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Biological Synthesis of Selenium Nanoparticles and Evaluation of their Bioavailability

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ABSTRACT

Nanoparticles due to their unique properties have attracted more attention and their bacterial biosynthesis is more favorable because is environmental friendly and the size and yield of nanoparticles can be optimized. The aim of the present study was biosynthesis of Selenium nanoparticles using Bacillus cereus. *For this purpose, bacterial culture was prepared in the presence of sodium selenate solution and incubated (30°C, 24 h). The produced nanoparticles were purified through consequent centrifugation, washing with 0.9% NaCl, sonication, washing with Tris- HCl containing Sodium dodecyl sulfate (SDS) and finally isolation with water- octanol two phase systems. Then using Ultraviolet-Visible spectroscopy, dynamic light scattering (DLS), scaning electron microscopy (SEM) and X-ray diffraction (XRD) analysis, nanoparticle production was confirmed. The bioavailability of nanoparticles was also investigated in rat. As a result of this study spherical selenium nanoparticles with a mean diameter of 170 nm were biosynthesized. MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of selenium for* Bacillus cereus *were same and equal to 75mM. Absorption and secretion of nanoselenium was significantly higher than bulk Selenium ($P<0.05$). In conclusion in the present study without any chemical substance, spherical Selenium nanoparticles were produced that do not have any environmental contamination. Furthermore, the metabolism of these particles suggests higher absorption rate of them that facilitates its application in medicine and also veterinary medicine.*

Key words: *Bacillus cereus*; Green chemistry; Selenium, nanoparticle.

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INTRODUCTION

Nanomaterials due to the size effect phenomenon have unique and new functions [1]. Increasing of surface to volume ratio leads to increase in function and reactivity of surface atoms [1]. Nanoparticles are made of several atoms or molecules with different size and morphology including spherical, layered, clustered, tube or rod shape [1, 2]. Among nanoparticles, metallic and metalloid nanoparticles have been widely considered because of their catalytic, photo catalytic, absorbent, optical, electrical and magnetic applications. Top-down and bottom-up methods are two main approaches for nanoparticle synthesis [3]. In bottom- up method the product is made gradually by assembly of atoms [4]. In this procedure, that can be accomplished chemically or biologically, it is possible that atoms interactions be controlled [5].The chemical method uses chemical stabilizers that leads to environmental pollution [4]. In contrast, biological synthesis of nanoparticles is superior because is a clean, cost effective, easy to operate and non–toxic approach which produces nanoparticles with novel properties. Microorganisms are of great importance with this respect [3, 6]. Following encountering with metallic ions, microorganisms accumulate them inside cell or on cell wall with different methods that is finally leads to nanoparticle synthesis [3]. In the field of biological synthesis of nanoparticles, bacteria have been more focused due to their high resistance to metals and surviving under harsh environment. In this procedure, the size and yield of nanoparticle production can be optimized by adjusting pH, temperature, substrate concentration and time of exposure to substrate [7]. Biosynthesis of gold and silver nanoparticles by *Lactobalillus* sp. and *Bacillus* sp. are two examples [3, 7].

Selenium (Se) is a metalloid element that is found with different oxidation status and properties in nature. It is commonly found as complexes with nutrient sulfides or Ag, Cu, Pb and Ni [8]. This element which has photolytic and conductor properties, is applied in pesticides, glass industry and as food additive in animals and poultries meal [9]. It is a trace element found as selenomethionine, selenocysteine and also in a variety of enzymes [10]. In addition, it plays an important role in glutathione peroxidase system [10, 11] that in association with vitamin E can act as antioxidant for prevention of harmful effects of metabolites on tissues [12, 13]. Furthermore, it is important in male fertility, immune system function, neurotransmitters production and prevention of malignancies in such a manner that its deficiency leads to multiple dysfunctions including thyroid dysfunction [11, 14-17]. With regard to unique properties of selenium nanoparticles, their synthesis has been focused [18]. For this purpose different methods have been investigated. Liu et al (2004), have synthesized selenium nanoparticle by a reverse microemulsion system using sodium selenosulfate as selenium source. They found that hydrochloric acid concentration and reaction temperature have great influence on morphology of selenium nanoparticles [19]. Abdelouas et al (2000), produced selenium nanoparticles through application of cytochrome C_3 obtained from *Desulfovibrio vulgaris*, a sulfate reducing bacterium. This cytochrome was able to reduce selenate to selenium and so production of selenium nanoparticles [20]. In the study of Zhang et al (2004) the reduction of selenium was performed by ascorbic acid in the presence of different polysaccharides such as chitosan, konjac glucomannan, acasia gum and carboxymethyl cellulose. Their results revealed that very stable spherical selenium nanoparticles were produced that are stable in solution for 6 months in the presence of polysaccharide [21]. However, in spite of different possible approaches for selenium nanoparticle production, its biosynthesis has been paid more attention because is an environmental friendly procedure [22]. The aim of the present study was biosynthesis of selenium nanoparticles using a selenium resistant bacterium and evaluating its absorption in mice.

MATERIALS AND METHODS

Isolation of selenium resistant bacterium

The bacterial strain used in this study for selenium nanoparticle production was isolated from eastern pond of Imam Khomeini petrochemical industries complex, located in Mahshahr (30.5589° N, 49.1981° E), khouzestan, Iran. This isolate was identified based on biochemical tests f Bergey's manual of systematic bacteriology [20] and confirmed by 16S rRNA gene sequencing. For this purpose, DNA was extracted from 48h culture with DNA extraction kit (Cinagene, Iran) [13]. The Forward (5́CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3́) and reverse primers (5́CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT3́) were used for amplification of target gene in a reaction containing PCR buffer (1X), dNTPs (10 mM), $MgCl_2$ (2 mM), forward and reverse primers (10 µM), Taq DNA polymerase (1.5 U) and 3µl of template DNA in a final volume of 25µl [13,24]. Thermal cycling program was denaturation (94°C, 60 S), 30 cycles each including denaturation (94°C, 60s), annealing (60°C, 40s) and extension (72°C, 150s) and a final extension (72°C for 20 min) [13]. The PCR product was evaluated by electrophoresis in 1% agarose gel containing DNA safe stain and subsequent sequencing (Macrogen, Korea).The sequence was compared in BLAST algorithm with available data in gene bank of NCBI.

Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC)

For MIC and MBC determination, 100µl of bacterial suspension with 0.5 McFarland turbidity was cultured in a series of 1 ml Muller-Hinton broth (Merck, Germany) containing 0.5-1200 mM sodium selenate (Merck, Germany). A tube without sodium selenate was also regarded as control and all of them were incubated for 24 h at 30°C and 120 rpm. The first dilution in the mentioned series that didn't show any bacterial growth was regarded as MIC. A culture was subsequently prepared from those growth negative tubes on Muller-Hinton agar (Merck, Germany) in the absence of sodium selenate and incubated at 30°C for 24h. The least concentration that inhibited colony formation was considered as MBC. All of these experiments were repeated three times [25].

Growth curve

The bacterial growth curve was obtained by inoculating nutrient broth (Merck, Germany) with 0.5 McFarland bacterial suspension and incubation (30°C, 120 rpm). The absorbance of culture was recorded as triplicates at 600 nm every 2h till 62h of incubation [9, 24, 26]. In addition the growth curve was obtained in the presence of 0.5, 1.75 and 3 mM sodium selenate, according to the method described above.

Production and isolation of selenium nanoparticles

In order to find the best method for selenium nanoparticle production four treatments were designed. In fist two treatments, 1 ml of fresh bacterial suspension (0.5 McFarland turbidity) was inoculated in two separate 100 ml nutrient broth (Merck, Germany). For other two treatments, the two nutrient broth (100 ml) was supplemented with sodium selenate solution (100 $^{mg}/_{ml}$) and only one of them was inoculated with 1 ml of fresh bacterial suspension (0.5 McFarland turbidity). All flasks were incubated at 30°C for 24 h. Then, one of the bacterial suspensions in

nutrient broth (from the first two experiments) was centrifuged (15 min, 6000rpm), the harvested supernatant was mixed in 1: 1 ratio with sodium selenate solution (100 $^{mg}/_{ml}$) and incubated for further 24 h at 30°C. Yellow to red color change was monitored in these experiments as an evidence for selenium nanoparticle production [24]. Based on the results of these experiments, the method of inoculation of nutrient broth containing 100 $^{mg}/_{ml}$ sodium selenate with 1 ml of 0.5 McFarland bacterial suspension was selected for selenium nanoparticle production. So, in order to selenium nanoparticle production this suspension was prepared and incubated at 30 °C and 120 rpm till reddish color change appearance, approximately, 24h, as an evidence for selenium nanoparticle production. After centrifugation (10 min, 5000 rpm), the precipitate was washed with 0.9 % NaCl solution and then centrifuged at 5000 rpm for 10 min. Three consequent freeze and thaw (-70 and 40°C) were done. Final precipitate was dissolved in 5 ml distilled water and sonicated. Following a centrifugation (5 min at 5000 rpm), the precipitate was washed three times with 1.5 M Tris – HCl (pH 8.3) containing 0.5% SDS (sodium dodecyl sulfate) for 10 min at 8000 rpm. In order to remove the remaining SDS, the precipitate that now contains selenium nanoparticles, was washed through three times dispersion in distilled water and centrifugation (8000 rpm, 10 min) . Finally, the precipitate was resuspended in distilled water.

In order to purify selenium nanoparticles from cell debris, 4 ml of prepared suspension was vigorously mixed with 2 ml of octanol, centrifuged (5 min, 3000 rpm) and incubated (24h at 4°C) for dissociation of two phases. In this manner, nanoparticles were accumulated in organic phase and impurities were remained in upper phase. The organic and aqueous phases were slowly separated and discarded and the remained nanoparticles were washed consequently with chloroform, absolute ethanol (Merck, Germany) and distilled water. These steps were repeated again in order to gain pure nanoparticles. The final suspension was stored at 4°C [9, 22, 24, 27].

Nanoparticles assay

In order to determine the size of nanoparticles, they were suspended in 5 ml distilled water and sonicated (5 min). The Uv/Vis analysis in 400-600 nm was performed on the obtained suspension. Then the size of nanoparticles was determined with particle size analyzer (England, MALVERN INSTRUMENTS- ZEN3600). The shape of these nanoparticles was investigated with scaning electron microscopy (SEM). One ml of the above mentioned suspension was dried and coated with Au and then analyzed with SEM (Germany،LEO 1455 VP) of Shahid Chamran University of Ahvaz.

The XRD analysis was also performed for these nanoparticles with x-ray instrument (France،NEL : EQuinox 3000) of Amir Kabir University [28].

Absorption of nano-selenium and bulk selenium in vivo

In order to determine the stability of produced selenium nanoparticles and also the effect of nano scale on its biological absorption in comparison with bulk selenium, its absorption was evaluated in mice. 24 female rats (220 gr average) were grouped in to 3 groups of 8 members and 100 $^{\mu gr}/_{Kg}$ of nano-selenium, bulk sodium selenate and distilled water were injected intra-peritoneal (IP) in to first, second and third groups, respectively. Blood samples then collected at 24h and 48 h after injection [29-31]. These samples were centrifuged (5 min, 2000 rpm) and 500µl of serum was collected [30]. The serum samples were diluted in 1: 2 ratios with 0.1% nitric acid (v/v) plus 0.1% Triton- X100 and their selenium concentration were measured using atomic absorption spectroscopy [32].

RESULTS

The results of biochemical tests and also 16S rRNA sequencing revealed that the bacterial isolate is *Bacillus cereus* which named as *B. cereus* BIPC04 (Fig 1).

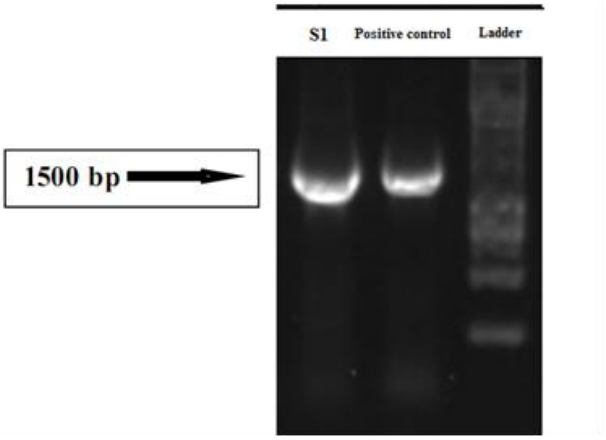

Figure1.Electrophoresis of 16S rRNA PCR product. 1500 bp product was amplified from *B. cereus* BIPC04

This isolate had 99% identity with registered *B. cereus* sequences in gene bank of NCBI. In the experiment for MIC and MBC, the yellow to red color change was obvious till 37.5 mM of sodium selenate but not in higher concentrations. As a result of culturing from higher concentrations on nutrient agar no bacterial growth and colony formation was happened. So the MIC and MBC of selenium for this bacterium was 75 mM (Fig 2).

In growth curve analysis, 2h after challenging the bacterium with 0.5 mM selenate, color change from yellow to orange-red was appeared that is a clue for selenate reduction to selenite and finally selenium. As we can find in (Fig 3), the isolate had much growth in the absence of sodium selenate and its growth curve was higher than when it encountered to 0.5 mM concentration of sodium selenate. This shows the effect of selenium on bacterium. However, in the 1.5 and 3 mM concentrations treatments, the higher growth curves were obtained for this bacterium. This higher absorbance do not means that this isolate had higher growth in 1.5 and 3 mM sodium selenate but is related to the color change and this fact that the absorption spectrum of orange color is in the 585-620 nm. So, the absorbance in these treatments were higher than control as well as 0.5 mM treatment.

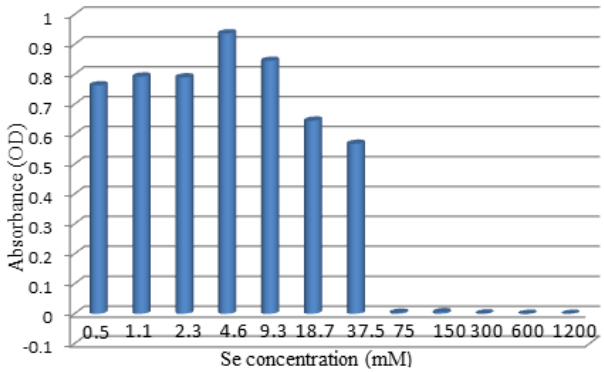

Figure 2.Growth of *B. cereus* in different concentrations of Se. The growth was inhibited at 75 mM concentration

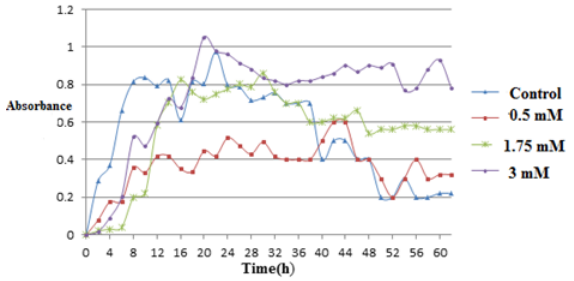

Figure 3.*B. cereus* BIPC04 growth curve(600 nm) in the presence and absence of sodium selenite.

Produced selenium nanoparticles have been evaluated by different methods. At Uv/Vis analysis in 400-600 nm, the absorption peak was obtained at 420-450 nm that is related to selenium nanoparticle (Fig 4).

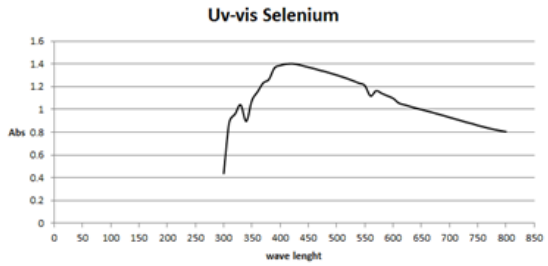

Figure 4.Uv/Vis analysis of produced Se nanoparticles.

The result of XRD analysis was in accordance with standard spectrum of selenium nanoparticle that confirms selenium nanoparticle production (Fig5).

The mean size of these nanoparticles was calculated by Deby- sharer equation (1):

$$D=0.9\lambda/\beta \cos\theta \quad (1)$$

According to this equation the 35.5 nm was obtained for the mean size of selenium nanoparticles [30]. The SEM results revealed that symmetrical and spherical nanoparticles were produced (Fig 6). Due to long time for purifying nanoparticles, it is possible that nanoparticle growth has been happened.

Table I presents the results of selenium absorption in mice. In control group no measurable concentration of selenium was present while in two test groups selenium was obviously measured at 24 and 48 h after injection. As it can be found, the serum level of seleniumin nano group was significantly ($p<0.029$) higher than bulk selenium group (Fig 7). One way variance analysis revealed significant difference between 3 groups. The results suggests that after 24h, absorption of selenium nanoparticles were higher than bulk selenium. Furthermore, the excretion rate of selenium was also higher in the group received seleniumnanoparticles.

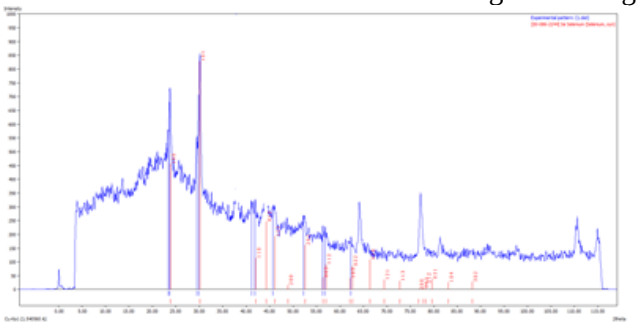

Figure 5.XRD analysis of biologically produced Se nanoparticles. These data are in accordance with standard X-ray diffraction (JCDPS: 00-086-2244)

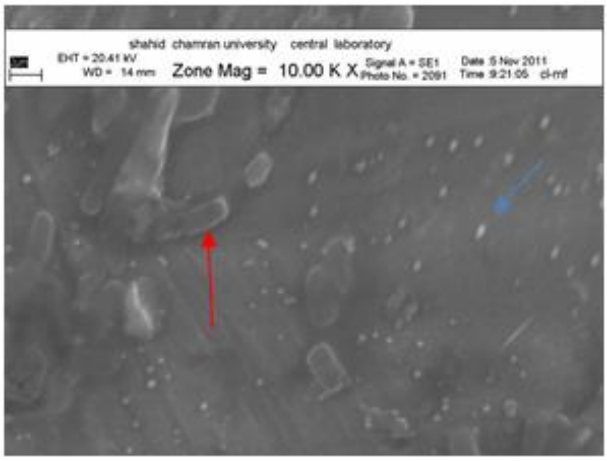

Figure 6(a). Se nanoparticles (blue arrow) before purification with aqueous/ organic biphasic method. Red arrow indicates bacterial debris.

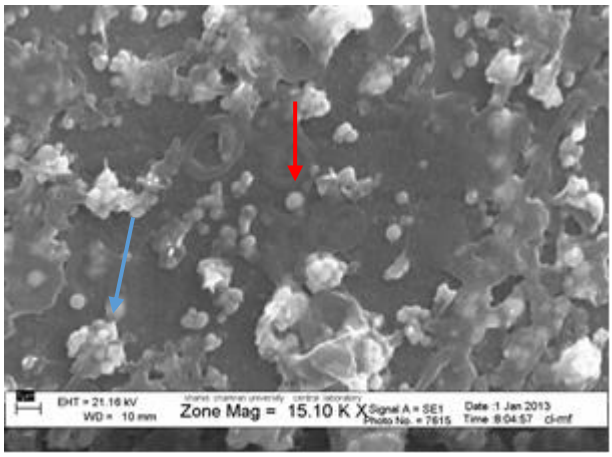

Figure 6 (b). Se nanoparticles after purification with aqueous/ organic biphasic method. Red arrow is Se nanoparticle and blue arrow shows their aggregation.

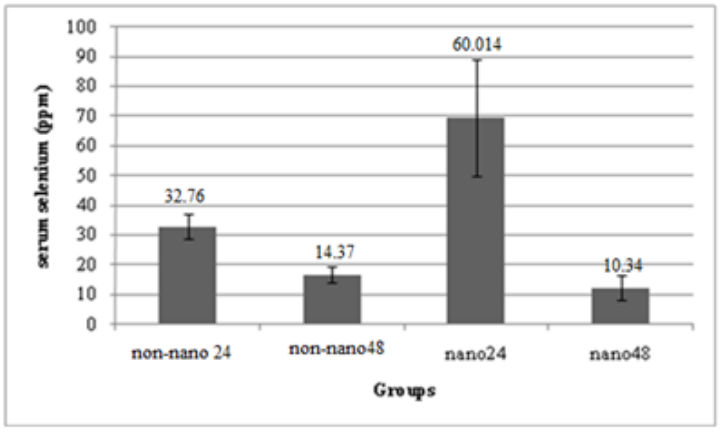

Figure 7. Bulk Se and nano Se concentrations in the serum of female wistar rat

Table I. Se concentration in the serum of female wistar rat.

Control (ppm)	**Bulk Se concentration (ppm)**		**Nano Se concentration (ppm)**	
-	**48(h)**	**24(h)**	**48(h)**	**24(h)**
-	12.73	29.52	20.29	17.71
-	21.90	70.14	17.33	100.23
-	15.19	43.41	2.59	74.52
-	7.66	27.62	18.12	74.52
Average	14.37	32.67	10.34	60.014

DISCUSSION

Microorganisms have oxido-reductive systems in order to use metals and also regulate their concentrations. This happen through altering metal charge that is accomplished by membrane electron transport system and reductive enzymes. In this manner, microorganisms can regulate metal ions diffusion and detoxify them [34]. Selenate can be metabolized through two possible metabolism pathways: dissimilatory selenate reduction that produces elemental selenium and assimilatory selenate reduction that leads to volatile selenium [35]. Two present allotropes of selenium in soil are red and black. selenium (0) in aqueous solution has red appearance and in temperature higher than 45°C it gradually becomes black [36]. The final products of dissimilatory reactions in different bacterial species are red and black allotropes that accumulates in medium [37].

Bacillus cereus BIPC04 that has been used in the present study due to gram positive cell wall structure and also spore formation is a good option for selenium nanoparticle production especially with regard to its resistance to selenium. Selenate is reduced in a two consequent steps from selenate to selenite and then elemental selenium. In the growth curve of *B. cereus* BIPC04 the color change was appeared at 2h that led to higher absorption at 600 nm. This confirms selenium nanoparticle production [9, 22, 24, 38, 39].

Bacillus cereus BIPC04 was resistant to 0.5-37.5 mM concentration of sodium selenate with MIC and MBC equal to 75 mM. Yadav *et al* (2008) and Anand *et al* (2005) reported the appearance of red color after 12h of bacterial culture (*Pseudomonas aeruginosa*) in a broth containing selenium and regarded it as a reason for selenium (0) production.

Pseudomans stutzeri has been reported in the study of Lorti *et al* (1992) that was able to tolerate 2.53 mM of sodium selenite [40]. Kessi *et al* (1999) reported the maximum tolerance to selenium for *Rhodosprillium rubrum* as 1.5 mM [36]. In the study of kashiwe *et al* (2000), *Bacillus* sp. was able to reduce 20 mM selenate to selenite and 2 mM of selenite to elemental selenium but the reduction rate of selenate was higher [41]. Roux *et al* (2001) reported that *Ralstonia metallidurans* can tolerate 6 mM selenite and reduce it to selenium (0). Zahir *et al* (2003) isolated *Enterobacter taylorae* from rice farm drainage that was able to grow at 500-5000 $^{\mu gr}/_{l}$ of selenate in such a manner that reduced 81-94 % of it during five days [42]. As it can be found *B. cereus* BIPC04 that has been used in this study can tolerate and metabolize higher concentrations of selenate than the reported for other bacteria in similar researches. However, in the study of Yadav *et al* (2008), *Pseudomonas aeruginosa* SNT1 isolated from soil was able to grow at 50 $^{mg}/_{l}$ [37] that is more than the tolerance of *B. cereus* BIPC04 but, with regard to the ability of spore formation

in *B. cereus* BIPC04, it can be considered as an advantage of the present study in comparison to the mentioned one.

In XRD analysis, hexagonal structures without amorphous shapes were found and the presence of prominent peaks in this spectrum reveals the high degree of nanoparticle crystallization. No peak that suggests impurity was present. The SEM analysis also showed symmetric and spherical nanoparticles.

B. cereus BIPC04 following to the changing the growth parameters was able to produce intracellular spherical Se nanoparticles with mean diameter of 170 nm. These nanoparticles were stable in the absence of any chemical stabilizer even after injection to rat. This stability is of great importance in medicine and pharmaceutical products production.

Shakibaei *et al* (2010), have produced intracellular selenium nanoparticles using *Bacillus* sp. Msh1, an isolate from Caspian sea, with 142- 255 nm in size but transmission electron microscopy (TEM) revealed spherical nanoparticles with 80-220 nm that had maximum absorbance at 450 – 500 nm in Uv/Vis analysis and confirmed by XRD analysis [24]. Debieux *et al* (2011), have produced spherical (150 nm) selenium nanoparticles using *Thauera selenatis* and confirmed it only by TEM analysis [39]. Dhanjal and Cameotra (2010), isolated *B. cereus* CM100B from soil that was able to produce intra- and extracellular selenium nanoparticles with 150- 200 nm mean diameter based on TEM analysis. These nanoparticles had maximum absorbance at 590 nm [22]. Lee *et al* (2007), reported that *Shewanella* sp. HN-41 is able to reduce selenium (IV) during respiration and produce selenium nanoparticles with 164- 181 nm size [38].

The results of seleniumabsorption in mice revealed that the rate of selenium nanoparticle absorption in first 24h and its excretion at second 24h is higher than bulk selenium. Therefore nano-selenium due to having higher absorption and excretion rate has less toxicity than bulk selenium. This has a significant effect in reducing the injection dose of selenium drugs and also hepatic and renal damages resulting from this element. Zhang *et al* (2005) have studied the toxicity of bulk selenium and selenium nanoparticles on rat liver. Their results revealed that selenite had increased the serum level of alanine aminotransferase and aspartate aminotransferase and inhibited the activity of catalase and superoxide dismutase while had no effect on these enzymes. Furthermore, selenite caused increase in liver malonaldehyde and seleniumnanoparticles decreased it. Both of them had same effect on glutathione peroxidase activity [31]. Benko *et al* (2012) studied the toxicity of selenium containing compounds in mice through treating mice with different selenium compounds and concentrations. As a result, they reported that maximum selenium accumulation was happened in liver and spleen of mice [43]. In the study of Chiba *et al* (1987), it was suggested that selenium and Sn have maximum accumulation in bone, liver and spleen of mice. [29]

CONCLUSION

In the present study using *B. cereus* BIPC04 and without any chemical substance, spherical selenium nanoparticles with mean diameter of 170 nm were produced that is preferable than chemical methods and have no environmental contamination. Furthermore, the metabolism of these particles suggests higher absorption rate that facilitates its application in medicine and also veterinary medicine.

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