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Synthesis of Selenium Nanoparticles by *Bacillus laterosporus* Using Gamma Radiation

A. I. El-Batal[1*], Tamer M. Essam[2], Dalia A. El-Zahaby[1] and Magdy A. Amin[2]

[1]Department of Drug Radiation Research, Biotechnology Division, National Center for Radiation Research and Technology, Cairo, Egypt.
[2]Department of Microbiology and Immunology Faculty of Pharmacy Cairo University, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. All authors designed the study, wrote the protocol, performed the experiments, managed the analysis of the study, managed the literature revise, wrote the paper. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: This study shows the possible synthesis of Selenium Nanoparticles (SeNPs) in aerobic optimized conditions using *Bacillus laterosporus (B. laterosporus)* bacterial strain.
Methodology: *B. laterosporus* was used to reduce selenium ions (selenite anions) to SeNPs by fermentation in Luria-Bertani Enrichment (EM) medium. Optimization of fermentation conditions using two-level full factorial design was performed. SeNPs were further characterized by UV-Vis., DLS, TEM, FT-IR, EDX and XRD analysis. SeNPs synthesis by Gamma irradiated *B. laterosporus* cells at different radiation doses was reported. Evaluation the probability of *B. laterosporus* to synthesis SeNPs by fermentation in skimmed milk aerobically. A microtiterplate assay was used to evaluate the ability of SeNPs to inhibit the biofilm formation of *Pseudomonas aeruginosa*. Evaluating the antimicrobial activity of some antibiotic agents upon addition of SeNPs was performed.
Results: *B. laterosporus* reduced the soluble, toxic, colorless selenium ions to the insoluble, non-toxic, red elemental SeNPs. Statistical analysis showed that the results were normally distributed. Temperature, incubation period and pH were significant factors in the fermentation process, in which the maximum SeNPs produced (8.37µmole/ml) was

**Corresponding author: E-mail: aelbatal2000@gmail.com;*

at temperature 37ºC, incubation period 48hr, pH7. The Gamma radiation exposure dose 1.5kGy gave the maximum SeNPs produced (10.01 µmole/ml). A pink color appear in the fermented milk revealing the formation of SeNPs-enriched milk. SeNPs inhibit the biofilm formation of *Pseudomonas aeruginosa* with a percentage reduction of 99.7%. SeNPs increase the antibacterial activity of fucidic acid by 13.6% and 28.5% against *Escherichia coli* and *Staphylococcus aureus* respectively. But with Gentamycin sulphate, no change in the antibacterial activity.

Conclusion: SeNPs can be synthesized aerobically by the probiotic *B. laterosporus* bacterial strain. SeNPs can be incorporated in nutraceuticals and functional foods like milk also can be used to inhibit the bacterial biofilm formation and can be added to some antibacterial creams to enhance their antimicrobial activity.

Keywords: Selenium nanoparticles; Bacillus laterosporus; Gamma radiation; Factorial design; Biofilm inhibition; Selenium fermented milk.

1. INTRODUCTION

Selenium is a trace element commonly found in materials of the earth's crust. Selenium in nanoparticle form, is well known for its photoelectric, semiconductor, free-radical scavenging [1], anti-oxidant [2] and anti-cancer properties [3-6]. Selenium occurs in different forms as red amorphous selenium (Se^{o}), highly water soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), and as gaseous selenide (Se^{2-}). Amongst its various forms, the SeO_3^{2-} is highly toxic, which adversely affect the cellular respiration and antioxidant system causes protein inactivation and DNA repair inhibition. [7-9]. Therefore, detoxification of SeO_3^{2-} has attracted a great deal of attention, particularly the reduction of this oxyanion by the microorganisms[10].

Most methods used to synthesize SeNPs are physical and chemical methods, they are characterized by elevated temperatures, long growth times, high pressures, low yields and are also environmentally hazardous [11]. There is an interesting and exciting biogenic synthesis to prepare SeNPs, the synthesis of SeNPs by biological systems occurs at close to ambient temperatures and pressures and at neutral pH [12]. This method is a clean, nontoxic and environmentally friendly procedure. In addition, biological synthesis can present extra advantages over chemical methods such as higher productivity and lower cost [13].

Attempts have been made to synthesize SeNPs from such microorganisms as bacteria, fungi [14] and yeast [15]. However, a bacterial system is preferred mainly due to reduced time of reaction, ease in handling, and easy genetic manipulation [16].

The majority of studies on the biogenesis of SeNPs have focused on anaerobic systems. However, anaerobic conditions have limitations, such as culture conditions and isolate characteristics that make optimization and scale up in bio-manufacturing processes tedious and challenging [17]. Selenium-tolerant aerobic microorganisms may provide an opportunity to overcome these limitations in the biosynthetic processes. Previous studies [18-22] have reported the aerobic formation of SeNPs by microorganisms, the generation of selenium nanospheres by soil bacteria *Pseudomonas aeruginosa, Bacillus* sp., *Enterobacter cloacae, Bacillus mycoides* SeITE01, *Duganella sp.* and *Agrobacterium sp.* under aerobic conditions.

So, the probiotic *Bacillus laterosporus* bacterial strain [23] was used for its aerobic cultivation.

Fermentation of Se-enriched milk is an interesting way of increasing the human intake of organic compounds of this element for a number of reasons: Milk is usually present in traditional meals, consumed regularly in moderate amounts, affordable, and already supplies the body with a significant amount of Se, at least 50% of the RDA(recommended daily allowance) [24]. So, Se-enriched fermented milk was reported.

It was reported that SeNPs prevent biofilm formation on polycarbonate medical devices [25]. Hence, a microtiter-plate screening method for biofilm inhibition using SeNPs was reported.

Although a number of different soluble metal ions or metal complexes show significant antimicrobial activity against a wide range of microorganisms, use of these materials as topical or systematic applications have been limited for various reasons, such as toxicity to biological systems. In the case of selenium, its antioxidant and pro-oxidant effects, or its bioavailability and toxicity, depend on its chemical form. Elemental selenium with a redox state of zero is not soluble in water and is generally considered to be biologically inert. Thus, the toxicity of elemental selenium is less than that of selenate or selenite ions. However, elemental selenium when supplied in the form of nanoparticles, have been reported for antimicrobial activity against pathogens causing nosocomial infection [15]. So can serve as a potent ingredient for the preparation of antifungal and antibacterial formulations[26]. According to these findings, the Antimicrobial activity of some antibacterial creams upon addition of SeNPs was reported.

The aim of the present investigation was to study the possible synthesis and characterization of SeNPs in aerobic conditions by Se-reducing probiotic *Bacillus laterosporus* bacterial strain.

2. MATERIALS AND METHODS

2.1 Materials

All the media, chemicals, reagents and sodium hydrogen selenite($NaHSeO_3$)used in the following experiments were of analytical grade and used without further purification.

B. laterosporus isolated and maintained on Luria-bertani (LB) agar slants, incubated at 37ºC for 3 days, then stored at 4ºC and subcultured routinely every month for culture maintenance. L.B medium and Enrichement(EM) medium(for the growth and synthesis of SeNPs) were prepared as described in [16]. LB medium: 1% tryptone, 0.5% yeast, 1% sodium chloride adjusted at pH7 with 5M NaOH. Enrichment (EM) medium: 0.05% sodium nitrate, 0.5% sodium chloride, 0.01% ammonium chloride, 0.27% dipotassium hydrogen phosphate, 0.3% tryptone, 0.1% beef, 0.05% yeast extract, 0.3% glucose) with pH7. The water used in this experiment was double distilled water.

2.2 Bacterial Growth Conditions and Synthesis of SeNPs Using *B. laterosporus*

Bacterial growth conditions and synthesis of SeNPs were prepared according to the method described in [16] with slight modification. Briefly, for the growth and activation of the

bacterium, seed culture was inoculated in 50ml sterile LB medium in 250ml Erlenmeyer flask. The flask was then incubated at 37ºC on (LAB-Line® Orbit Environ) rotatory shaker (150rpm). After 24hr of bacterial growth, the *B. laterosporus* was collected for further experiments. Then, for synthesis of SeNPs, 4mM sodium hydrogen selenite (molecular weight 150.96 contain 25.3% elemental Se) and 1ml activated *B. laterosporus* approximately 1-6×10^8 CFU/ml were added into 50ml sterile EM medium. This reaction solution was allowed to react completely for 48hr at 37ºC on (LAB-Line® Orbit Environ) rotatory shaker (150rpm).

2.3 Measurement of Elemental Selenium (SeNPs) Produced by Bacterial Reduction of Selenite

This method employs the use of 1M Na_2S to convert the insoluble SeNPs to a red-brown solution and with this method there is a direct correlation between concentration of SeNPs and the absorption at 360nm (λmax of bacterial SeNPs). Both intracellular and extracellular SeNPs would be measured by this method. A standard for elemental selenium (Se^o) was constructed using black powdered selenium metal as described in [27]. Selenium was placed in test tubes to give values ranging from 2 to 14µmol/ml of elemental selenium. One milliliter of 1M Na_2S was added to each test tube and absorption was measured at 360nm. In order to determine the amount of selenium nanoparticles produced by *Bacillus* strains, the bacterial fermented culture (50ml) along with the insoluble red SeNPs were gently mixed and transferred to 50ml centrifuge tubes. After centrifugation at 6,000rpm for 10 minutes at 6ºC, bacterial cells and SeNPs were collected as a pellet. To remove non-metabolized Selenite, pellets were washed twice with 10ml of 1M NaCl. This high salt concentration of NaCl was employed because 1M NaCl was effective in collection of colloidal elemental sulphur [28]. The red SeNPs in the pellet of the centrifuge bottle were dissolved in 10ml of 1M Na_2S and after centrifugation to remove bacterial cells, absorption of the red-brown solution was measured at 360nm (λmax of bacterial SeNPs) using (JASCO V-560 UV-visible spectrophotometer).

2.4 Optimization of Medium Components for SeNPs Production

The influence of physical factors on SeNPs production represented by optical densities(O.D) (correlated to their corresponding concentrations), was evaluated using a full two-level factorial design which mean seven factors and two levels (low -, high +), with a total of 128 runs. The independent variables include: pH (6, 7), temperature (30, 37ºC), incubation period (24, 48hr), sodium chloride (0.5, 1%), sodium nitrate (0.05, 0.1%), ammonium chloride (0.01, 0.02%), yeast extract (0.05, 0.1%) against the dependent variable the optical density of SeNPs. The statistical analysis and results of optimization were analyzed by using the Software Minitab 16.

2.5 Gamma Irradiation Process

The process of irradiation was carried out at the National Center for Radiation Research and Technology (NCRRT), Egypt. The facility used was Co-60 Gamma chamber 4000-A-India. Irradiation was performed using Co-60 Gamma rays at a dose rate of 0.919Gy/s and a specific activity of 3496.8 curie at the time of the experiment.

The bacterial cells were exposed to different doses: 0.5, 1, 1.5, 2, 3, 4, 5 and 6 kGy [29]. The effect of radiation was estimated by measuring the concentrations of Selenium nanoparticles produced by the irradiated cells used in fermentation.

2.6 Characterization of SeNPs.

The aqueous selenium ions were reduced to SeNPs which can be detected visually by a color change from yellow to red.

UV/Vis spectra of SeNPs were recorded as a function of wavelength using JASCO V-560 UV/Vis spectrophotometer from 200–900nm operated at a resolution of 1nm.

Average particle size and size distribution were determined by PSS-NICOMP 380-DLS particle sizing system St. Barbara, California, USA.

FT-IR measurements were carried out in order to obtain information about chemical groups present around SeNPs for their stabilization and understand the transformation of functional groups due to reduction process. The measurements were carried out using JASCO FT/IR-6300 infra-red spectrometer by employing KBr pellet technique.

The size and morphology of the synthesized nanoparticles were recorded by using TEM model JEOL electron microscope JEM-100 CX. TEM studies were prepared by drop coating SeNPs onto carbon-coated TEM grids. The film on the TEM grids were allowed to dry, the extra solution was removed using a blotting paper.

X-Ray Diffraction patterns were obtained with The XRD-6000 series, including stress analysis, residual austenite quantitation, crystallite size/lattice strain, crystallinity calculation, materials analysis via overlaid X-ray diffraction patterns Shimadzu apparatus using nickel-filter and Cu-Ka target, Shimadzu Scientific Instruments (SSI), Kyoto, Japan.

Elemental composition analysis with energy dispersive x-ray was carried out to ascertain the reduction of Selenite to elemental selenium [19]. EDX spectrum was measured at 10 KV accelerating voltage using EDX-model-OXFORD spectroscopy coupled to scanning electron microscope SEM- JEOL-JEM-5400 that is equipped with an EDX detector.

2.7 Aerobic Formation of SeNPs-enriched Fermented Milk Using Probiotic *B. laterosporus*

In an Erlenmeyer flasks (250 ml) each containing 100ml pasteurized skimmed milk, different concentrations of a filter-sterilized sodium hydrogen selenite 100, 200, 400, 500ppm (1ppm=1µg/ml) were added before each flask was inoculated with 5ml activated *B. laterosporus* approximately 1-6×10^8 CFU/ml. Incubation was aerobically for 24hr in rotatory shaker at 37ºC and concentrations of SeNPs produced were evaluated.

2.8 Microtiter-plate Assay for Testing Biofilm Inhibition by SeNPs

The assay was done according to the method described in [30-32] with slight modification. Briefly, activated *Pseudomonas aeruginosa* culture medium was diluted to 100 fold to be approximatly 10^6CFU/ml. Using sterile 96-well U-bottomed microtiter plate [30]. 50µl of the diluted microbial culture was added into each well. In case of control wells another 50µl of

the sterile fermentation media without SeNPs was added. while in Test wells 50µl of 100 ppm (1ppm=1µg/ml) SeNPs was added. Test was done in triplicate by mixing the contents of each well using pipett. This was incubated aerobically at 37°C for 48hr. The planktonic cells were removed then the plate was washed and dried. Fixation of the adherent biofilm by adding 100µl 95% ethanol. Staining with 100µl crystal violet 1% w/v. In order to quantify adhered cells, solubilize the dye by adding 200µl 95% ethanol as decolouring solution. Then 125µl was drawn from each well to measure the optical density at 540nm (λmax of crystal violet) using ELISA reader.

2.9 Antimicrobial Activity of Some Creams Containing Antibacterial Agents upon Addition of SeNPs

The effect of SeNPs on the antimicrobial activity of some antibacterial creams (2% Fucidic acid, 0.1% Gentamycin sulphate) was evaluated by agar well diffusion method against different kinds of pathogenic bacteria, the Gram negative *E. coli* (ATCC 7839) and the Gram positive *Staphylococcus aureus* (ATCC 6538). Standardized suspension of each tested strain 2-4×10^8CFU/ml for bacteria was swabbed uniformly onto sterile nutrient agar plates using sterile cotton swab. Wells of 10mm diameter were bored into the agar medium using cork borer. Using a pipette, 1ml of antibiotic cream emulsion (0.5gm/ml) was added to one well and 1ml of mixed solution of antibiotic (0.5gm/ml) and SeNPs (50ppm) was added to the other well. After incubation at 37ºC for 24hrs, the different levels of zone of inhibition were measured and interpreted using the CLSI zone diameter interpretive standards [33].

3. RESULTS AND DISCUSSION

3.1 Bacterial Synthesis of SeNPs

Bacillus laterosporus formed reddish cell suspension, which indicated its ability to reduce the toxic, colorless, soluble Selenite (SeO_3^{2-}) ions to nontoxic, red elemental, insoluble SeNPs [19]. The characteristic red color of SeNPs produced is due to excitation of the surface Plasmon vibrations of Selenium particles and provided a convenient spectroscopic signature of their formation [16].

3.1.1Localization of reduced Selenite in the bacterial cells

Studies have shown that active efflux of the metal is a frequently utilized strategy to produce tolerance by lowering the intracellular concentration to subtoxic levels [34]. However, in the study, intracellular SeNPs were formed, suggesting that efflux pumps probably do not mediate the metalloid tolerance mechanism in strain *Bacillus laterosporus* since Selenite tolerance is associated to an intracellular reduction of these oxyanions and then by their accumulation inside the cytoplasm or periplasmic space of the bacterial cell and subsequent exudation by the bacterial cell.

3.1.2 Proposed mechanism of Selenite detoxification and formation of SeNPs

It is proposed that selenite anions are being reduced in the cell protein fractions (cytosolic and membrane-associated). The reduction seems to be initiated by electron-transfer from NADPH/NADH by NADPH/NADH dependant selenate reductase enzymes as electron carrier, bring about the reduction of selenium (selenite/selenate) oxyions [19]. Another proposed mechanism is that, selenite can be reduced to SeNPs by reaction with reactive

thiol groups of protien/peptides (activated at the plasma membrane) which has been suggested as a general microbial detoxification reaction to oxyanions. One of these protien/peptides is glutathione (GSH)/glutathione reductase (GR) system responsible in the formation of SeNPs from selenite. The other protien/peptides is thioredoxin/thioredoxin reductase which is induced in bacilli when exposed to selenite also responsible for the formation of SeNPs. These protien/peptides may function as oxido-reductase enzymes or proton antitransporters [20]. SeO_3^{2-} would be reduced to form Seº seeds by interacting with these proteins. Sequentially, Seº seeds would grow into large SeNPs by further reduction of SeO_3^{2-} and aggregation of Se atoms through an Ostwald ripening mechanism [35]. Another proposed mechanism is that, the conversion of unstable enol pyrovate to stereo chemically stable keto pyruvate in glycolysis remains continuously operative in the cytosol there by making H^+ available all the time. This availability of hydrogen atoms might facilitate the synthesis of SeNPs [36]. All this provides an amenable environment for synthesis of SeNPs.

3.2 Measurement of Elemental Selenium Produced by Bacterial Reduction of Selenite Using Na_2S Solution

A red-brown liquid without any turbidity resulted from the addition of Na_2S to the red SeNPs. The absorption spectra of the red-brown selenium-sulfide liquid revealed that there was a distinctive peak at 360nm (Fig. 5). Both intracellular and extracellular SeNPs would be measured by Na_2S, as the alkalinity of the 1M Na_2S solution would dissolve cell membranes and account for measurement of intracellular SeNPs deposits as well as SeNPs attached to extracellular protein [27].

As seen in Fig. 1, the correlation between different concentrations and optical densities was directly proportional till 8µmol/ml (linear relationship) then a plateau was observed, the direct proportionality alone gives a linear straight line, by using the linear equation (Eq. 1) we got the concentrations of different optical densities which is

$$y = 0.306\,x + 0.4465 \quad \text{..... (1),}$$

In which y is the optical density and x is the concentration in µmol/ml.

3.3 Optimization of Medium Components for SeNPs Concentrations by Statistical Analysis

The results of statistical analysis for optimization of physical factors in SeNPs production using the software Minitab 16. The maximum value for SeNPs production was 8.35µmole/ml which was observed in the following conditions; (gm%) sodium chloride:1, sodium nitrate: 0.1, ammonium chloride: 0.02, yeast extract : 0.1, pH:7 (which is the most effective pH showed highest growth rate for *Bacillus*) [37], Temp: 37ºC, Incubation period: 48hr (Table 1).

3.3.1 Normal probability plot

The normal probability plot show that the results are normally distributed which mean that the whole experiment is stable (Fig. 2).

Table 1. Effect of seven variables and their interactions using two level full factorial design on SeNPs concentration. Produced by *Bacillus laterosporus* under liquid state fermentation

No. of run	pH	Temp. °C	Incubation period (hr)	Na Cl %	$NaNO_3$ %	NH_4Cl %	yeast ext.%	Concentration of SeNP in *(µmol/ml)
1	+	+	+	-	+	+	+	5.86
2	-	-	+	+	+	+	+	2.02
3	+	+	-	+	+	+	+	6.94
4	+	-	+	-	-	-	+	5.75
5	+	+	-	+	-	-	-	3.65
6	-	+	-	+	-	+	-	5.17
7	+	-	+	-	+	+	+	3.30
8	+	+	+	-	+	-	+	8.04
9	+	+	+	+	-	+	-	7.21
10	-	-	+	-	-	-	-	3.65
11	+	-	+	-	+	+	-	3.40
12	-	+	-	+	-	+	+	5.57
13	+	+	-	+	+	-	+	7.95
14	-	+	+	-	-	+	+	7.87
15	-	+	+	-	-	-	-	6.74
16	+	-	-	+	-	+	-	4.78
17	+	-	+	+	+	-	-	7.77
18	+	+	-	-	+	-	+	7.66
19	-	+	+	+	-	-	+	7.64
20	+	-	-	+	+	+	+	4.45
21	-	+	-	-	-	-	-	6.01
22	-	+	+	-	-	-	+	7.54
23	-	-	+	+	-	-	-	4.20
24	-	+	-	-	+	+	-	4.78
25	+	-	+	+	-	+	+	4.18
26	+	+	+	+	+	-	+	7.70
27	-	+	+	+	-	-	-	6.90
28	+	-	-	-	-	-	+	3.25
29	-	-	-	+	+	+	+	3.67
30	+	+	-	+	-	+	+	6.66

31	+	+	-	-	-	+	+	4.78
32	+	-	-	+	-	+	+	2.72
33	+	-	-	-	-	+	-	5.32
34	-	+	-	-	-	+	-	5.91
35	-	-	+	+	+	-	+	4.78
36	+	+	+	-	-	+	+	7.91
37	-	+	+	-	+	+	+	7.05
38	-	-	-	-	-	-	-	2.22
39	-	-	-	-	+	+	-	3.80
40	+	-	+	+	-	+	-	6.55
41	-	+	-	+	+	-	-	4.86
42	-	-	+	-	+	-	-	7.05
43	-	+	-	+	+	+	+	5.33
44	+	+	+	+	-	+	+	6.86
45	+	+	-	+	-	-	+	6.84
46	+	+	+	-	+	+	-	7.54
47	+	+	-	-	+	-	-	7.11
48	-	+	-	-	+	-	+	6.43
49	+	+	-	-	-	-	+	6.36
50	-	-	+	+	+	+	-	4.99
51	-	-	+	-	+	+	-	7.62
52	+	+	-	-	+	+	+	6.17
53	+	+	+	+	+	+	-	8.02
54	+	-	+	-	+	-	-	5.90
55	-	+	+	+	+	+	-	6.78
56	-	+	+	+	-	+	-	7.21
57	+	-	-	+	-	-	+	3.82
58	-	-	-	-	-	+	-	1.92
59	+	+	+	-	-	-	-	6.78
60	-	+	-	+	-	-	+	6.42
61	-	+	+	-	-	+	-	7.76
62	+	-	+	-	-	+	-	7.94
63	-	+	-	-	-	+	+	5.07
64	+	+	-	+	+	+	-	8.09
65	-	-	-	-	-	-	+	2.75

66	+	-	-	-	+	-	+	4.45
67	-	+	-	-	+	+	+	6.47
68	+	-	-	-	-	-	-	3.62
69	-	-	-	+	+	+	-	5.41
70	+	-	+	+	+	+	+	7.96
71	-	+	-	+	+	+	-	6.35
72	+	+	+	-	-	+	-	7.58
73	-	+	-	-	-	-	+	6.56
74	-	-	-	-	+	-	+	3.13
75	+	-	-	+	+	-	-	4.78
76	-	-	-	+	-	+	-	3.72
77	+	-	-	-	+	+	+	2.61
78	+	-	+	-	-	+	+	6.23
79	-	+	+	+	+	+	+	7.28
80	+	+	-	+	-	+	-	7.76
81	-	-	-	+	+	-	+	3.06
82	+	+	+	-	-	-	+	7.35
83	-	-	-	+	-	-	+	3.38
84	+	+	-	-	-	-	-	6.61
85	-	-	-	-	+	-	-	2.84
86	+	-	-	+	+	+	-	2.26
87	+	-	+	+	+	+	-	7.95
88	-	-	+	+	+	-	-	7.26
89	+	-	+	+	+	-	+	7.69
90	+	+	-	-	+	+	-	6.75
91	+	-	-	-	-	+	+	6.01
92	-	+	+	-	+	-	+	6.47
93	-	-	-	-	-	+	+	7.34
94	-	-	+	-	+	+	+	4.26
95	+	-	-	-	+	-	-	6.79
96	+	-	+	-	+	-	+	6.45
97	+	+	-	-	-	+	-	7.09
98	-	-	-	-	+	+	+	6.37
99	-	+	-	-	+	-	-	6.45
100	-	-	+	-	-	+	-	7.63

101	-	+	+	+	+	-	-	6.71
102	-	+	+	+	+	-	+	6.11
103	-	+	+	+	-	+	+	5.73
104	+	+	+	+	-	-	-	7.95
105	+	-	-	+	-	-	-	7.64
106	+	+	-	+	+	-	-	7.14
107	-	-	-	+	-	-	-	7.76
108	-	-	-	+	-	+	+	6.36
109	+	+	+	+	-	-	+	8.12
110	+	-	+	-	-	-	-	7.84
111	-	-	+	-	-	-	+	6.23
112	+	+	+	+	+	-	-	3.91
113	+	-	+	+	-	-	+	7.44
114	-	-	+	+	-	+	+	4.45
115	-	+	+	-	+	-	-	7.09
116	+	+	+	-	+	-	-	8.34
117	-	+	-	+	-	-	-	6.31
118	-	+	+	-	+	+	-	6.90
119	-	-	-	+	+	-	-	6.66
120	-	-	+	+	-	-	+	7.45
121	-	-	+	+	-	+	-	6.42
122	+	-	+	+	-	-	-	3.91
123	-	-	+	-	-	+	+	6.27
124	-	-	+	-	+	-	+	6.16
125	-	+	-	+	+	-	+	6.24
126	+	-	-	-	+	+	-	6.15
127	+	-	-	+	+	-	+	6.32
128	+	+	+	+	+	+	+	8.35

***The different variables and their levels used for optimization of SeNP productivity**

Levels	pH	Temp ºC	Incubation period(hr)	NaCl %	$NaNO_3$ %	NH_4Cl %	Yeast ext. %
low(-)	6	30	24	0.5	0.05	0.01	0.05
High(+)	7	37	48	1	0.1	0.02	0.1

**(µmol/ml): Concentration of SeNPs from 50ml fermented medium extracted in 10ml Na_2S solution*

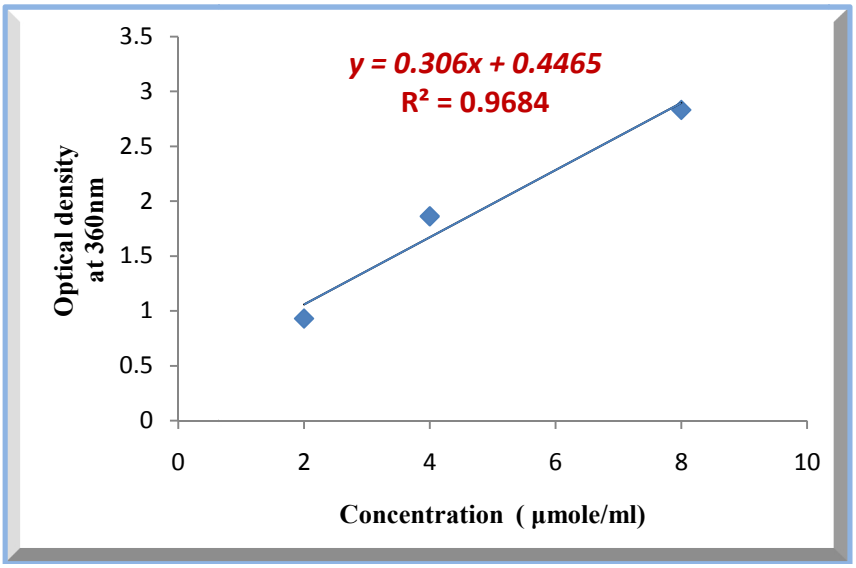

Fig. 1. Standard curve for Se^0NP

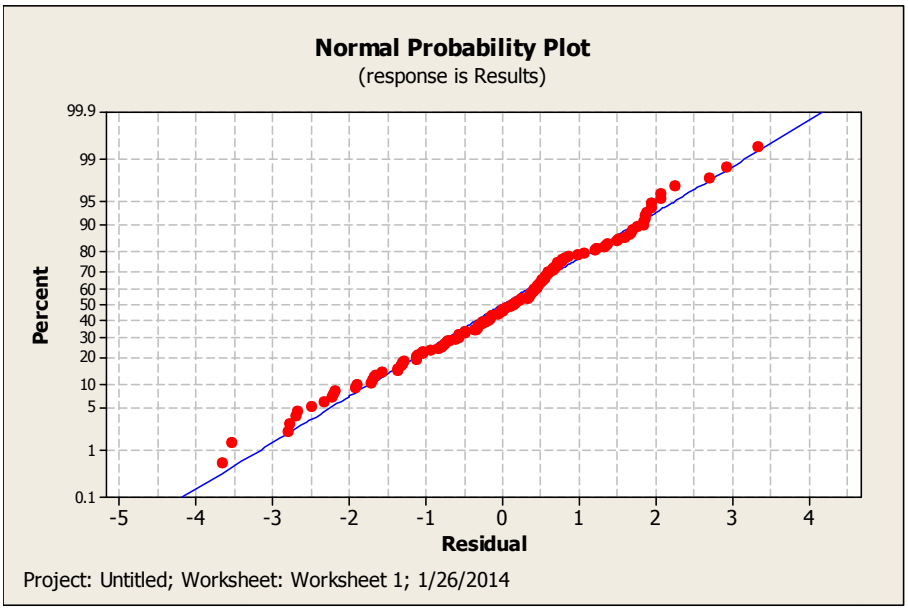

Fig. 2. Normal probability plot of the experiment

3.3.2 Normal plot of the standerdized effects

The results of the statistical analysis show the factors which have significant effect when $p<0.05$. It was found that the significant factors are Temperature, Incubation period and pH (Fig. 3) (Table 2).

Table 2. Regression coefficients for SeNPs concentration under physical parameters

Term	Effect	Coef	SECoef	T-value	P-value
Constant	---------	5.9911	0.1286	46.57	0.000
pH	0.05278	0.2639	0.1286	2.05	0.043*
Temp	1.4803	0.7402	0.1286	5.75	0.000*
Period	1.1431	0.5716	0.1286	4.44	0.000*
NaCl	0.0669	0.0334	0.1286	0.26	0.795
NaNO3	0.0238	0.0119	0.1286	0.09	0.927
Amm.Cl	-0.1428	-0.0714	0.1286	-0.56	0.580
Y.extract	-0.1809	-0.0905	0.1286	-0.70	0.484
pH*Temp	0.0778	0.0389	0.1286	0.30	0.763
pH*Period	-0.0413	-0.0206	0.1286	-0.16	0.873
pH*NaCl	0.1337	0.0669	0.1286	0.52	0.604
pH*NaNO3	0.2044	0.1022	0.1286	0.79	0.429
pH*Amm.cl	-0.1559	-0.0780	0.1286	-0.61	0.546
pH*Y.extract	-0.0678	-0.0339	0.1286	-0.26	0.793
Temp*Period	-0.2737	-0.1369	0.1286	-1.06	0.290
Temp*NaCl	-0.1694	-0.0847	0.1286	-0.66	0.512
Temp*NaNO3	0.0688	0.0344	0.1286	0.27	0.790
Temp*Amm.cl	0.1053	0.0527	0.1286	0.41	0.683
Temp*Y.extract	0.3016	0.1508	0.1286	1.17	0.244
Period*NaCl	-0.2234	-0.1117	0.1286	-0.87	0.387
Period*NaNO3	-0.2234	-0.1117	0.1286	-0.87	0.387
Period*Amm.Cl	-0.0400	-0.0200	0.1286	-0.16	0.877
Period*Y.extract	-0.0381	-0.0191	0.1286	-0.15	0.882
NaCl*NaNO3	0.1016	0.0508	0.1286	0.39	0.694
NaCl*Amm.Cl	-0.2063	-0.1031	0.1286	-0.80	0.425
NaCl*Y.extract	-0.0250	-0.0125	0.1286	-0.10	0.923
NaNO3*Amm.Cl	-0.3081	-0.1541	0.1286	-1.20	0.234
NaNO3*Y.extract	-0.1544	-0.0772	0.1286	-0.60	0.550
Amm.Cl*Y.extract	-0.2772	-0.1386	0.1286	-1.08	0.284

*(*significant)*

3.4 Effect of Gamma irradiated *Bacillus laterosporus* Cells on the Synthesis of SeNPs

The bacterial cells were exposed to different doses : 0.5, 1, 1.5, 2, 3, 4, 5 and 6 kGy [29]. It was observed that by increasing the dose of radiation on the bacterial cells, SeNPs concentration increase with maximum 10.01ppm at a dose of 1.5kGy (Fig. 4) and then increasing the dose more lead to a decrease in production. This may be attributed to that at lower doses of gamma radiation, generation of reactive oxygen species (ROS) is well documented, the elimination of which by induction of reductases enzymes confers radiation protection on the cells. One of the reductases enzymes is glutathione (GSH)/glutathione reductase (GR) which is an abundant cellular thiol, has been implicated in numerous cellular processes and in protection against stress caused by xenobiotics, carcinogens and radiation [38,39], this enzyme system was proposed to has a role in reduction of selenite and formation of SeNPs [20]. The maximum SeNPs synthesized at radiation dose 1.5kGy, further increase in the radiation dose decreases SeNPs, due to gradual decrease in the enzyme activity production till complete inhibition of the enzyme activity. This could be explained by

damage or deterioration in the vitals of the microorganism as radiation causes rupturing in the cell membrane. This major injury to the cell allows the extracellular fluids to enter in to the cell. Inversely, it also allows leakage out of ions and nutrients which the cell brought inside [40].

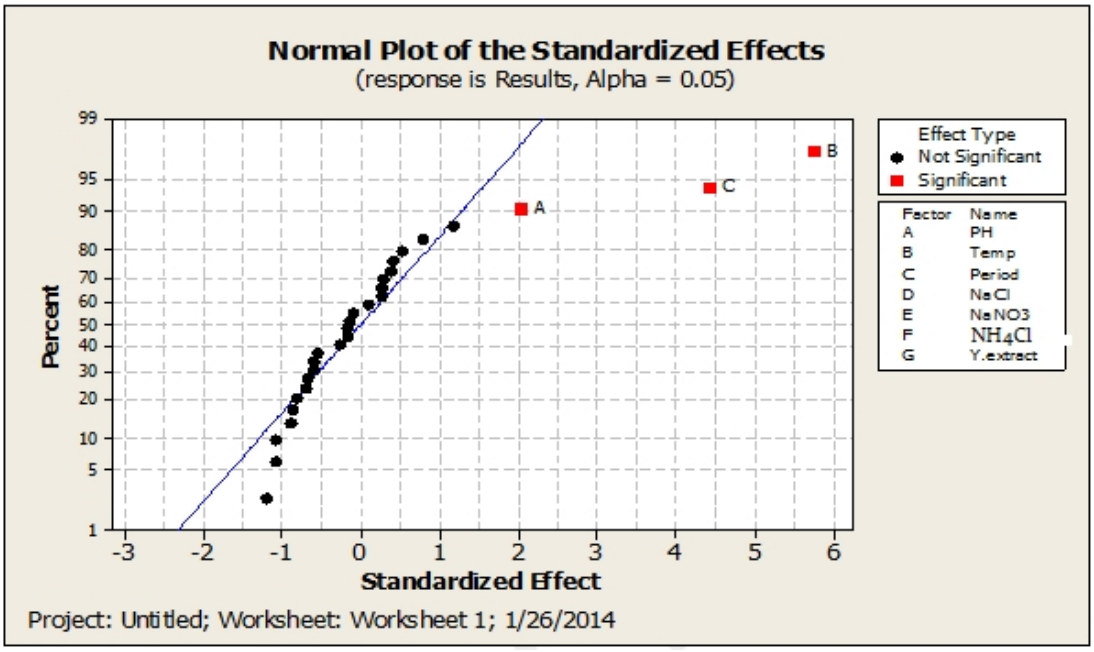

Fig. 3. Normal plot of the standardized effects

3.5 Characterization of SeNPs synthesized by *B. laterosporus*

3.5.1 UV-Visible spectrophotometer (UV-Vis.)

The reddish-brown SeNPs extracted by Na_2S have characteristic optical absorption spectrum in the UV-visible region, strong, broad peak located at 360nm (Fig. 5).

3.5.2 Transmission Electron Microscopy (TEM) for bacterial SeNPs

The TEM image shows dispersed SeNPs with spherical shape with size ranging from 40-70nm (Fig. 6).

3.5.3 Dynamic light scattering(DLS) for bacterial SeNPs

The size distribution was determined by DLS method and the average particle size of SeNPs was 74.8nm (Fig. 7), which is larger than TEM imaging and this is due to DLS analyze measures the hydrodynamic radius which take into consideration the native functional group of the protein on the surface of SeNPs.

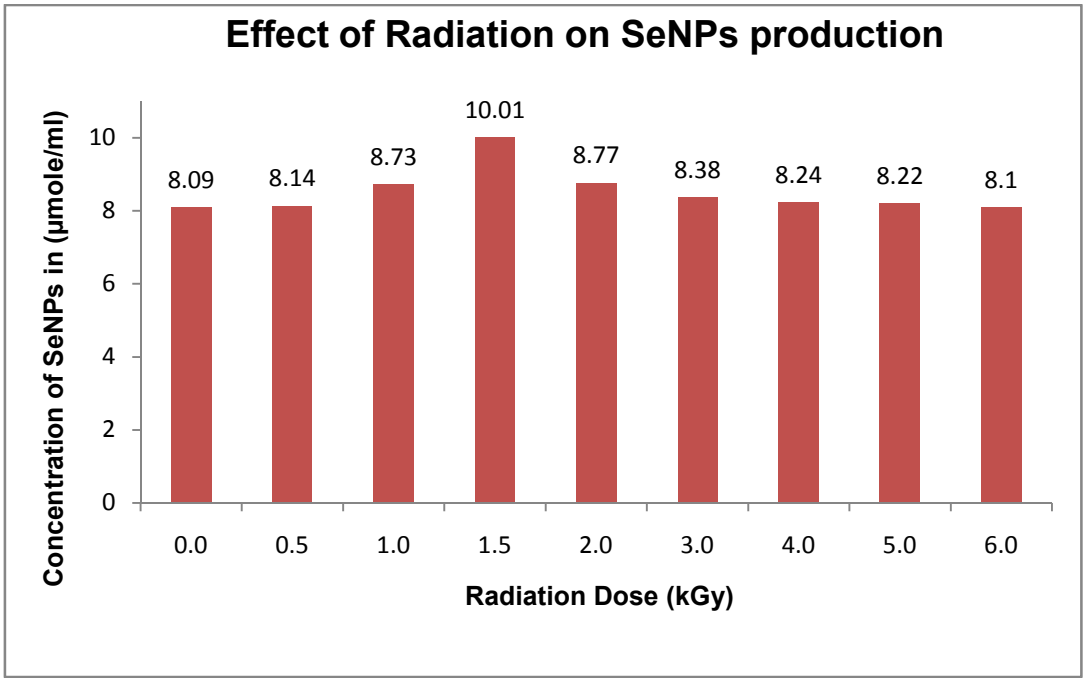

Fig. 4. Effect of radiation on the synthesis of SeNPs by *B. laterosporus*

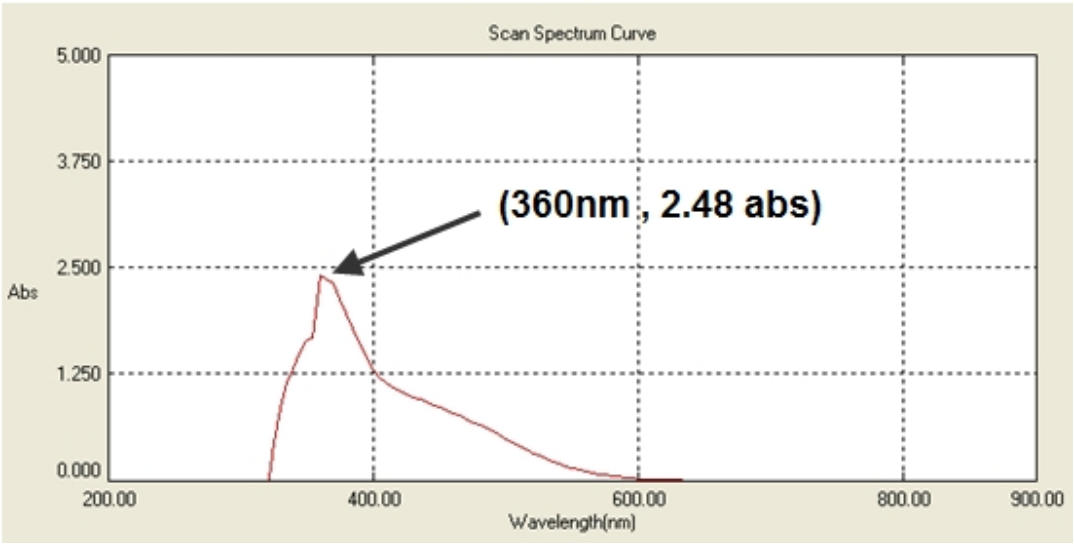

Fig. 5. The UV-Vis of SeNPs synthesized by *B. laterosporus*

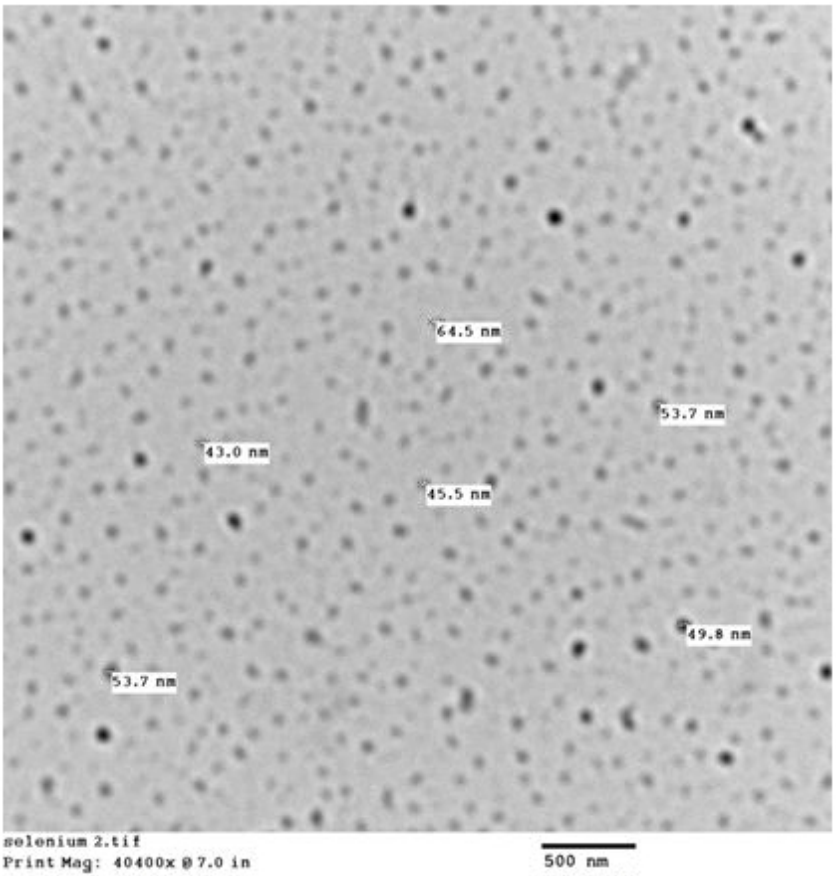

Fig. 6. TEM image of SeNPs synthesized by *B. laterosporus*

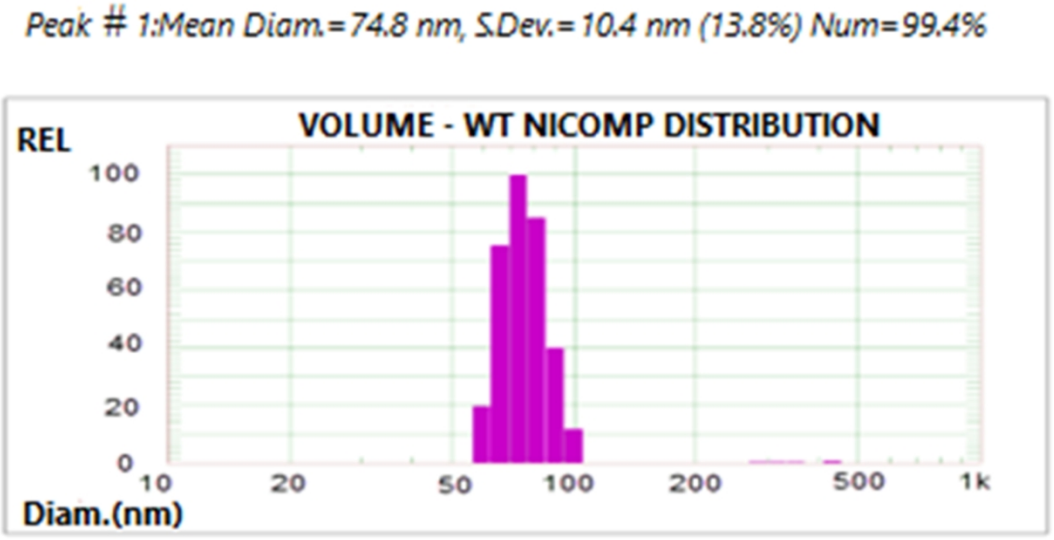

Fig. 7. DLS of SeNPs synthesized by *B. laterosporus*

3.5.4 Fourier transform infrared spectrometer (FT-IR) for bacterial SeNPs

The FT-IR spectra of the fermented culture media without SeNPs (Fig. 8a) and the fermented culture media with SeNPs (Fig. 8b) show weak transformation (Table 3), that revealing a weak interaction between Se atoms and the NH, C=O groups [35]. According to these results, it seemed that proteins were involved in the process of SeNPs formation.

Table 3. Peaks appeared in FT-IR spectra for fermented extract with and without SeNPs [40]

Peak number	Extract without SeNPs at (cm^{-1})	Extract with SeNPs at (cm^{-1})	Comment
1	3274.54	3548.38	The broad peaks are characteristic to the presence of $–NH_2$ amino group and –OH stretching groups.
2	2499.29	2090.46	Corresponding to aliphatic C-H stretching**.**
3	1631.48	1627.63	Characteristic to the carbonyl group.
4	1434.78	1419.35	May be ascribed for the presence of primary amine groupC-N stretching.
5	1137.8	1133.94	Corresponding to a primary amine (NH stretch vibrations of the proteins).
6	678.82	674.96	Signifies the presence of R-CH group.

3.5.5 X-Ray Diffraction analysis (XRD) of SeNPs

The XRD pattern obtained for the intracellular SeNPs with three intense peaks in the whole spectrum of 2θ values ranging from (0) to (80) is shown in (Fig. 9). The diffractions at 31.64º, 45.37º and 56.41º can be indexed to the (101), (111) and (112) planes of the face-centered cubic (fcc) SeNPs, respectively [10].

The full-width-at-half-maximum (FWHM) values measured for 101, 111 and 112 planes of reflection were used to calculate the size of the nanoparticles. The calculated average particle size of the intracellular produced SeNPs was determined to be 17.1nm, 20.81nm and 26.43nm respectively by using scherrer's relationship (Eq. 2).

$$D=K\lambda/\beta\cos\theta \quad \text{..... (2)}$$

where, D is the crystal size, k is constant equal 0.94, λ is wavelengh equal 15.4056nm, β is the full-width-at- half-maximum (FWHM) of the peaks, θ is the angle of diffraction.

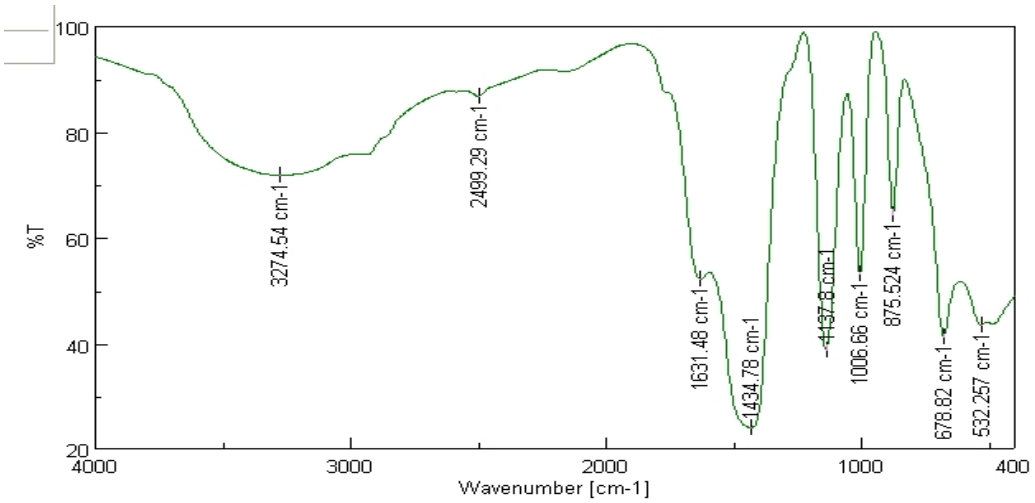

Fig. 8a. FT-IR spectra of the fermented culture media without selenium

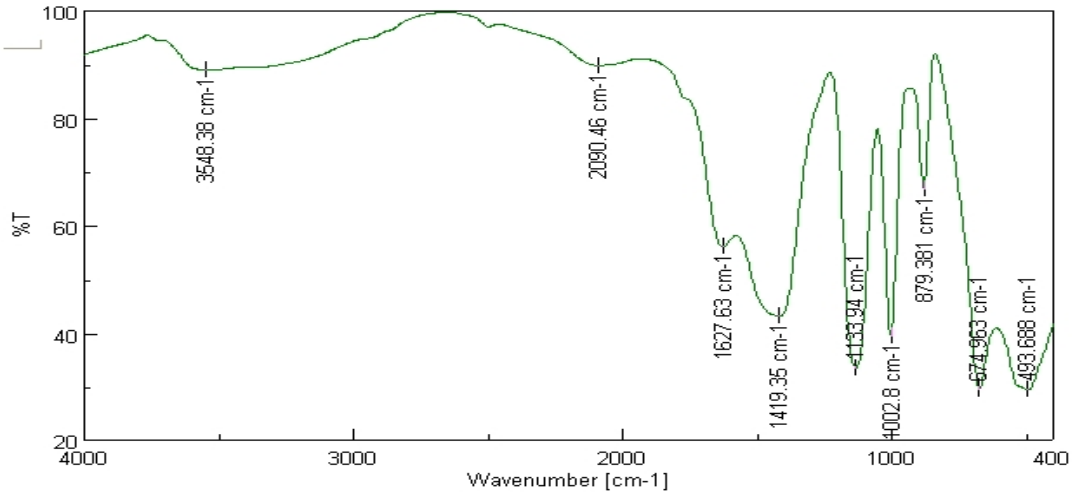

Fig. 8b. FT-IR spectra of the fermented culture media with SeNPs formation

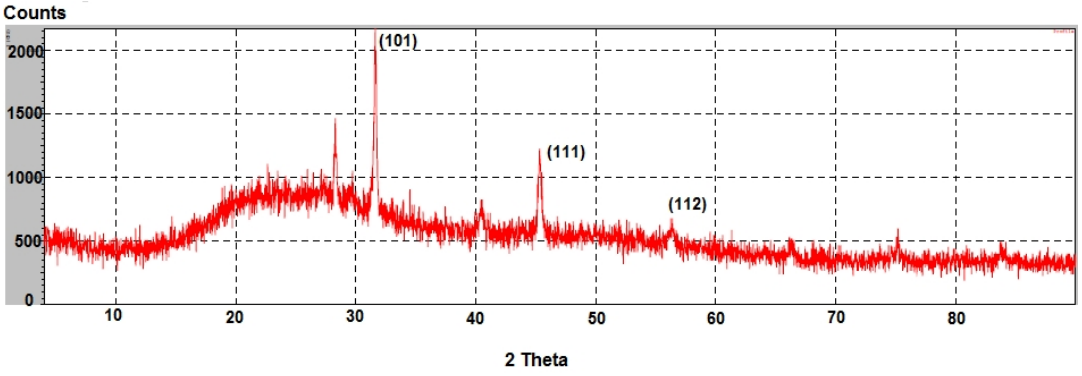

Fig. 9. XRD pattern of SeNPs synthesized by *B. laterosporus*

3.5.6 Energy Dispersive X-ray (EDX) analysis

In EDX analysis specific absorption peaks for SeNPs at 1.37 keV (peak SeLα), 11.22 keV (peak SeKα) and 12.49 keV (peak SeKβ) was produced. which indicate bacterial uptake and transformation of selenite to SeNPs that accumulate intracellular [19] (Fig. 10).

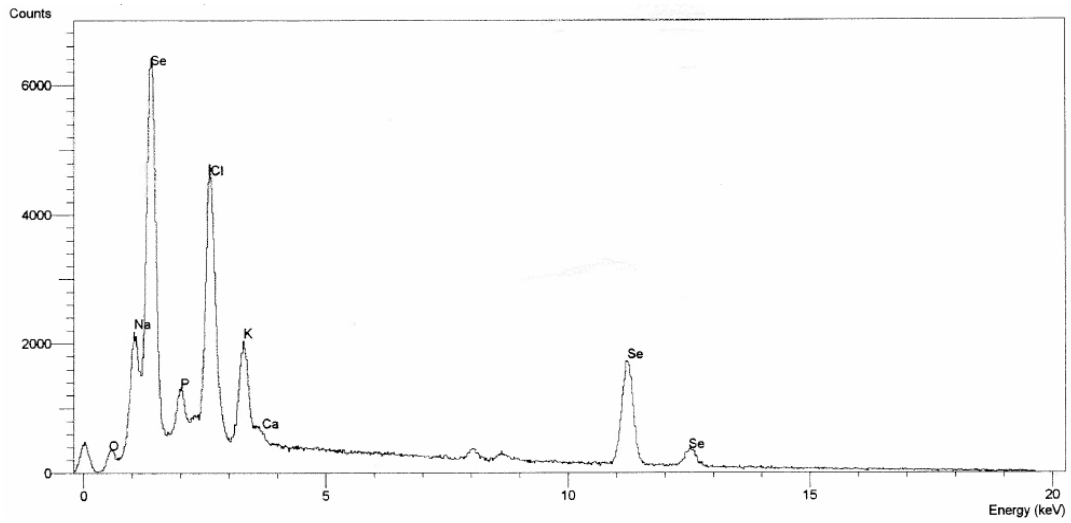

Fig. 10. EDX of *B. laterosporus* cells containing SeNPs

3.6 SeNPs Enriched Fermented Milk

After 24hr, reduction of Selenite in milk resulting in a pink color which reveal the formation of SeNPs (red color in white milk). By measuring the produced SeNPs concentrations, it was observed that the produced quantity decreased as the concentration of sodium hydrogen selenite used increased (Table 4). This may be due to the toxicity of selenite that lead to reduction of bacterial mass cells so less SeNPs produced. Formation of SeNPs-enriched fermented milk using probiotic *B. laterosporus* has advantage over probiotic Lactic acid bacteria [41] as it can be cultivated aerobically.

Table 4. Effect of increasing Selenite (SeO_3^{2-}) ions on SeNPs produced in fermented milk

Selenite concentration (ppm)	Weight of produced cells (mg/ml)	SeNPs (µmol/ml)
100 ppm	31.6	2.58
200 ppm	29.6	1.76
400 ppm	7.6	1.14
500 ppm	5.6	0.62

**Total volume of fermentation media 100ml*

3.7 Biofilm Inhibition by SeNPs Using Microtiterplate Assay

SeNPs were found to inhibit 99.7% of the biofilm formation of *Pseudomonas aeruginosa* by using a measure of efficacy called percentage reduction. The percentage reduction was

calculated from the blank, control, and treated absorbance values on a plate (Eq. 3) [31]. let B denote the average absorbance per well for blank wells (no biofilm, no treatment), C denote the average absorbance per well for control wells (biofilm, no treatment), and T denote the average absorbance per well for treated wells (biofilm and treatment). Since, Average control was 0.51, Average blank was 0.037 and Average test was 0.038.

$$\text{Percentage Reduction} = \{(C - B) - (T - B)/\, C - B\}\ 100\% \quad \text{.....(3)}$$

So, the percent reduction was 99.7%.

A possible mechanism toward inhibiting bacteria growth in biofilm, is that, SeNPs may serve as a catalyst oxidizing thiol groups and reducing oxygen to superoxide [42]. As thiol is an essential substance for bacteria cell function, selenium can inhibit bacteria by depleting their thiol levels. This intracellular thiol depletion mechanism is significant because healthy cells are more resilient to this effect than bacteria cells. However, the mechanism of selenium-inhibited bacteria growth in biofilms is likely complicated, and further studies are certainly required [25].

3.8 The Antimicrobial Activity of Some Antibacterial Cream upon Addition of SeNPs

It was found that adding SeNPs, increase the antibacterial activity of fucidic acid by 13.6% and 28.5% against *Escherichia coli* and *Staphylococcus aureus* respectively. But with Gentamycin sulphate, no change in the antibacterial activity (Table 5).

This may be attributed to the high surface areas of SeNPs that allow for more active sites for interacting with biological entities such as cells. The higher surface areas of nanoparticles compared with conventional micron-size particles also offer more sites for functionalization with other bioactive molecules [43].

Table 5. Effect of adding SeNPs to antibacterial agents represented by zone of inhibition

Tested strain	Average diameter of inhibition zone(mm)			
	F	FS	G	GS
E. coli	22	25	30	30
S. aureus	37	45	30	30

F: Fucidic acid, FS: Fucidic acid + SeNPs, G: Gentamycin sulphate, GS: Gentamycin sulphate + SeNPs

4. CONCLUSION

Bacillus laterosporus can reduce selenite (SeO_3^{2-}) ions to red SeNPs, which can be incorporated in nutraceuticals and functional foods like milk. SeNPs can inhibit the biofilm formation of *Pseudomonas aeruginosa,* and owing to the fact that selenium is a normal element in the body so, SeNPs can be used as coating for medical devices to prevent attachment of biofilm forming bacteria. SeNPs can be added to some antibiotic preparations to increase their antimicrobial activity.

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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