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Generation of Selenium Containing Nano-Structures By Soil Bacterium, *Pseudomonas aeruginosa*

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Abstract: The aim of the study was to explore the biogenesis of nano-structures through reduction of selenium by soil bacterium *Pseudomonas aeruginosa*. The strain under study indicated tolerance of selenium upto 50 mg L^{-1}, using reductive mechanisms for detoxication, thus biotransforming selenium oxyanions to elemental red selenium. Partial characterization using AFM of the biotransformed selenium was carried out which indicated formation of spherical amorphous allotropic elemental selenium.

Key words: Selenium, bacteria, reduction, nano-structures

INTRODUCTION

Nanotechnology is the study, control and manipulation of materials at the nanoscale level. The field of nanotechnology has recently witnessed spectacular advance in the methods of nanomaterial fabrication and the utilization of there exotic physiochemical and optoelectronic properties (Shanker *et al*., 2004). The synthesis of nanoparticles of different chemical compositions, sizes and controlled monodispersity is an important area of research in nanotechnology.

There are various conventional chemical (Murray *et al*., 2000) and physical (Ayyub *et al*., 2001) methods to synthesize nanoparticles, but these routes for synthesis of particles/crystallites require tedious and environmentally challenging techniques. As an alternative to conventional methods, biological methods are considered safe and ecologically sound for nanomaterial fabrication (Shanker *et al*., 2004). The use of biological materials offers many advantages over traditional processing methods to construct the next generation of miniaturized electronics devices, particularly involving spatial control on the nanometer scale, parallel self assembly of multiple electronic components on a single device and correctability (Dameron *et al*., 1989; Seeman and Belchar, 2002). The critical factors in developing a bio-directed self-assembly approach are identifying the appropriate compatibilities and combinations of biological-inorganic materials. In natural biological systems, macromolecules exert exceptional control over inorganic nucleation, phase stabilization, assembly and pattern formation, in addition to nanostructural topography of inorganic crystals (Belcher *et al*., 1996; Falini *et al*., 1996). Biological systems also assemble nanoscale building blocks into complex and functionally sophisticated structures with high perfection, controlled size and compositional uniformity. Among variety of microorganisms, only a few groups had been confirmed to be able to selectively reduce certain metal ions (Klaus *et al*., 1999; Mukherjee *et al*., 2001; Nair and Pradeep, 2002; Oremland *et al*., 2004). Some of the significant findings in the recent past include reduction of palladium (II) to palladium (0) and concomitant generation of palladium nanoparticles (De Windt *et al*., 2005; Pollmann *et al*., 2006), formation of tellurium nanocrystals by anaerobic bacteria (Baesman *et al*., 2007), zircon and silicon enrichment in nanostate (Bansal *et al*., 2007).

The potential of microorganisms to biotransform metals has lead to another new dimension of exploring the biological mechanisms towards generation of zero-valent elements, bi/multi elemental quantum dots, metal-containing nanoparticles and further exploit for variety of applications.

The biogenesis of selenium nanomaterials was demonstrated by Oremland *et al.* (2004) and Baesman *et al.* (2007) who reported effectively reduction of two chalcogenide oxyanion species viz., selenite/nate and tellurite/rate, to elemental selenium and tellurium by two anaerobic bacteria, *Bacillus selenireducens* and *Sulfurospirillum barnesii*. In the case of selenium, extracellular granules formed consisting of stable, uniform

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nanospheres (diameter ~300 nm) of Se^0 having monoclinic crystalline structures. In the case of tellurium, *B. selenireducens* initially as nanorods (~10 nm) that cluster together forming larger rosettes (~1000 nm) composing of numerous individual shards. In contrast, *S. barnesii* forms extremely irregular shaped nanospheres (diameter <50 nm) that coalesce in to large composite aggregates. The microbial synthesis of Se^0 nanospheres results in unique, complex, compacted nanostructural arrangements of Se atoms. These arrangements probably reflect a diversity of enzymes involved in the dissimilatory reduction that are subtly different in different microbes. Remarkably, these conditions cannot be achieved by current methods of chemical synthesis reported (Oremland *et al*., 2004). However, the aforesaid synthesis was carried out with anaerobic organisms intracellularly. Present study demonstrates the synthesis by aerobic bacterium, *Pseudomonas aeruginosa* (SNT1) that is tolerant upto 100 mg L^{-1} of selenium as selenate and selenite in the growth medium.

With reference to the biological importance of selenium and its nano forms, recent studies (Ip, 2006) reveal that, compared to various forms of selenium, elemental selenium at nano size (nano-Se) possesses equal efficacy in increasing the activities of glutathione peroxidase and thioredoxin reductase but has much lower toxicity as indicated by median lethal dose, acute liver injury and short-term toxicity. Report of Wang *et al.* (2007) suggest that nano-Se can serve as an antioxidant with reduced risk of selenium toxicity. Gao *et al.* (2002) have reported the anti-oxidant properties of hollow sphere selenium nanoparticles using ESR spectroscopic techniques. They demonstrated the free-radical scavenging activity through reduction in formation of hydroxyl radical from 5,5-dimethyl-1-proline oxide (DMPO). Researchers working on characterization and efficacy of Nano-Se envisage the application of nano-selenium in special anti-oxidative stress drug therapy in medicine.

There are limited and scarce studies on nano-chalcogenide synthesis especially in the case of selenium. With the growing importance of selenium and selenide containing nanomaterials both in health and engineering applications, the cost-effective route of nanomaterial synthesis using selenium tolerant microbes becomes more so important. The objectives of the project were based keeping this lacunae and importance in view.

MATERIALS AND METHODS

Pseudomonas aeruginosa (SNT1) was isolated during a study carried out on isolation and characterization of rhizospheric bacteria from seleniferous soils. The strain was inoculated into the autoclaved tryptone soy broth (pH 5.5-6.0) (TSB-Himedia) in sterile conditions. The flasks were kept on a shaker at 150 rpm for the first 2 h and then at 120 rpm at 28°C for 3 days. For the growth experiment, 0.1 mL of inoculum was taken from actively growing culture in TSB (OD_{600} 2.0) and re-inoculated in 100 mL of TSB in 250 mL Erlenmeyer flasks with different concentrations (5, 15 and 25 mg L^{-1}) of sodium selenite (SDFine MW 173) in TSB. Two different controls were simultaneously maintained-one with inoculum but without selenium and the other as positive control without inoculum and with selenium, to check if there is any chemically induced transformation of selenium in the medium devoid of the organism. The growth experiments were carried out for 12 h with observations taken at 2 h interval at OD_{600}. Observations beyond 12 h were avoided due to interference of red colour of elemental selenium with the optical density.

For examining the uptake, test organism (*P. aeruginosa*) exposed upto 25 mg L^{-1} of selenium as selenite further processed. Samples were collected at 2 h interval and centrifuged at 8000 rpm for 10 min. The biomass pellet as well as supernatant of 0 to 12 h samples were subjected to Se analysis by Analytika-Jena graphite furnace-AAS (NOVAA-400). Stock solution of sodium selenite was prepared from AR grade sodium selenite and AAS standards were prepared from Perking-Elmer AAS stocks along with AR-sodium selenite as cross-reference. Furnace temperature programme involved drying at 110 and 130°C, pyrolysis at 1300°C, atomization/read step at temperature 1900°C, cleanout temperature 2400°C.

X-ray Florescence (XRF) analysis was carried out to validate the Se uptake by examining the excitation peaks in the presence of X-ray. The grown bacterial culture (*P. aeruginosa*) spiked with 50 mg L^{-1} of selenite was taken and filtered through 0.22 μm Whatman filter paper. This paper was then oven dried at 70°C and powdered in a ball-mill (Giegerflex, Germany). The powdered sample was analysed for XRF. Fresh dried and powdered paper was used as control. XRF counting was carried out upto 140 units across 400-600 channels with search specifically orientated for SeKα.

The grown bacterial culture was taken and lysed using SDS and lysozyme. The lysed biomass was then centrifuged at 10000 rpm. The pellet was lyophilized and powdered followed by layering on mica plate for observing using AFM (Vicco, USA).

RESULTS

Growth profile of *P. aeruginosa* was carried out to examine the tolerance of these organisms against different

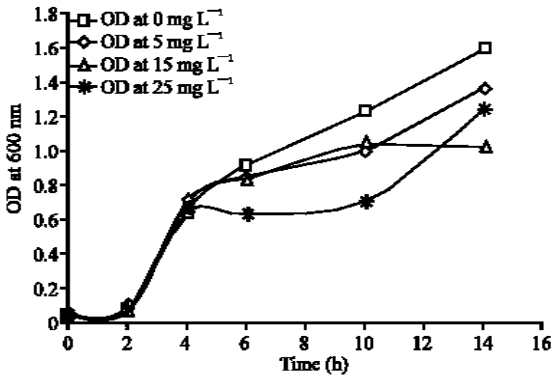

Fig. 1: Growth profile of strain *P. aeruginosa* in the presence of selenium

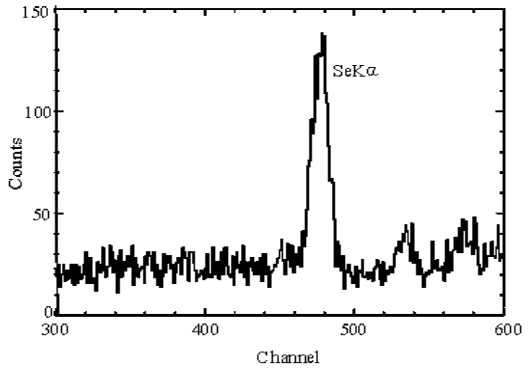

Fig. 2: X-Ray fluorescence (XRF) spectrum of biomass exposed to 25 mg L^{-1} Se indicating significant presence of Se

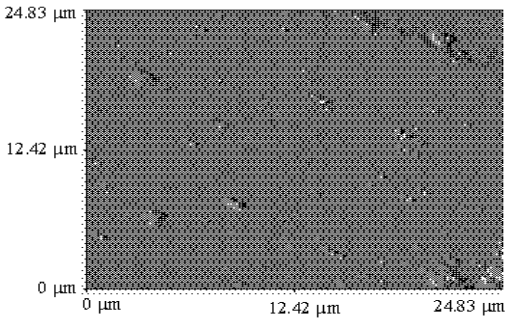

Fig. 3: Atomic force microscopic images of spherical nanospheres in cell free cell medium after lysozyme treatment

concentrations of selenium (5, 15 and 25 mg L^{-1}) exposed as selenite. The profile over 12 h are represented in the Fig. 1. As observed, the growth profile was similar to that of control, which was not exposed to selenium impact.

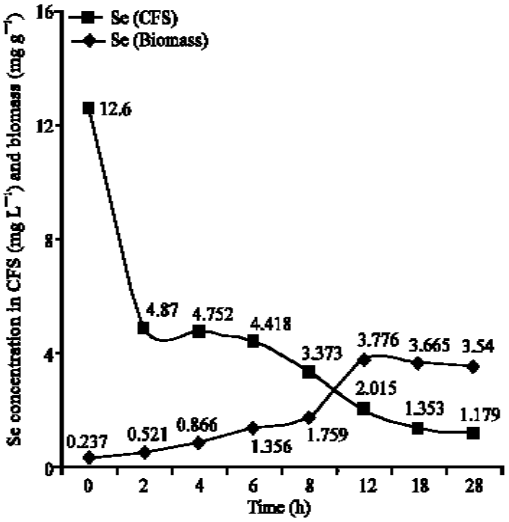

Fig. 4: Selenium uptake in *P. aeruginosa* with reference to Se levels in CFS

During the present study, the formation of red-selenium was quite distinct within 10 h of exposure period. The unique nature of Se-resistant bacterial isolates examined is the aerobic nature and potential to reduce selenium as a mechanism to detoxify the impact of selenium.

The uptake and tolerance exhibited by the organism was further validated using XRF (Fig. 2). The intense peak of SeKα indicate the prominent presence of Se fraction.

Preliminary examination of the reduced selenium was carried out by processing the *P. aeruginosa* biomass and subjecting the samples to atomic force microscopy (AFM) (Fig. 3) indicated spherical Se^0 formations both intra and extracellularly. The Se^0 was observed to be amorphous based on XRD (Rieger-Flux) examination (data not shown).

The Se^0 particles may represent the ultimate repository of the selenite added at the start of incubation. This was indicative from the analysis of Se uptake in bacteria cells vis-à-vis the total selenium concentration in cell biomass and the cell free supernatant (Fig. 4), as examined by total selenium estimation using GF-AAS.

DISCUSSION

Biological systems have a unique ability to be self-organized and synthesize molecules that have highly selective properties. These properties make them a prospective tool that can be used to synthesize nano-scale particles (Chi and Gao, 2003). Many biological systems are able to create an interface with these materials

to use them. The present work was focused on examining the potential of bacterium *P. aeruginosa*, which was found to be tolerant to metal, accumulate selenium and generate metal particulates in periplasmic space.

The purpose of the growth profile studies was to understand the response of the test organism to the presence of selenite. Apparently, Se does not present a toxicity problem to *P. aeruginosa* at the mentioned Se concentrations, which are about four orders of magnitude higher than in the environment from which the organism has been isolated. Dungan *et al.* (2003) reported formation of Se^0 after 28 h during studies with *Stenotrophomonas maltophilia* in the presence of selenite. The Se^0 precipitation was also reported in *Enterobacter taylorae* when exposed to 2000-5000 μg L^{-1} (Zahir *et al.* 2003). The precipitation of Se was noted both in growth medium as well as on the surface of the *E. taylorae* cells. The observation in the present study, is supported by the report of Zahir *et al.* (2003), where in the Se^0 precipitation was evident both in biomass and Cell Free Supernatant (CFS). In a study on selenium biotransformation in the culture medium by *E. cloacae* cells, Losi and Frankenberger (1997) reported that Se^0 was of <0.1 μm in diameter either free in the solution or protruding from the outer surface of the cells. Kessi *et al.* (1999) suggested that the presence of selenium particles on surface and in solution is an indication of vesicular mechanism to expel the biotransformed selenium. Report of Losi and Frankenberger (1997) also indicated that the precipitation profile is dependent upon the selenium concentration in the medium. Diverse species of *Bacteria* and *Archea* reported by Stolz and Oremland (1999) showed that the end products of dissimilatory selenium reduction reactions result in red, amorphous or monoclinic allotropes of Se^0. The greater stability of allotropic form of Se^0 produced by bacteria or precipitated into cell-free medium obtained from the stationary phase culture implies that Se^0 is tightly bound to some substances produced by cells and is protected from further transformation to black form (Kessi *et al*., 1999).

Switzer-Blum *et al.* (1998) examined the formation of small spheres or Se^0 on the cell surface of a gram-positive rod, *Bacillus selenireducens* strain MLS10, after respiratory growth of selenite. Reports of such formations are noted from *Wollinella succinogenes* (Tomei *et al*., 1992), *Enterobacter cloacae* (Losi and Frankenberger, 1997) and *Stenotrophomonas maltophilia* (Dungan *et al*., 2003). However, the present study differs from that of Kessi *et al.* (1999) and is in agreement with Tomei *et al.* (1992) that majority of the elemental selenium in the medium was observably due to lysis of cells with lesser extent of exudation by viable cells. Pioneering study in characterizing the nano-hollow Se spheres was carried out by Oremland *et al.* (2004) who proposed the nanospheres to compose of interconnected three-dimensional nets of selenium in which both the chain and ring structural aspects are maintained, factors that should result in the spherical shape. The characterization of spherical nanostructures obtained in AFM analysis indicated diverse sized aggregates of Se (0) nanospheres across the medium as reported.

The uptake and transformations were correlated to precipitation of selenium intra and extracellularly by Lortie *et al.* (1992) and other researchers. The results are significantly similar to the observations by Kessi *et al.* (1999) and Roux *et al.* (2001), which have indicated intense Electron Dispersion Spectroscopy (EDS) spectral peaks for SeKα at 11.22 KeV. Elemental selenium (Se^0) in recent past has been envisaged to have immense medical (free radical scavenging, anti-cancer and anti-oxidative drug applications in medicine) and industrial (glass and optical lens coatings) applications. The growing importance of nanoselenium in diverse variety of applications has further enhanced the interest of researchers to determine if the diverse properties of the biologically based selenium nanospheres and other nanostructures can be reliably reproduced biosynthetically and if they can be, whether they have a practical application in the field of nanotechnology.

CONCLUSION

The present study, thus, demonstrates a preliminary step forward in the mentioned approaches towards biosynthesis of selenium nanomaterials. The study elucidates the synthetic route using aerobic organisms and the partial characterization of the synthesized nanomaterials through aforesaid approach. Because of different and unique spectral of the biogenic selenium nanospheres and other materials, further reproduction, purification and characterization of these nanoparticulates becomes more so important for future applications.

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