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RESEARCH ARTICLE

Selenium reducing *Citrobacter fruendii* strain KP6 from Mandovi estuary and its potential application in selenium nanoparticle synthesis

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Abstract The present study foregrounds the isolation of selenium tolerant bacteria from Mandovi estuary of Goa, India, known to be contaminated with mining and industrial waste. The isolate was tentatively identified as *Citrobacter fruendii* strain KP6, which can tolerate up to 60 mM of Na_2SeO_3 in mineral salt medium (MSM). Bacterial strain KP6 when grown in 60 mM Na_2SeO_3 in MSM precipitated soluble toxic selenite (Se^{4+}) into colloidal elemental selenium (Se^0, red colored) using glucose as a sole source of carbon. Interestingly, scanning electron micrograph (SEM), transmission electron micrograph (TEM) and X-Ray diffraction (XRD) analysis also confirmed this bioconversion reaction in the formation of nano –sized material extracellularly with the size ranging from 45 to 70 nm. This bioconversion ability of strain KP6 can be exploited for eco-friendly bioremediation of selenite contaminated estuarine sites as well as for synthesis of Se^0 nanoparticles having its significance in nanobiotechnology.

Keywords Estuarine waters · *Citrobacter fruendii* strain KP6 · Biogenic selenium nanoparticles · Bioremediation

Introduction

In recent years industrialization and mining activities are accounted to release heavy metals and metalloids in terrestrial and marine environment exhibiting their toxic effects on micro and macro biota [1]. Metals and metalloids are persistent in nature and can cause toxicity for years to come. This further opens a gateway for extensive research opportunities in remediation of metal polluted regions along with significant metal conversion reactions carried out by diverse group of microorganisms [2].

Selenium is a metalloid, widely distributed in mining ores and aquatic zones on the earth, existing as reduced form (selenide, Se^{-2}), as an element form (Selenite, Se^0), and as water soluble form (Selenite, SeO_3^{-2}/Selenate SeO_4^{-2}). It is an important structural component of many enzymes such as thioredoxin reductase and glutathione peroxidase [3]. However, at higher concentration in drinking water it can negatively affect human health due to its bio-magnification [4, 5]. Conversely, dietary deficiency of selenium causes Keshan disease [6] but higher concentration of >400 μg ml^{-1} selenium/selenite in body causes selenosis [7]. Selenosis causes diffuse necrosis and hemorrhage resulting from capillary damage and chronic poisoning by degenerative and fibrotic changes in liver and skin [8]. WHO has recommended safe level of selenite in drinking water as 40 μg l^{-1} [9].

Industrial discharge from tanneries, glass production industry, plastic industry, paint and pigment industry, oil refineries and power sources are the main sources of water soluble selenium contamination in aquatic life forms [10] along with mining industry which releases selenium as a major contaminant in aqueous waste streams [11]. Direct discharge of selenium polluted effluent in marine environment may result in biomagnification of selenium in fishes and thus, ultimately reaching to humans [12]. Therefore, there is a pressing need to bioremediate selenium from marine sites polluted with selenite. Alternatively, cost effective and eco-friendly technologies should be employed since existing chemical methods themselves

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contribute to pollution. Besides being toxic to macro and micro biota some natural microbial strains can survive at very high concentration of selenite without having any impact on their growth and metabolism [13, 14].

Selenium reducing bacteria are ubiquitous occurring in diverse terrestrial and aquatic environments [15]. Moreover, microorganisms that have been well characterized for their ability to reduce selenate and selenite oxyions into non-toxic elemental form Se^0 under anaerobic conditions include *Thauera selenatis* [16], *Aeromonas salmonicida* [17], and *Shewanella oneidensis* MR-1 [18] and under aerobic conditions they show diverse species of *Rhizobium* sp. B1 [19], *Pseudomonas* sp. CA5 [20], *Duganella* sp. and *Agrobacterium* sp. [21] to list a few, respectively. Although these studies explored the terrestrial soils and aquatic waters, there are few reports on selenium reduction by a marine estuarine habitant microbe [22].

It is well known fact that Mandovi estuary is heavily contaminated with mining waste containing metals such as iron, manganese, cobalt, copper, zinc, lead and chromium [23] along with metalloid arsenic [24]. Moreover, it is also ascertained that the bacteria isolated from Mandovi estuary demonstrate cross resistance to multiple heavy metals such as lead, mercury, cadmium and organo-metal tributyltin [25]. In the view of this, the authors attempted to investigate the selenium reducing ability of an estuarine eubacteria isolated from Mandovi estuary contaminated with mining and industrial rejects.

Material and methods

Enrichment and isolation of selenite reducing bacteria from Mandovi estuary

Surface estuarine water sample was collected in sterile polycarbonate bottles from Mandovi estuary, Goa, India (Latitude: 15°09′ and 15°33′N, Longitude 73°45′ and 74°14′E, temperature 30 °C) and 1 ml of this water sample was added in 50 ml nutrient broth amended with 1 mM of sodium selenite (Na_2SeO_3) and kept at 30 °C on rotary shaker (150 rpm) for 48 h for enrichment. Nutrient broth (50 ml) with 1 ml of estuarine water sample without Na_2SeO_3 served as a control. This was followed by isolation of selenite reducing bacteria by dilution of enriched sample on MSM plates [26] incorporated with 2 mM Na_2SeO_3 and glucose as sole source of carbon. Red colored colonies that appeared on MSM plates were transferred on MSM plates with varying concentrations of Na_2SeO_3. Bacterial colony which appeared on MSM plate with highest concentration of Na_2SeO_3 was selected for further characterization study and designated as strain KP6. Bacterial strain KP6 was routinely subcultured on MSM plates with 1 mM of Na_2SeO_3 at 30 °C.

Determination of maximum tolerance concentration (MTC) and minimum inhibitory concentration

Maximum tolerance concentration of selenium reducing bacterial isolate was performed in MSM broth with varying concentrations of Na_2SeO_3 such as 10, 20, 30, 40, 50, 60,70 and 80 mM respectively, at 30 °C and pH 7.5 with constant shaking at 150 rpm. Stock solution of 1 M of sodium selenite was prepared by dissolving 172.95 g of Na_2SeO_3 in 1 l of sterile distilled water followed by filter sterilization. Hence, 100 ml of MSM broth in 250 ml of Erlenmayer flask with 0.2 % glucose as sole source of carbon with respective concentrations of Na_2SeO_3 was inoculated with 5 % v/v overnight grown culture and incubated for 24 h at 30 °C with constant shaking at 150 rpm. The growth was measured every 2 h for 24 h (stationary phase) by taking absorbance at 600 nm using UV–visible spectrophotometer (Shimadzu 2470). Un-inoculated MSM media with appropriate concentration of selenite served as a control. MTC was calculated as maximum concentration of Na_2SeO_3 at which growth was seen while MIC was calculated as minimum concentration of Na_2SeO_3 that inhibited the growth of KP6 completely.

Selenite reduction studies

MTC was selected to study selenite reduction by the estuarine bacterial isolate strain KP6. Here, the culture was grown in 1 l MSM medium with 0.2 % glucose as sole source of carbon amended with 60 mM of Na_2SeO_3 with constant shaking (150 rpm) at 30 °C for 24 h. After 24 h, the red colored reduced selenium formed aggregate that either got settled at the bottom or was collected by centrifuging at 2000 rpm for 5 min. The red precipitate layer was scooped off using clean and dry spatula and was collected on a clean watch glass. The red colored pellet was washed thrice with sterile distilled water to remove any media components and then centrifuged at 1000 rpm. This process was repeated five times to remove media components and cell impurities. The red pellet was then dried at 80 °C in an oven for 2 h till constant weight and then used for characterization study. The dry weight of red pellet was also used to calculate conversion efficiency of selenite to selenium.

Morphological characterization

Scanning electron microscopy (Zeiss EVO18) was used to examine morphological alterations in strain KP6 when exposed to 60 mM Na_2SeO_3 during exponential growth.

Strain KP6 was grown with 60 mM Na_2SeO_3 in MSM media with 0.2 % glucose as sole source of carbon while culture grown without selenium in MSM media served as control. The cell pellet was harvested at exponential growth phase followed by centrifugation at 10,000 rpm for 15 min at 4 °C. Thin smear was made on a glass coverslip and fixed with 3 % gluteraldehyde for 24 h at 4 °C followed by dehydrating the fixed bacterial cells with series of alcohol gradients (10, 30, 50, 70, 90 and 100 %, respectively.) and finally sputter coated with gold and examined using SEM.

Identification of selenite reducing bacteria

Identification of Na_2SeO_3 tolerant bacterial strain KP6 was done based on morphological characterization and biochemical test following Bergey's Manual of systematic bacteriology [27].

Characterization of red precipitate

TEM

TEM micrographs of mineral was obtained by drop coating of homogenous solution obtained by sonicating the red pellet in 200 µl of milliQ water for 5–10 min onto 2 mm carbon coated copper grids of 200–300 mesh followed by air drying. The grid was then placed in the sample chamber of the TEM (Philips CM200 Supertwin STEM) operated with the voltage of 200 keV and imaged.

SEM

SEM was performed of a thin smear of homogenous solution obtained by sonicating the red pellet in 200 µl of milliQ water for 5–10 min on glass coverslip. The air dried smear was then sputter coated with gold to a thickness of 10 nm approximately and examined under SEM (Zeiss EVO18) operating at 30 kV.

XRD

The dried red pellet was finely powdered and was analyzed by Rigaku Miniflex powder diffract meter equipped with a Ultima IV solid- state detector at a voltage of 40 kV and current of 20 mA using CuKα radiation ($\lambda = 1.5406$ A) in the range of $5° \leq 2\theta \leq 80°$ at 40 kV. The particle size (D) of the sample was calculated using the Scherrer's relationship ($D = 0.9\ \lambda/\beta\ \cos\theta$) which has been used, where λ is the wavelength of X-ray, β is the broadening of the diffraction line measured half of its maximum intensity in radians and θ is the Bragg's diffraction angle.

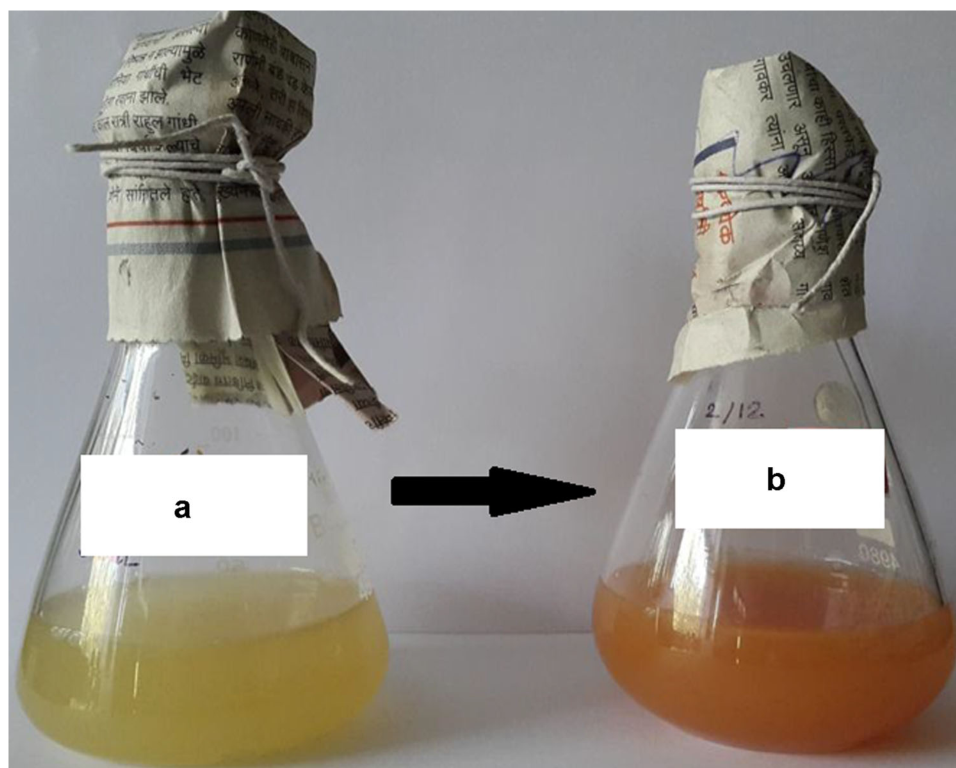

Fig. 1 Reduction of selenite (Se^{4+}, flask **a**) to elemental selenium ($Se^{0,}$ flask **b**) in nutrient broth amended with 1 mM Na_2SeO_3

Results and Discussion

Selenium is bio-essential element in trace amount but at higher concentrations of selenium it is toxic to the natural biota including humans [28]. Se exist in marine water in water soluble form (Selenite SeO_3^{2-}/selenate SeO_4^{2-}), and as an element form (Se^0). Although it exists in natural environment in trace quantity (0.09–2 ppm) anthropogenic activities such as mining and release of industrial effluents in water bodies have led to the increase in selenium concentration (0.15–19 ppm) causing chronic effect on flora and fauna of marine environment [29, 30]. Therefore, scientists around the world are looking for alternate eco-friendly and cost-effective method over chemical method which generates toxic chemical waste.

Appearance of reddish color in enrichment broth after 24 h indicated that selenite reducing bacteria has been enriched which converted soluble selenite (Na_2SeO_3) into red colored elemental selenium (Se^0) when compared to control flask which failed to show a change in color when incubated under same conditions (Fig. 1). After dilution plating of enriched broth on MSM agar plates amended with 1 mM of Na_2SeO_3, 20 morphologically distinct red colored selenite reducing bacterial colonies appeared (data not shown). These 20 bacterial isolates were then transferred to MSM agar plates with varying concentration of selenite (10, 20, 30, 40, 50, 60, 70 and 80 mM). Only one bacterial isolate that could grow on 60 mM Na_2SeO_3 was selected for further study and designated as bacterial strain KP6.

Moreover, the selenite reducing bacterial strain KP6 that could grow on MSM plate amended with 60 mM Na_2SeO_3 was found to be gram negative, short, rod shaped, motile and fermentative (Table 1). It showed the presence of enzyme such as catalase, urease, and nitrate reductase. Indole and VP tests were found to be negative, but bacterial isolate showed methyl red test positive and was able to utilize citrate. Based on the morphology and biochemical analyses followed by Bergey's Manual of Systematic Bacteriology, Volume I bacterial strain KP6 was tentatively identified as *Citrobacter fruendii*. The MTC of *Citrobacter fruendii* strain KP6 was found to be 60 mM in MSM broth and MIC was recorded as 61 mM (Fig. 2). Consequently, *Citrobacter fruendii* strain KP6 isolated from contaminated water sample from Mandovi estuary was able to tolerate 60 mM of selenite by reducing selenite (soluble) to insoluble elemental selenium as revealed by a change in color of MSM broth from white to red. Similar studies of bioconversion of selenite to selenium have been reported by Hunter and Manter [20] in *Pseudomonas* sp., *Bacillus cereus* [31] and *Bacillus* sp. MSh-1 [32]. Moreover, Dhanjal and Cameotra [31] also reported that aerobic bacterial reduction of selenite and selenate to elemental selenium is suggestive of a detoxification mechanism. Various detoxification mechanisms consisting of enzymatic systems have been proposed to catalyze the reduction of selenite in bacteria. According to Losi and Frankenberger [33], the selenite reduction in *Enterobacter cloacae* occurs close to the membrane as the result of membrane associated reductases followed by rapid expulsion of Se^0 particles expelled by the membrane efflux pump. Similar

Table 1 Biochemical tests for identification of bacterial strain KP6

Biochemical tests	Observed results
Morphology	Rod
Arrangement	Single
Pigments	–
Motility	+
Catalase	+
Oxidase	–
Indole	[–]
MR	+
VP	–
Citrate	+
Urease	D
PPA	–
Ornithine	D
Gelatinase	–
H_2S	+
Nitrate red	+
Starch	–
Glucose	+
Lactose	+
Sucrose	D
Gas	+
Mannitol	+
O/F	FA

FA, Facultative anaerobe; OA, obligate aerobe; +, 90 % or greater positive; –, 90 % or greater negative; D, 26–75 % strains positive; [+], 76–89 % positive; [–], 76–89 % negative

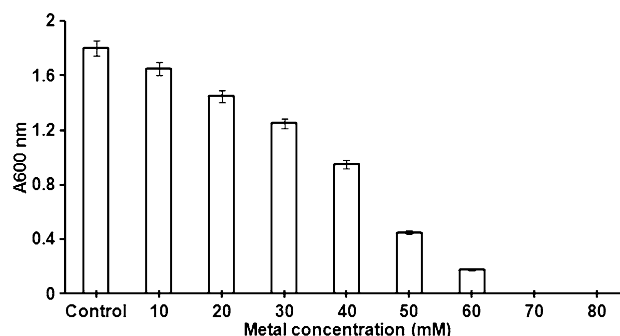

Fig. 2 Growth of bacterial strain KP6 at different concentrations of Na_2SeO_3 in MSM media

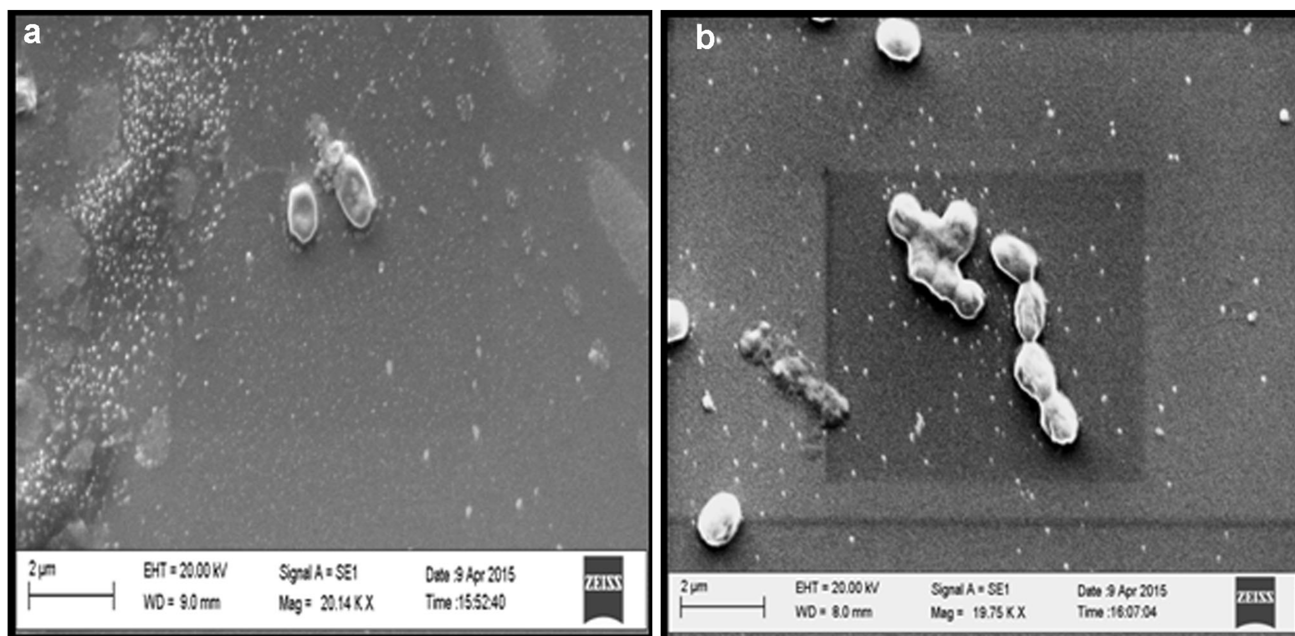

Fig. 3 Scanning electron micrograph of cells of bacterial strain KP6, **a** control cell grown without selenite, **b** cells grown in 60 mM selenite, in MSM broth with glucose as a sole carbon source

detoxification mechanism is also demonstrated in *Bacillus selenatarsenatis*, a facultative anaerobe that grows aerobically by oxidative respiration using arsenate, selenate or nitrate as electron acceptor [34]. It comprises of membrane bound selenate reductase complex (srdBCA) that couples quinone oxidation with selenate reduction. Thus, reducing the toxicity of selenium oxyions along with peroxiredoxin having its role in degrading the reactive oxygen species. Likewise, *Bacillus selenitireducens* is also reported to produce an enzyme capable of reversing the oxidation of selenate and arsenate ions to their less toxic "reduced" forms [35]. The key ingredient in these enzymes is the transition element molybdenum that grants the ability to reduce certain unusual elements. Moreover, as arsenate contamination and arsenate reducing bacteria are reported in Mandovi estuary [24], bacteria isolated from these estuarine waters will possibly have selenate reducing property. Also, there are electronic industries along the coastal belt of Mandovi estuary which may possibly be a responsible source of selenite contamination in Mandovi.

Further, the well separated and dried selenium nanoparticles weighed 3.6953 mg l^{-1}, therefore the bioconversion efficiency of selenium was found to be 77 %. Additionally, the SEM analysis revealed that *Citrobacter fruendii* strain KP6 is single short rod arrangement (Fig. 3a) but when exposed to 60 mM selenite cells it was found to form aggregate and produce exopolysaccharide like substance surrounding the bacterial cell (Fig. 3b) and hence was characterized as *Citrobacter* sp. The formation of cell aggregate and exopolysaccharide (EPS) production reveals the protection strategy of *Citrobacter fruendii* strain KP6 from toxic levels of selenite. This result corroborates to that reported by Naik et al. [36] which stated that *Enterobacter cloacae* in the presence of toxic levels of lead produced EPS and showed aggregation to withstand the metal stress.

Furthermore, the surface topology and morphological features of red precipitate obtained after bacterial reduction of soluble selenite to selenium was studied using SEM and particle size was analysed using TEM. The average particle size confirmed using TEM (Fig. 4a) was found to range from 45 to 70 nm while the SEM images revealed spherical shaped nanomaterials (Fig. 4b). Moreover, the powder XRD pattern (Fig. 5) of the red precipitate substantiated the formation of Se nanoparticles showing peaks with d values 3.30, 2.82, 1.99, 1.70 and 1.63 which are characteristic for nano selenium (JCPDS 27-601 and 27-1202) indicating that the Se particles were crystalline in nature [37, 38]. The nanocrystallites mean size analysis performed using Scherer's formula indicated the average size of mineral formed to be 40 nm. XRD thus confirmed the bioconversion of selenite by *Citrobacter fruendii* strain KP6 to red coloured elemental selenium. This is the first report of estuarine *Citrobacter* species isolated from Mandovi estuary of Goa, India, bioconverting selenite as elemental Se^0 nanomaterial. As compared to previous studies on aerobic bacterial reduction of selenite and selenate to elemental selenium [20, 31, 32] which lacks the detailed characterization of selenium nanoparticles.

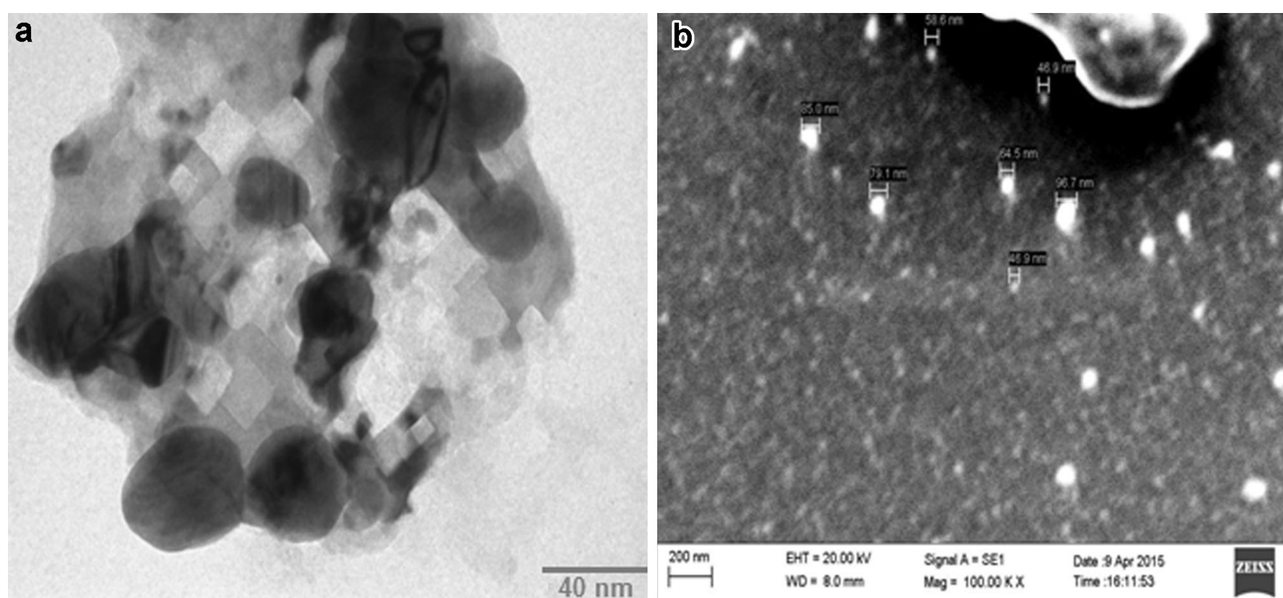

Fig. 4 TEM **a** and SEM **b** micrograph of selenium nanoparticles produced by bacterial strain KP6

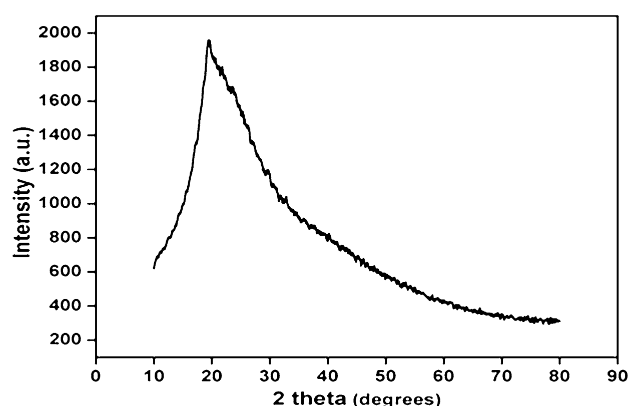

Fig. 5 X-ray diffraction pattern of selenium nanoparticles produced by bacterial strain KP6

The present study reported for the first time that the MIC and MTC of selenite as well as nanocrystallite size determination of selenium nanoparticles synthesized by an estuarine isolate can also be used for bioremediation of selenite contaminated sites. Moreover, the studies in an estuarine environment so far emphasized mainly on the anaerobic reduction of selenite and selenate to elemental selenium [15, 39] making the present study more advantageous for being carried out in aerobic environment at room temperature (30 °C) and easier separation of selenium nanoparticles which the former is deficient. The biologically synthesized selenium nanoparticles are recognized for their significant applications in electronics, optics, catalysis and sensors [40] as well as having biomedical applications [41].

Future prospective

Deducing the fundamental mechanism of selenite resistance in estuarine, *Citrobacter fruendii* strain KP6 by enzyme mediated detoxification of selenite (soluble and toxic) to selenium (insoluble and non toxic) would prove useful in designing bioremediation of estuarine polluted with toxic selenite in near future. The combination of genetic engineering of bacterial catalysts with judicious eco-engineering of polluted sites will be of paramount importance in future bioremediation strategies. Genetic engineering and high level expression of selenite reducing enzyme in estuarine bacteria has proved to be a very promising strategy in near future to deal with metalloid pollution. Ecological and environmental concerns and regulatory constrains are major obstacles for testing GEMs in the fields therefore their practical impact and delivery under field conditions need to be studied in details.

Conclusion

Citrobacter fruendii strain KP6 can be exploited for bioremediation of estuarine sites contaminated with selenite into non-toxic selenium while the green synthesis of Se nanoparticles could be further employed for its use in the field of nanotechnology and biotechnology which is more economical and eco-friendly solution to chemically synthesized Se nanoparticles.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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