785±0.003**
LSD*	**0.006**	**0.000**	**0.001**	**0.061**	**0.132**

Each value is Mean ± SD (n=3), Optical density (OD)
***The mean difference is significant at the 0.05 level.**

Isolate number 6, according to the spectrophotometer readings, this isolate can reduce selenium salt at concentration 1g/l and the red color began to appear after 48 hours from incubation but it cannot reduce it at high concentrations.

Table 5: Reduction of selenium by isolate number 6 at different concentrations of sodium selenite

Se conc	0	1	5	7	9
Day 0	0.761±0.002	0.765±0.002	0.771±0.002	0.774±0.002	0.781±0.002
Day 1	0.762±0.003	0.858±0.002	0.773±0.002	0.775±0.002	0.782±0.001
Day 2	0.763±0.002	0.976±0.002	0.774±0.002	0.775±0.002	0.784±0.002
Day 3	0.763±0.002	1.018±0.002	0.774±0.002	0.776±0.002	0.785±0.002
Day 4	0.764±0.002	1.043±0.003	0.775±0.002	0.776±0.002	0.785±0.002
Day 5	0.764±0.002	1.115±0.002	0.775±0.001	0.777±0.002	0.785±0.002
Day 6	0.765±0.001	1.146±0.003	0.775±0.002	0.777±0.002	0.785±0.003
LSD*	0.220	0.000	0.127	0.185	0.132
Each value is Mean ± SD (n=3), Optical density (OD) *The mean difference is significant at the 0.05 level.					

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According to the spectrophotometer readings of isolate number 38, this isolate can reduce selenium salt at concentration 1g/l , 5g/l and 7g/l and the red color began to appear after 24 hours from incubation butit cannot reduce it at other high concentrations.

Table . Reduction of selenium by isolate number 38 at different concentrations of sodium selenite.

Se conc	0	1	5	7	9
Day 0	0.748±0.002	0.738±0.002	0.748±0.002	0.799±0.006	0.775±0.002
Day 1	0.749±0.002	0.803±0.002	0.818±0.002	0.865±0.001	0.775±0.002
Day 2	0.752±0.003	0.927±0.004	0.908±0.002	0.919±0.002	0.776±0.002
Day 3	0.754±0.002	1.019±0.004	1.028±0.001	0.987±0.002	0.777±0.001
Day 4	0.756±0.002	1.096±0.002	1.114±0.002	1.075±0.002	0.778±0.001
Day 5	0.757±0.002	1.119±0.002	1.225±0.002	1.086±0.002	0.778±0.001
Day 6	0.758±0.003	1.148±0.002	1.316±0.003	1.165±0.002	0.778±0.001
LSD*	0.000	0.000	0.000	0.000	0.008

Each value is Mean ± SD (n=3), Optical density (OD)
*The mean difference is significant at the 0.05 level.

Optimization of different factors affecting selenium reduction by bacteriaEffect of different media on selenium reduction

Different media were prepared such as Nutrient broth media, lauria broth media, Bennet medium, Glucose- asparagine broth media, Glycerol -asparagine broth media, Minimal salt media. After 7 days from incubation of bacterial metabolites produced on different media with sodium selenite to obtain final concentration 5g/l under aseptic conditions the spectrophotometer readings at wavelength 300nm as the following:

Isolate 20

After 7 days from incubation of metabolites with selenium salt at 30C and 90 rpm , we noticed thatthe metabolites produced on Bennet medium was the best and the most rapid in selenium reduction than other media.

Table . Effect of different media on selenium reduction for isolate 20

Media	NB	LB	MSM	Bennet	Glu asp	Gly asp	P value
Day 0	0.665 ± 0.002^{b}	0.365 ± 0.001^{d}	0.532 ± 0.001^{c}	0.925 ± 0.001^{a}	0.273 ± 0.002^{e}	0.197 ± 0.001^{f}	0.000
Day 1	1.131 ± 0.001^{b}	0.415 ± 0.001^{d}	0.714 ± 0.001^{c}	1.212 ± 0.001^{a}	0.396 ± 0.001^{e}	0.301 ± 0.002^{f}	0.000
Day 2	1.216 ± 0.056^{b}	0.612 ± 0.094^{d}	0.755 ± 0.082^{c}	1.634 ± 0.250^{a}	0.760 ± 0.085^{e}	0.324 ± 0.203^{f}	0.000
Day 3	1.235 ± 0.001^{b}	0.745 ± 0.001^{d}	0.882 ± 0.082^{c}	1.826 ± 0.001^{a}	0.802 ± 0.001^{e}	0.574 ± 0.002^{f}	0.000
Day 4	1.302 ± 0.001^{b}	0.865 ± 0.001^{d}	0.935 ± 0.001^{c}	1.954 ± 0.002^{a}	0.892 ± 0.001^{e}	0.756 ± 0.001^{f}	0.000
Day 5	1.355 ± 0.001^{b}	0.905 ± 0.001^{d}	1.016 ± 0.001^{c}	2.303 ± 0.003^{a}	0.978 ± 0.001^{e}	0.942 ± 0.001^{f}	0.000
Day 6	1.358 ± 0.001^{b}	0.906 ± 0.001^{d}	1.018 ± 0.001^{c}	2.312 ± 0.002^{a}	0.983 ± 0.002^{e}	0.946 ± 0.001^{f}	0.000

Isolate 38

After 7 days from incubation of metabolites with selenium salt at 30C and 90 rpm , we noticed that the metabolites produced on Bennet medium was the best and the most rapid in selenium reduction than other media.

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Table . Effect of different media on selenium reduction for isolate 38

Media	NB	LB	MSM	Bennet	Glu asp	Gly asp	P value
Day 0	0.417±0.001^{c}	0.308±0.002^{d}	0.255±0.001^{e}	0.578±0.001^{a}	0.241±0.002^{f}	0.517±0.001^{b}	0.000
Day 1	0.662±0.001^{c}	0.415±0.002^{d}	0.337±0.001^{e}	1.122±0.001^{a}	0.268±0.001^{f}	0.963±0.002^{b}	0.000
Day 2	0.758±0.001^{c}	0.467±0.001^{d}	0.401±0.002^{e}	1.372±0.001^{a}	0.286±0.001^{f}	0.987±0.002^{b}	0.000
Day 3	1.208±0.001^{c}	0.987±0.001^{d}	0.701±0.001^{e}	1.474±0.001^{a}	0.607±0.015^{f}	1.286±0.002^{b}	0.000
Day 4	1.253±0.001^{c}	1.067±0.001^{d}	0.853±0.001^{e}	1.522±0.002^{a}	0.992±0.001^{f}	1.307±0.002^{b}	0.000
Day 5	1.318±0.001^{c}	1.115±0.001^{d}	0.931±0.001^{e}	1.566±0.001^{a}	0.995±0.001^{f}	1.357±0.001^{b}	0.000
Day 6	1.320±0.002^{c}	1.117±0.001^{d}	0.934±0.001^{e}	1.566±0.002^{a}	0.997±0.001^{f}	1.358±0.001^{b}	0.000

Isolate 68

After 7 days from incubation of metabolites with selenium salt at 30C and 90 rpm , we noticed that the metabolites produced on Nutrient broth medium was the best and the most rapid in selenium reduction than other media.

Table . Effect of different media on selenium reduction for isolate 68

Media	NB	LB	MSM	Bennet	Glu asp	Gly asp	P value
Day 0	0.508±0.001^{b}	0.373±0.001^{c}	0.323±0.002^{d}	1.202±0.002^{a}	0.317±0.001^{e}	0.212±0.002^{f}	0.000
Day 1	1.015±0.002^{b}	0.457±0.001^{e}	0.457±0.001^{e}	1.224±0.001^{a}	0.886±0.001^{c}	0.786±0.001^{d}	0.000
Day 2	1.477±0.001^{a}	0.478±0.001^{e}	0.478±0.001^{e}	1.366±0.001^{b}	0.955±0.002^{c}	0.797±0.001^{d}	0.000
Day 3	1.668±0.001^{a}	0.798±0.001^{e}	0.798±0.001^{e}	1.388±0.001^{b}	1.273±0.001^{c}	1.095±0.001^{d}	0.000
Day 4	1.738±0.002^{a}	0.965±0.002^{e}	0.968±0.001^{e}	1.416±0.002^{b}	1.408±0.001^{c}	1.163±0.002^{d}	0.000
Day 5	1.992±0.003^{a}	0.973±0.001^{e}	0.973±0.002^{e}	1.607±0.002^{b}	1.615±0.001^{c}	1.167±0.002^{d}	0.000
Day 6	1.993±0.002^{a}	0.974±0.001^{e}	0.976±0.002^{e}	1.609±0.001^{b}	1.616±0.001^{c}	1.165±0.001^{d}	0.000

Phytochemical studies

Qualitative and quantitative analysis of some secondary metabolites were carried out on the studied isolates. Bacterial metabolites were the raw material for phytochemical studies. After incubation period, we made centrifugation and filtration by bacterial filter to ensure absence of bacterial cells.

The highest production of secondary metabolites was at glucose asparagine media as the bacterial isolate secretes flavonoids ,tannins and cardiac glycosides.

Table . Preliminary phytochemical screening for isolate 20

	NB	LB	MSM	Ben	Gly-asp	Glu-asp
Flavonoids	+	+	-	+	-	+
Tannins	-	-	+	-	+	+
Card glyco	-	-	-	-	-	+
Saponin	-	-	-	-	-	-
Phlobtannins	-	-	-	-	-	-

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Isolate no 38, the highest production of secondary metabolites was at glucose asparagine,Bennet and nutrient media as the bacterial isolate secretes flavonoids ,tannins and saponin.

Table . Preliminary phytochemical screening for isolate 38

	NB	LB	MSM	Ben	Gly-asp	Glu-asp
Flavonoids	+	-	-	+	-	+
Tannins	+	+	-	+	+	+
Card glyco	-	-	-	-	-	-
Saponin	+	+	-	+	-	+
Phlobtannins	-	-	-	-	-	-

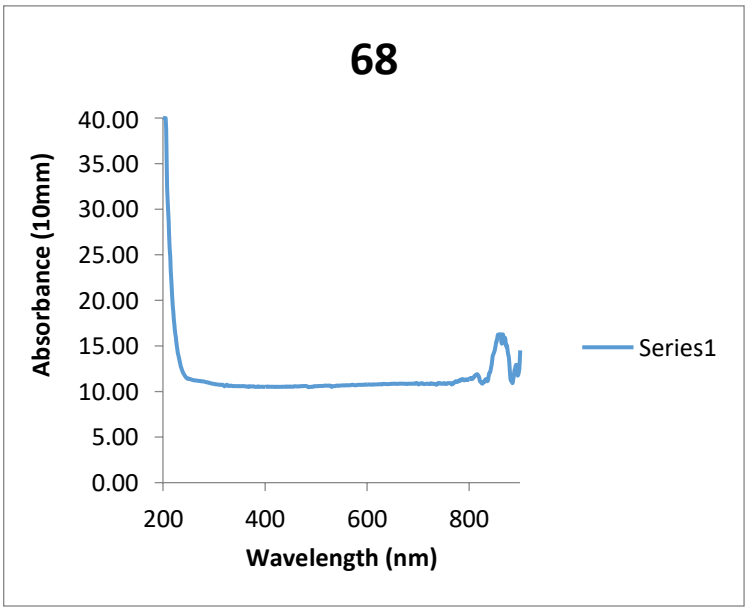
Isolate no 68, the highest production of secondary metabolites was at nutrient broth media as the bacterial isolate secretes flavonoids ,tannins ,saponin and cardiac glycosides.

Table . Preliminary phytochemical screening for isolate 68

	NB	LB	MSM	Ben	Gly-asp	Glu-asp
Flavonoids	+	-	-	+	-	-
Tannins	+	-	-	-	-	-
Card glyco	+	+	-	-	-	+
Saponin	+	+	-	+	-	-
Phlobtannins	-	-	-	-	-	-

UV–visible absorption spectroscopy

It is well known that selenium nanoparticles exhibit strong absorption band and generate specific color in solution. The formation of SeNPs was monitored by color change observation and UV–vis absorption spectroscopy. Figures 1 , 2 and 3 display the color change and UV–visible spectra of the mixture after reaction. The reduction of selenium ions into SeNPs during the presence of sodium selenite with bacterial metabolites was evidenced by the change of color from colorless to red which is attributed to the excitation of SeNPs.



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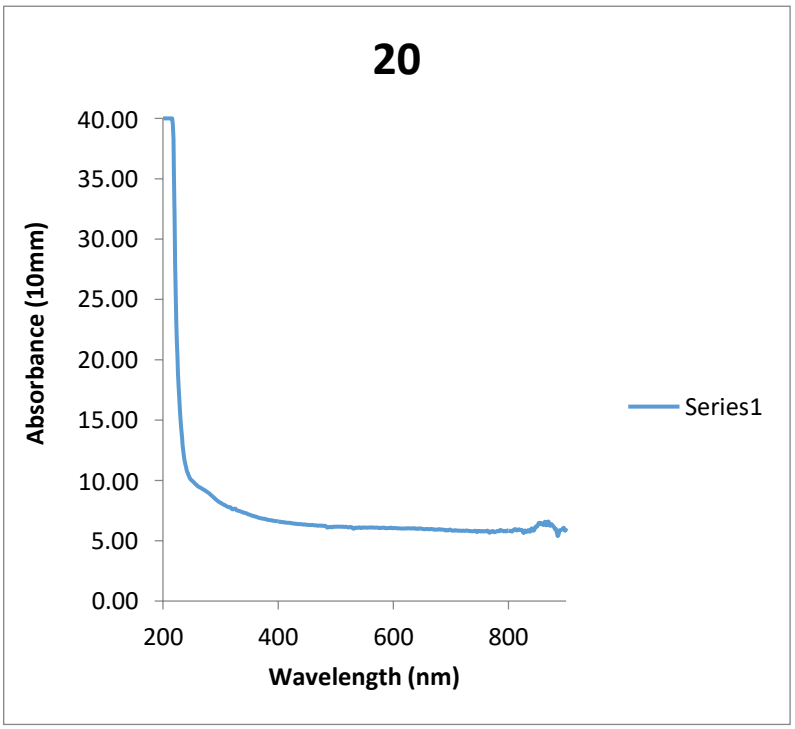

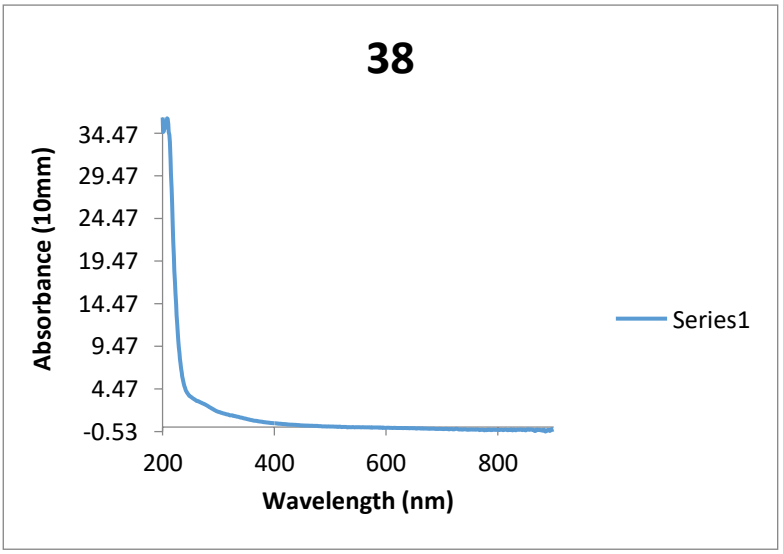

CONCLUSION

An eco-friendly and cost-effective protocol for the synthesis of selenium nanoparticles by utilizing natural source such as bacteria isolated from soil and waste water was proposed. a Gram +ve selenite reducing bacterial strains were used for the green biosynthesis of selenium nanoparticles from metalloid precursor sodium selenite. Bacteria reduce selenium ions to their respective nanoparticles which is confirmed by the change in colour of the medium and by UV-Vis spectroscopy. The proper condition for biosynthesis of selenium nanoparticles using the most tolerated isolates for high concentration of sodium selenite(7g/l) was optimized. It was found that the selenium nanoparticles synthesis reaction tended towards completion within 48 hour at room temperature, under aerobic conditions and slightly alkaline medium were beneficial for the formation of nanoparticles. These results are of great importance due to the low culture requirements with

the subsequent low cost of biologically active selenium nanoparticles production. The biosynthesised selenium nanoparticles were characterised by UV–visible absorption spectroscopy, EDX, TEM, XRD and FITR. The selenium nanoparticles were spherical in shape, and their average particle sizes determined by TEM were between 15 to 30 nm. The biogenic selenium nanoparticles exhibited good antibacterial activities against *Escherichia coli, Staphylococcus aureus, Staphylococcus MRSA, Bacillus subtilis, Serratia and Klebsiella penumoniae* . Further research on the selenium nanoparticles biosynthesised using bacteria could bring a promising application in the fields of medicine and hygiene. Further studies are needed in probing the enzymatic mechanisms of metalloid reduction.

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