Selenium and Lead Tolerance in Marine *Aspergillus Terreus* for Biosynthesis of Nano Particles – Quantum Dots/ Rods

Jaya Mary Jacob, Raj Mohan B*, Soubhik Kumar Bardhan, P E JagadeeshBabu, and M Aruna

***Abstract*—**The concentrations of lead and selenium in sea water samples near industrial areas located in Mangalore, Karnataka, India were determined using atomic absorption spectrophotometer. Sea water samples in proximity to petro chemical industries were analyzed with high lead and selenium concentrations. A marine fungus, *Aspergillus Terreus* was isolated from this lead and selenium contaminated seawater sample and screened for their resistance to these elements. Although, the marine fungi exhibited a dwarfed growth in comparison to the control (isolates grown in agar medium without heavy metals) in typical shake flask studies in the presence of the metals, the tolerance index to lead and selenium was found to be remarkably high in *Aspergillus Terreus* analogous to marine *Penicillium sp*. Further, the growth of the marine fungi in presence of heavy metals was characterized by a longer stationary phase in comparison to the control flasks. The tolerance of *Aspergillus Terreus* to concentrations of lead and selenium as high as 25 mM, projects the fungi as an attractive potential candidate for further investigations regarding their ability to synthesize Lead Selenide quantum dots with high quantum confinement effects.

Keywords*—Aspergillus Terreus*, Seawater, Lead, Selenium, Tolerance Index, Quantum dots.

I. Introduction

INDUSTRIALIZATION in the current century has pioneered extensive research in the field of environmental impact concerned with heavy metals and metalloids. Of particular interest are the chalcogen group and the related salts with heavy metals. Of these, the salt of Selenium (Atomic number 34) with Lead (Atomic number 82) is of environmental relevance owing to the promising applications of Lead Selenide Quantum Dots bound with excellent quantum confinement effects as conducive photo catalysts and as leaders in future energy crisis. While the principal lead emission sources include lead water pipes, lead-based paints and leaded petrol; selenium is a by-product of various electrolytic metal refining, paint and petroleum industries. The effluents from these industries usually find way to water bodies resulting in increased levels of these metals. The contamination of natural sources due to the effluents accompanied by its toxicological and physiological importance, has led to an increasing interest in the determination of these elements [1]. Long term accumulation of heavy metals and metalloids in natural sources of water from the industries not only lead to bio magnification and eutrophication but also turn these water bodies as sources for isolation of heavy metal tolerant microbial species. Prior research reports the isolation of microbes from various contaminated water bodies including lakes, well, sea, industrial effluents and domestic sewer [2, 3]. The bio-sorption of heavy metals using fungal strains has been studied by researchers world-wide. Sastry *et.al* (2003) studied the bio-synthesis of metal nano particles using fungi and actinomycetes. Because of their tolerance and metal bioaccumulation ability, fungi are taking the centre stage of studies on biological generation of metallic nanoparticles [4]. Marine fungi belonging to the genera *Aspergillus niger, Fusarium sp*. and *Penicillium sp*. are reported to exhibit tolerance to lead and selenium [5a]. Review suggests that these biological entities with metal tolerance could serve as agents for the cost effective biosynthesis of quantum dots [6].The present study attempts to determine the concentration of Selenium and Lead from sea water samples near industrial areas. The presence of heavy metal resistant fungus is probable in these heavy metal contaminated sites. The above samples were thus used to isolate heavy metal tolerant fungi and to quantify their tolerance index.

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II. Materials And Methods

A. Collection of samples

Sea water and sediment samples were collected in clean sampling containers from beaches near Mangalore Refineries and Petrochemicals Pvt. Ltd, Mangalore (Fig.1). These samples were brought to laboratory and kept in refrigerator at $4^{o}C$ for further processing.

III. Lead and Selenium Analysis in Samples

Water and sediment samples were analyzed for their total content of lead and selenium. The sediment samples were oven dried at 105°C. A sample of 1 g was digested with 5 ml HNO_3 and 10ml HCl. Digestion was carried out on a hot plate until dense fumes evolved and a clear solution was obtained. The clear solution was filtered through a Millipore filter (0.45 µm) and diluted to 50 ml with distilled water prior to analysis. As for liquid samples, 50 ml of sea water was directly used for analysis using double beam GBC932 plus Atomic Absorption Spectrometer with selenium and lead lamps. Stock Solutions of Lead and Selenium for AAS were purchased from HiMedia Laboratories Pvt Ltd. Working standard solutions of Selenium and Lead were made by appropriate dilution of the respective 1000ppm standard stock solutions

Fig.1 Localisation of sampling sites

IV. Isolation of Micro-organisms from Polluted Sites

The water sample with highest contamination was enumerated for micro-organisms employing a serial dilution technique using Potato Dextrose Agar (Hi-Media, Mumbai, India) containing 20ppm of Pb and Se. The 1000ppm stock solutions of Pb and Se were made in double distilled water using Lead Nitrate and Sodium Selenite. The stock solution of heavy metals was sterilized separately and added to sterilized potato dextrose agar (PDA) medium under sterile conditions. Sea water samples were serially diluted up to 10^{-8} and 1ml of diluted sample (10^{-8} and 10^{-6}) was added in sterilized petri plates. 20 ml of PDA medium containing 20ppm of the heavy metals was poured in these petri plates and incubated for 3 days at 25°C. Developed colonies were randomly picked and isolates were purified by streaking the colonies repeatedly in PDA medium. Pure cultures of isolated micro-organisms were identified using the keys of Pitt (1979) and Domsch *et.al.* (1980) [7],[8]. The cultures were characterized on the basis of macroscopic characteristics like colonial morphology (color, appearance of colony, shape).

V. Growth Response Study

To compare the tolerance index of the isolated fungus, the radius of colony extension in PDA medium containing 1ml of 20 ppm Pb and Se in combination was measured against the control (medium without metal) after at least 7 days of incubation and compared with the reported tolerance indices of other isolated marine fungi. The index is defined as the ratio of the extension radius of the treated colony to that of the untreated colony.

The growth characteristic of heavy metal tolerant (20 ppm) fungal isolate was further screened using typical shake flask experiments in the presence and absence of Pb and Se in Potato Dextrose Broth (PDB). Owing to the remarkably high tolerance index of the fungus, the fungal spores were seeded on 100ml PDB medium containing 50ml of 1mM concentration of the metals in combination. Seeding of fungal isolates on normal PDB medium served as control (normal growth). Observations on growth of fungal isolate were made by measuring the weight of biomass every day after 72 h of incubation for 10 consecutive days.

VI. Tolerance Ranges of Fungal Isolate towards Pb and Se in Liquid Media

Metal tolerant *Aspergillus Terreus* isolate was subjected to broth studies in PDB medium containing Pb and Se in a concentration range of 5mM-50mM. Potato dextrose broth containing 50ml of the metals in their respective concentrations was dispensed in 100 ml lots to 250 ml conical flasks and sterilized at 15 lbs/psi for 15 min. These flasks were inoculated with 1 ml of freshly prepared spore suspension of the fungal isolate and put on shaker at 150 rpm at 28^0C for 5 days. Fungal growth was harvested after 5 days through filtration using Whatman filter No. 42. The harvested fungal biomass was rinsed with double distilled water 3–4 times and dried in hot air oven at 80^oC for 18h. The dry weight of the biomass was calculated.

VII. Biosynthesis of PbSe Quantum Rods in Marine Aspergillus Terreus

Lead and selenium tolerant *Aspergillus Terreus* isolated from sea water were cultured in PDB medium for 96 hours and this was treated as source culture for biosynthesis. 20 ml of 0.25M Lead Nitrate ($PbNO_3$) solution was taken and 5 ml aqueous solution of 0.25M Sodium selenite (Na_2SeO_4) was added to the diluted source culture and it was heated on steam bath upto 60^{o}C for 10-20 mins until fluffy yellow-white deposition starts to appear at the bottom of the flask, indication the initiation of transformation. Now the culture solution was cooled and allowed to incubate at room temperature in the laboratory ambience overnight. Next day, the culture solution was observed to have distinct coalescent yellow-white clusters deposited at the bottom of the flask leaving the colloidal supernatant at the top. It was filtered, the residue was subjected to several cycles of oven-drying and freezing and the resultant powder was subjected to characterization.

VIII. CHARACTERIZATION

Preliminary characterization for the formation of PbSe nanoparticles was checked by analysis under Scanning Electron Microscopy (SEM) using JEOL JCPDS Analysis station (Japan) along with EDAX analysis to confirm the elemental composition of the nano particles.

IX. RESULTS AND DISCUSSIONS

A. Sediment and water analysis

Common sources of lead and selenium include discharge from industries such as electroplating, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes [9]. The effluents from these industries are consequently discharged into the environment. Introduction of metals in various forms into the environment can induce numerous modifications in microbial communities thereby affecting their activities [10, 11]

In the present study, a metal tolerant fungus was isolated from sea water near industrial areas after analyzing the lead and selenium content in the same. The selenium and lead content in the collected sea water and sludge samples is listed in Table I. Results indicate high concentrations of lead as well as selenium in sea water samples in proximity to industrial areas. Sea water in proximity to petrochemical industries had selenium and lead concentrations of 1.191 ppm and 1.144 ppm respectively. Our findings draw relevance as research states that selenium exposures at even concentrations below the current European Union and World Health Organization upper limit of 10μg/l can result in health risks. Analysis of lead content in the water and sludge samples shows a similar trend with the sea water showing relatively higher concentrations of the heavy metal. According to EPA (2011), the permissible limit of lead in water samples in 15ppb [12]. The samples analyzed in the current study manifest higher levels of lead and selenium owing to the proximity of the sampling sites to petrochemical and refinery industries.

B. Isolation of Heavy Metal Tolerant Fungi

Fungi and yeast biomasses are known to tolerate heavy metals [13]. In the present study, *Aspergillus Terreus* fungus tolerant to heavy metals were isolated from sea water sample contaminated with lead and selenium (Fig.2).

Prior studies report an appreciable tolerance index of marine *Penicillium sp* towards lead and selenium [5b]. Plate studies using PDA was thus conducted to compare the tolerance of *Aspergillus Terreus* isolated from the same source. Results reveal a significantly high tolerance index in *Aspergillus Terreus* when compared to other reported marine fungi (Fig.3). The index of tolerance showed significant difference between isolates from the same source with the indices ranging from 0.46-1.11 in other marine fungi and an index of 1.235 in *Aspergillus Terreus*.

TABLE I
METAL CONTENT IN SAMPLED SITES

Sampling sites and description	Water Content		Sediment Content	
	Metals Content (mg/l)			
	Pb	**Se**	**Pb**	**Se**
S1 (Sea water, 6km from Refinery)	1.144	1.191	0.774	2.537
S2 (Sea water, 8km from Refinery)	0.469	0.721	0.762	2.133
S3 (Sea water, 12km from Refinery)	0.408	0.581	0.614	1.414
S4 (Treated Refinery Effluent)	0.292	0.229	0.86	1.558

Fig. 2 Aspergillus Terreus mother culture on PDA amended with 20ppm Pb & Se

Our results are in agreement with the findings by Iram *et.al* (2012) who confirmed that the response of isolates to heavy metals depended on the metal tested, its concentration in the medium and on the isolate under consideration [14]. Our observations project the importance of the isolated strain as a potential fungus with high lead and selenium tolerance from a relatively less polluted source.

C. Fungal growth responses towards Pb & Se

The growth of the fungi in the presence and absence of lead and selenium was further studied using shake flask experiments to quantify the growth and to study the phases of growth with respect to biomass weight. The growth of *Aspergillus Terreus* in the presence and absence of lead and selenium is shown in Figure 4. Although the dried biomass weight showed a cogent increase in the absence of metals in liquid medium, the stationary phase of the growth curve in presence of lead and selenium was eloquently extended. Surprisingly, the decline phase in presence of selenium and lead is not so evident comparing to the control. Maximum biomass dry weight was observed on the 6th day for control and on the 5th day in case of the growth of *Aspergillus Terreus* in presence of the metals.

Similar growth patterns were exhibited by *Penicillium* at lower concentrations of metal amendments in the media. The tolerance of Penicillium to heavy metals is explained by the findings of Sun and Shao (2007) who demonstrated that both intracellular bioaccumulation and extracellular bio-sorption contributed to the high resistance of *Penicillium sp* to heavy metals. As for the high tolerance in *Aspergillus Terreus*, prior researchers highlight *Aspergillus sp* isolated from industrial waste water as promising biological entities with metal tolerance. The mechanism of lead and selenium resistance in the fungus isolated in the present study could be explained by the early onset of an extended stationary phase. The release of reductases during this phase may account for the significant metal resistance in *Aspergillus Terreus*. Another possible mechanism of tolerance may be explained by the reports on the existence of heavy metal tolerant genes in *Aspergillus Terreus* [16]. Furthermore, reports by Dias *et.al* (2002) on heavy metal removal by *Aspergillus Terreus* isolates from steel foundry effluents in Brazil seconds our observations on the metal tolerance in the fungi and signifies the importance of these as an important bio sorbent of heavy metals [17].

Based on the high index of tolerance showed by *Aspergillus Terreus*, the metal tolerant strain was subjected to higher concentrations of the metals in typical broth studies using PDB. As the concentration of the metals increased from 5 mM to 35 mM, there was a reduction in biomass of the fungi as shown in Figure 5. The proportional reduction fungal biomass was observed till a concentration increment of 25mM. As the concentration of Pb and Se was further increased, the fungal biomass dropped drastically as is seen in the reduction of fungal dry weight from 377.9 mg at 25 mM to 97.2 mg at 35mM. Results clearly indicate that the fungi could not tolerate metal concentrations beyond 35mM as indicated by a drastic reduction in fungal dry weight.

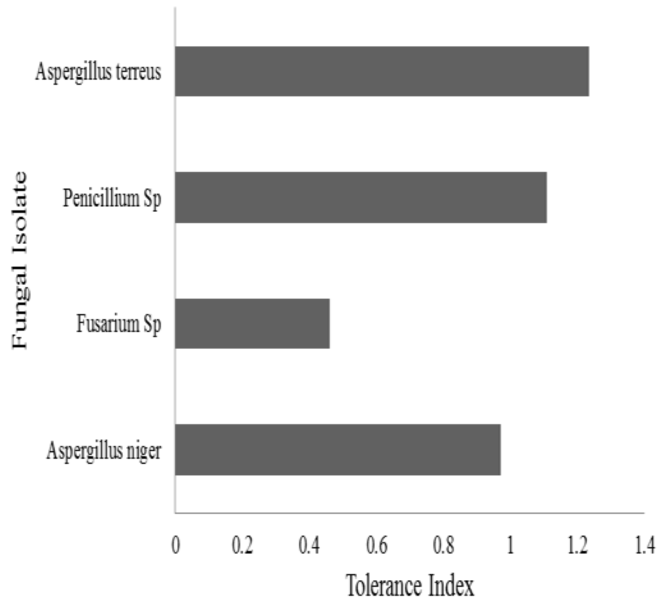

Fig. 3 Comparison of tolerance indices of marine fungi

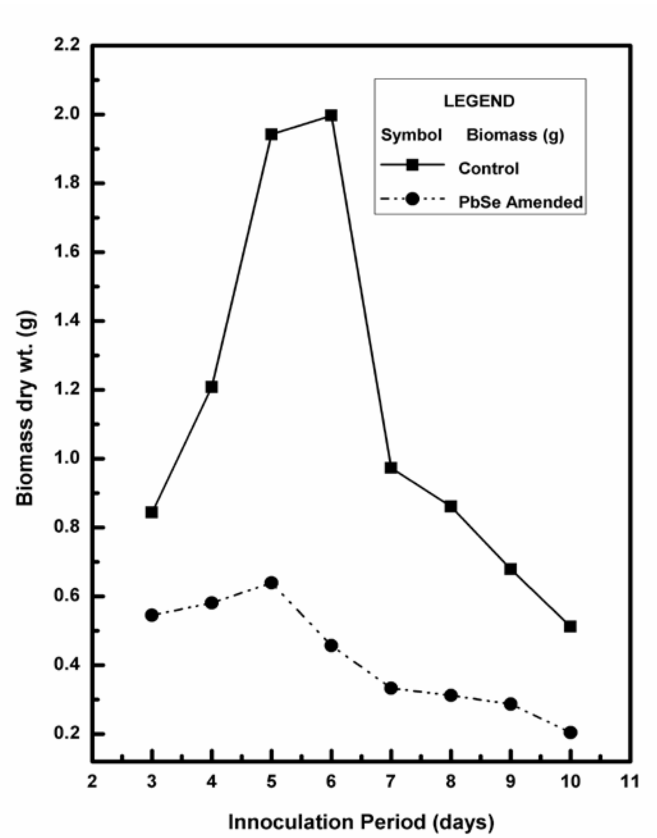

Fig. 4. Growth curve of *Aspergillus Terreus* in the absence and presence of lead and selenium

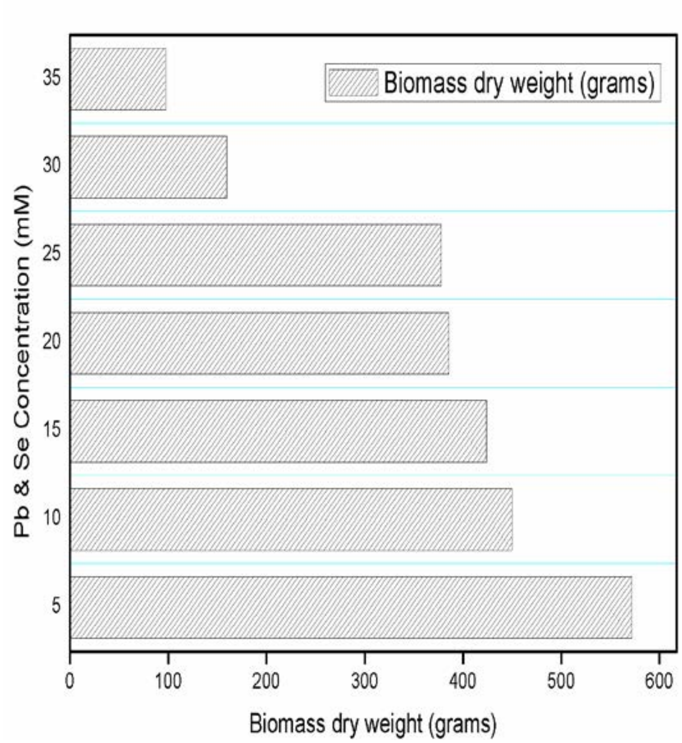

Fig. 5 Effect of higher concentrations of Pb-Se on *Aspergillus Terreus* biomass

D. Biosynthesis and Characterization of PbSe nanoparticles

Prasad and Jha (2009) reported the biosynthesis of CdS quantum dots from Lactobacillus from buttermilk using a similar protocol described in our experiments [18]. Authors report the formation of fluffy orange-yellow deposits of CdS after a reaction period of 20 min at 60^oC using $CdCl_2$ and H_2S gas. In our investigation, a fluffy white deposition of PbSe was found in the source culture solution treated with salts of Pb & Se in their respective concentrations after a reaction period of 20 mins in steam bath at 60^oC. SEM analysis confirmed the formation of nanorods of PbSe by marine *Aspergillus Terreus* isolates as shown in Figure 6.

The aspect ratio of the biosynthesized nanoparticles ranged between 10 and 70 with an average diameter of 95nm. Literature reports the synthesis of PbSe quantum rods with comparable aspect ratios [19]. Our findings have an edge on the prior findings owing to the eco-friendly and cost effective biosynthesis by a marine fungi. Further, EDAX analysis confirmed the composition of the biogenic quantum rods. The nano size range of PbSe imparts attractive quantum properties to these nano rods for use in solar panels and as photo catalysts and bio-sensing agents. The temperature and other reaction conditions could be varied to result in a size reduction of these biosynthesis products to improve their quantum confinement effects.

Preliminary results on biosynthesis of PbSe quantum rods directs attention on the potential of these marine fungi as effective agents for the shape controlled biogenesis of quantum dots under controlled reaction conditions.

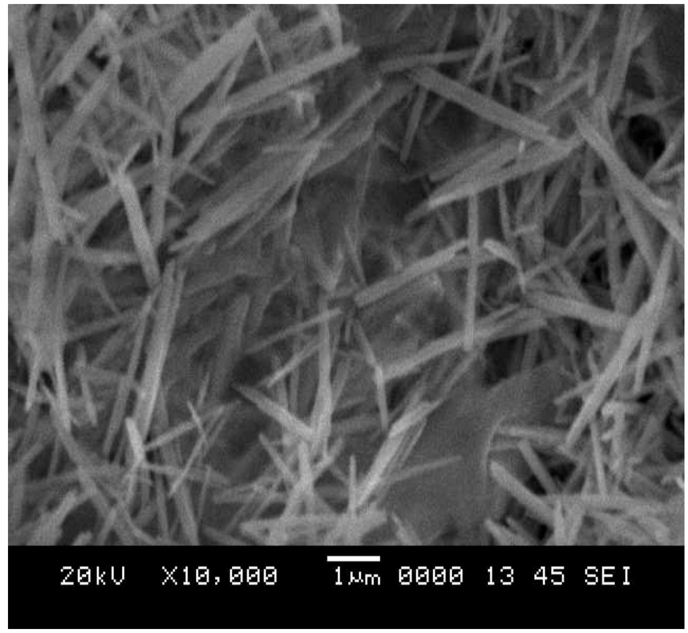

Fig. 6 SEM Image of biosynthesized PbSe nano rods

X. Conclusion

The present study concludes that sea water near industrial areas contain lead and selenium salts in high concentrations. Abundance of these metals in sea water makes it potential candidates for the isolation of lead and selenium tolerant fungal species. Marine fungus, *Aspergillus Terreus* was isolated from sea water. Growth studies in the presence and absence of metals and tolerance studies revealed that this species of fungi is more physiologically adapted to higher concentrations up to 25mM of Pb and Se. Hence *Aspergillus Terreus* isolated from Pb-Se contaminated sea water can serve as promising biological agents for the synthesis of Lead Selenide based nano materials that is likely to lead the future solar energy sector.

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