# Chapter 31
# Biomineralization of Metallic Tellurium by Bacteria Isolated From Marine Sediment Off Niigata Japan

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**Abstract** Three facultative anaerobe mesophilic bacteria were isolated from marine sediment collected off Niigata, Japan. Sequencing of complete 16S ribosomal DNA revealed 99% homology with *Shewanella algae*, *Pseudomonas pseudoalcaligenes*, and *P. stutzeri*. Phylogenetic analyses suggest novel strain status thus new strains were designated as *S. algae* strain Hiro-1, *P. pseudoalcaligenes* strain Hiro-2, and *P. stutzeri* strain Hiro-3. Minimum inhibitory concentration assays using increasing concentrations of $Na_2TeO_3$ revealed resistance of *S. algae* strain Hiro-1 at 15 mM, and *P. pseudoalcaligenes* strain Hiro-2 and *P. stutzeri* strain Hiro-3 both showed resistance at 4 mM. Transmission electron microscopy revealed intracellular aggregation of metallic tellurium nanorods with a minimum unit size of 60-nm nanoparticle.

**Keywords** Marine sediment · *Pseudomonas pseudoalcaligenes* · *Pseudomonas stutzeri* · *Shewanella algae* · Tellurite reduction · Tellurium nanorods

## 31.1 Introduction

Tellurite is a strong oxidizing agent that is highly toxic to most microorganisms (Fleming 1932; Taylor 1999; Chasteen et al. 2009; Arenas-Salinas et al. 2016). The compound's toxicity was reported to induce oxidative stress, which eventually leads to cell death. Some microorganisms can resist this toxicity, either by enzymatic reduction with the aid of nitrate reductase or by overexpression of glutathione (GSH) to maintain homeostasis inside the cell (Avazeri et al. 1997; Sabaty et al. 2001; Turner 2001; Turner et al. 2012; Pugin et al. 2014). Tellurate ($TeO_4^{2-}$) and tellurite ($TeO_3^{2-}$) oxyanions can also serve as electron acceptors in anaerobic

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respiration by purple sulfur bacteria (Csotonyi et al. 2006; Baesman et al. 2007). Reduction of tellurite into pure metallic elemental tellurium (Te⁰) can be observed as formation of black tellurium precipitate (Tucker et al. 1962).

Production and recovery of this scarce metalloid is required for the sustainability of green technologies such as solar cells in the future. However, the exact mechanism of tellurium reduction is still unknown. Most studies conducted on tellurite reduction (TR) employed bacterial species with very low resistance to the element, and the poor survival of cells in the presence of tellurite is a major impediment to fully elucidating the enigmatic reduction mechanism (Arenas-Salinas et al. 2016). Biomineralization of tellurium nanoparticles (TeNPs) has been documented in several bacterial genera including *Rhodobacter*, *Escherichia*, *Shewanella*, *Geobacter*, *Sulfurospirillum*, *Bacillus*, *Pseudomonas*, *Erwinia*, *Agrobacter*, *Staphylococcus*, and *Selenihalanaerobacter* (Moore and Kaplan 1994; Avazeri et al. 1997; Trutko et al. 2000; Sabaty et al. 2001; Di Tomaso et al. 2002; Borsetti et al. 2003; Oremland et al. 2004; Csotonyi et al. 2006; Baesman et al. 2007, 2009; Turner et al. 2012; Borghese et al. 2014). Some species within these bacterial genera possess innate resistance to the toxic metalloid and are thereby potential candidates for microbiological reduction and recovery of the valued rare Earth element. Here, we report the isolation and identification of tellurite-resistant and tellurite-reducing bacterial strains that can be used for the development of efficient metal recovery strategies. Further analyses of these strains may add additional insights into factors prerequisite for efficient bioremediation.

## 31.2   Materials and Methods

### 31.2.1   *Isolation and Cultivation*

A marine sediment sample collected at a depth of 100 m off Niigata, Japan (38°05′N, 139°04′E) was generously provided by Dr. Takeshi Terahara of the Tokyo University of Marine Science and Technology. The sediment was inoculated and incubated at a final volume of 50 mL with RCVBN medium (Burgess et al. 1991) in completely filled 50-mL centrifuge tubes (Falcon) under continuous illumination for 1 month. The culture showed prominent growth of a biofilm-forming purple bacteria as observed by the deep purple pigment production in the media. A 1-mL sample of the resulting culture was transferred into 7-mL fresh RCVBN medium supplemented with 1-mM sodium tellurite and cultured in completely filled 8-mL screw-capped tubes at 24 °C under continuous illumination (38 μmol/m²/s). TR activity was visually recorded by the formation of black tellurium precipitate. The successive pour plate method and streak plate method were used to isolate tellurite-resistant and tellurite-reducing bacteria on RCVBN agar media (pH 7.6, 37 °C). Purified colonies were re-streaked in RCVBN agar plates with 1 mM $Na_2TeO_3$ to observe TR activity. Colony characteristics were observed after 72 h of growth. To determine the TR activity and minimum inhibitory concentration (MIC), the cultures were incubated with varying concentrations of $Na_2TeO_3$ (1, 2, 4, 6, 8, 10, 12, and 15 mM). Inoculum

was standardized by using culture with similar OD values for each strain. Bacterial growth in pH and temperature optimum experiments was measured using spectrometer at $OD_{550}$ nm (WPA CO-7500 Colorimeter, Biochrom Ltd., UK).

### 31.2.2    16S rDNA Amplification, Cloning, and Sequencing

The 16S rDNA was amplified using PCR mix including 10X KOD Plus-Neo Buffer, 2 mM dNTPs, 25 mM $MgSO_4$ (TOYOBO Co., Ltd., Osaka, Japan), 10 µM each forward and reverse primer 16S 27F (F-5′-AGAGTTTGATCNTGGCTCAG-3′) and 16S ipRSR2 (R-5′-AAGGAGGTGATCCANCCGC-3′) (Eurofins Genomics, Tokyo, Japan), 0.05 U/µL KOD Neo POL (TOYOBO Co., Ltd., Osaka, Japan), and 2 µL genomic DNA. Thermal cycling conditions were as follows: pre-denaturation 2 mins at 94 °C for 1 cycle, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 68 °C for 60 s, and final extension at 68 °C for 60 s (T100™ Thermal Cycler, Bio-Rad, CA, USA).

After PCR, amplified fragments were excised from the gel. Purified 16S rDNA fragments were cloned into the ZERO-Blunt TOPO vector following the kit protocol (Invitrogen, USA). Clones able to grow on LB/Kanamycin plates were used for colony PCR to verify insertion using M13 primers (F-5′-TGTAAAACGACGGCCAGT-3′; R-5′-CAGGAAACAGCTATGACC-3′) (Eurofins Genomics, Tokyo Japan). Thermal cycling conditions were as follows: pre-denaturation 5 mins at 94 °C for 1 cycle, followed by 28 cycles of denaturation at 94 °C for 10 s, annealing at 70 °C for 20 s, and extension at 72 °C for 45 s. Positive clones with verified inserts were sub-cultured and plasmids were extracted using a Fastgene plasmid mini kit (Nippon Genetics Co., Ltd., Tokyo, Japan) following the manufacturer's protocol. The sequences of the resulting clones were analyzed by Eurofins Genomics Co., Ltd. (Tokyo, Japan). SnapGene (SnapGene Software, www.snapgene.com) was used to check chromatogram quality and to guide quality trimming. High quality reads were assembled using CodonCode Aligner (CodonCode Corporation, www.codoncode.com) and assembled sequences were used for BLAST analysis (NCBI).

### 31.2.3    Phylogenetic Analysis

With MEGA6 software, Neighbor-Joining, Maximum-Likelihood, Minimum Evolution and Maximum Parsimony with Kimura-2-parameter distance correction and 1000 bootstrap value were used to identify the three pure cultures (Kimura 1980; Felsenstein 1985; Saitou and Nei 1987; Rzhetsky and Nei 1992; Nei and Kumar 2000; Tamura et al. 2013). NCBI GenBank database was surveyed for closely related strains with partial and complete 16S ribosomal DNA sequences included in the phylogenetic analyses. The 16S rDNA sequences of all species identified were aligned with the muscle DNA alignment method in MEGA 6 software.

Aligned sequences were visually evaluated to ensure that the subsequent cladogram would result from polymorphisms rather than sequence errors, gaps, and branch pulling due to differences in sequence directions or sizes. A total of 26 *Shewanella* and 24 *Pseudomonas* species and strains were included in the phylogenetic analyses with equal length of final quality trimmed sequences of 1360 bp and 1391 bp, respectively.

### 31.2.4   Benchmarking of New Isolates with Type Strains

Type strains *Shewanella algae* (NBRC 103173[T]), *Pseudomonas pseudoalcaligenes* (NBRC 14167[T]), and *Pseudomonas stutzeri* (NBRC 14165[T]) were obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC) in Japan. The type cultures were initially revived using the prescribed growth medium for each culture, then were grown in RCVBN media to compare colony morphology with our new isolates. Cultures were streaked onto plates to observe single pure colonies of the type strains and new isolates. RCVBN agar spread with 200 μL 1 mM $Na_2TeO_3$ was used to evaluate the TR activity of the type strains. Plates were incubated at 37 °C for 6 days were used for observation of morphology and TR activity under stereomicroscope (TW-360, WRAYMER, Japan).

### 31.2.5   Electron Microscopy of New Isolates

Transmission electron microscopy (TEM) was performed on the three new isolates to observe their crystal morphologies and localizations. Enrichment cultures of each strain exposed at 1 mM $Na_2TeO_3$ incubated for 101 days were used for TEM. The long incubation period would allow for the observation of all possible crystal morphologies. A 1-mL culture of each strain was washed twice with milliQ water by centrifugation at $10,000 \times g$ for 5 min. The harvested cells were resuspended in milliQ water and mounted on 150-mesh copper grids coated with collodion (Nisshin EM Co., Ltd., Tokyo, Japan).

## 31.3   Results

### 31.3.1   Three Marine Mesophiles with Tellurite Resistance and Reduction Activity

The mesophiles isolated into pure culture included two *Pseudomonas* species and one *Shewanella* species. BLAST search using the full-length 16S rDNA sequences of the three isolates revealed 99% homology to *P. pseudoalcaligenes* (Query length: 1529 bp),

*P. stutzeri* (Query length: 1529 bp), and *S. algae* (Query length: 1538 bp) with E-value of 0.00. Based on phylogenetic analyses using four different building methods, the three isolates were found to be unique strains. Representative trees are shown in Fig. 31.1. Thus, we designate new strains on *S. algae*, *P. pseudoalcaligenes*, and *P. stutzeri* with strain Hiro-1 (DDBJ Accession no.: LC339942), Hiro-2 (DDBJ Accession no.: LC339940), and Hiro-3 (DDBJ Accession no.: LC339941), respectively. The two *Pseudomonas* isolates showed TR activity up to 4 mM tellurite with a MIC of 6 mM tellurite.

The *S. algae* isolate showed TR activity up to 10 mM tellurite with a MIC of 15 mM tellurite (Fig. 31.2). Negative control samples containing only RCVBN (Fig. 31.2, tube C1) or RCVBN spiked with 1 mM $Na_2TeO_3$ (data not shown) showed no visible TR or bacterial growth indicating no spontaneous reduction or contamination, respectively.

### 31.3.2 Colony Characteristics of New Isolates

*S. algae* strain Hiro-1 formed colonies with round form, entire margin, convex elevation, mucoid consistency, and orange-brown color. *P. pseudoalcaligenes* strain Hiro-2 formed colonies with irregular form, undulate margin, raised elevation, viscid consistency, and translucent color. *P. stutzeri* strain Hiro-3 formed colonies with wrinkled form, undulate margin, raised elevation, rugose consistency, and yellow color. The morphology and TR activity were also compared to the type strains, and the new isolates showed similar colony morphology and TR activity to the type cultures (Fig. 31.3). All three new strains grew optimally at pH 7.0. Growth at pH 7.0 was significantly higher than growth at all other pH levels tested based on unpaired *t*-test with 95% confidence level (*p*-value <0.05). Optimum growth temperature coincided with mesophilic cardinal temperatures. For all three strains, there was no significant difference between the growth at 25 °C, 37 °C, and 45 °C based on unpaired *t*-test with 95% confidence level (*p*-value >0.05). Little to no growth was observed at 4 °C.

### 31.3.3 Intracellularly Deposited Tellurium Particles

TEM revealed that tellurium particles were deposited within the cell for all three strains (Fig. 31.4). In *S. algae* strain Hiro-1, 60-nm minimum units of nanorod particles were conjugated along the crystallographic axis, forming long rod crystals that finally assembled into a bundle within the cell (Fig. 31.4a). However, this culture was too old, and the cells were easily lysed. External, inorganically growing crystals were also found (data not shown). Strain Hiro-2, interestingly, formed needle-shaped particles 60 nm in size (Fig. 31.4b), whereas strain Hiro-3 formed rod-shaped crystals resembling the nanorods from the strain Hiro-1 (Fig. 31.4c).
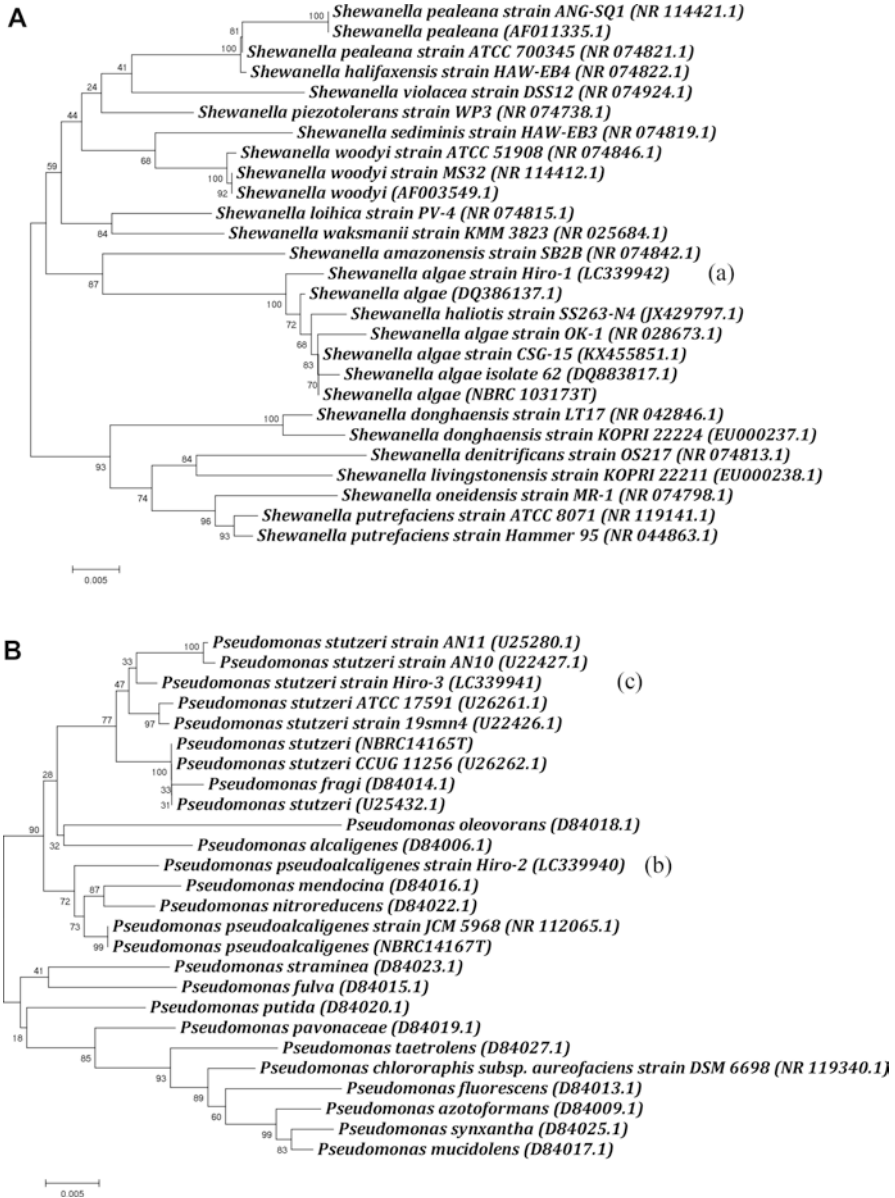
**Fig 31.1 Unrooted neighbor-joining tree.** (**a**) *S. algae* strain Hiro-1 (a). (**b**) *P. pseudoalcaligenes* strain Hiro-2 (b). P. stutzeri strain Hiro-3 (c). Accession numbers are in parentheses. Numbers at nodes represent bootstrap values from 1000 resampled datasets. Scale bar indicates 0.5% sequence divergence. Kimura-2-parameter was used for distance correction
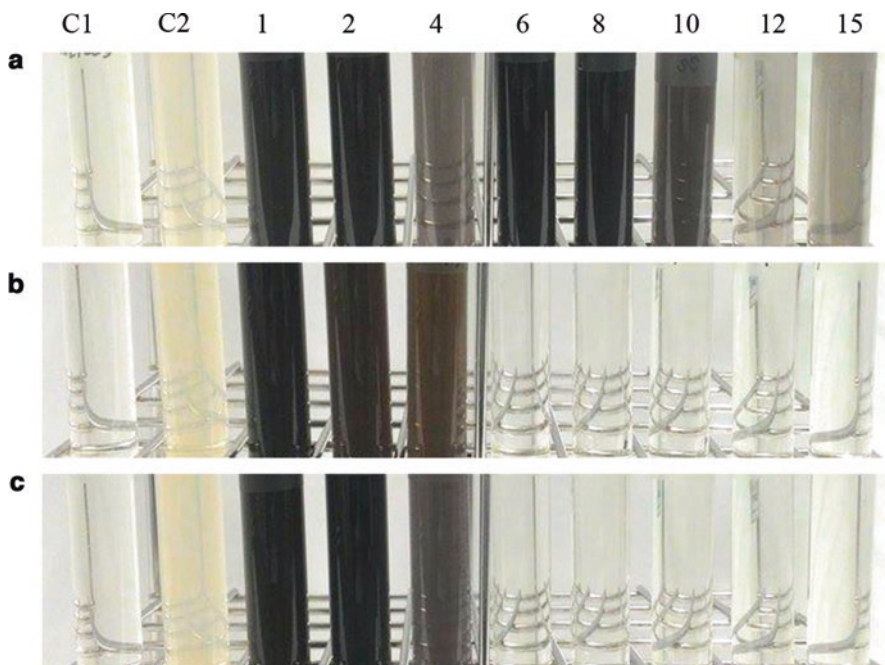
**Fig. 31.2** Tellurite reduction activity (TR activity) and minimum inhibitory concentration (MIC) of tellurite. Control tube C1 is RCVBN media alone and control tube C2 is RCVBN with culture inoculum. The other tubes are labeled with the concentration (mM) of $Na_2TeO_3$ added to the RCVBN media inoculated with the culture. Image shown is representative of two replicate reactions taken after 3 weeks of incubation. (**a**) *S. algae*, (**b**) *P. pseudoalcaligenes*, and (**c**) *P. stutzeri*

Both *Pseudomonas* spp. maintained cell integrity, and both showed smaller and scattered nanoparticles within the cell. Long crystals were observed in ripped cells, suggesting that the length of tellurium crystal must be regulated within the cellular environment.

## 31.4  Discussion

Deep marine sediments support the growth of anaerobic microorganisms that may use various metals as final electron acceptors in the respiratory chain, such as purple sulfur bacteria. *S. algae* has bioremediation potential against uranium, plutonium, tellurite ions, nitrite, and halogenated organic compounds (Almagro et al. 2005; Klonowska et al. 2005). Mucoid, round colonies are common colony morphologies for *Shewanella* species (Simidu et al. 1990; Nozue et al. 1992; Venkateswaran et al. 1998; Holt et al. 2005; Gao et al. 2006; Kim et al. 2007) which is also evident in our isolate. *P. pseudoalcaligenes* and *P. stutzeri* have bioremediation potential against cyanide and tellurite (Romero et al. 1998). *P. pseudoalcaligenes* subsp. *citrulli*,
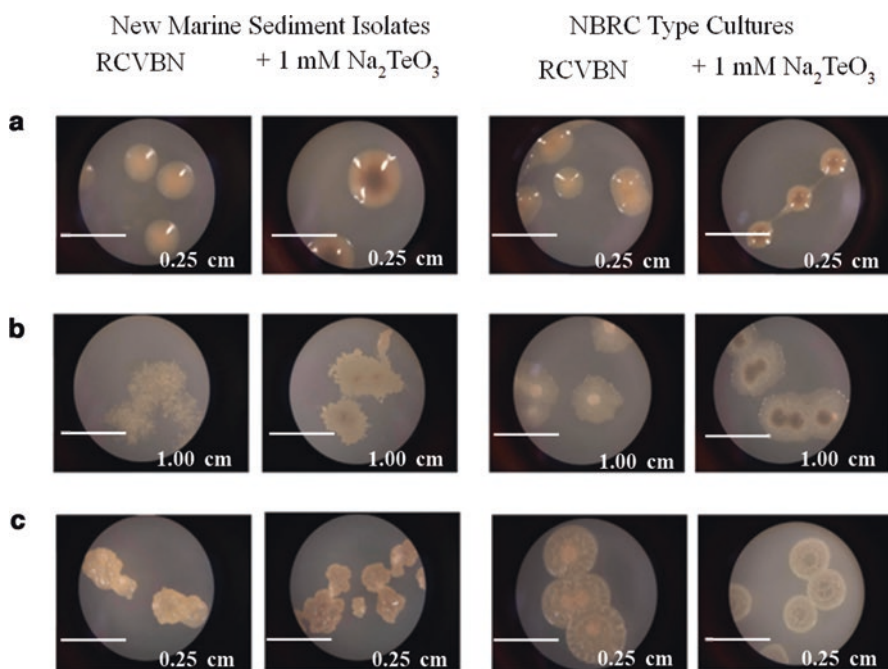
**Fig. 31.3** Colony morphology and TR activity of the three new isolates and their corresponding type cultures, *S. algae* (**a**), *P. pseudoalcaligenes* (**b**), and *P. stutzeri* (**c**)
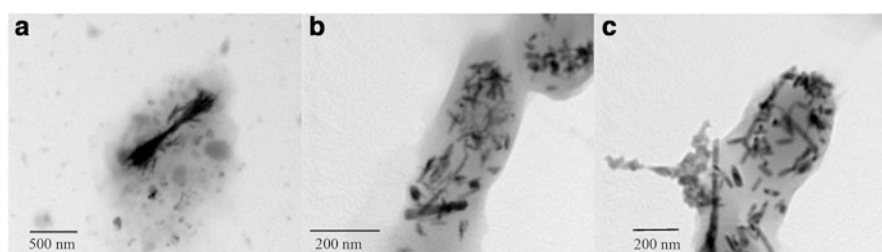


**Fig. 31.4** TEM observation of intracellular tellurium particles in *S. algae* strain Hiro-1 (**a**), *P. pseudoalcaligenes* strain Hiro-2 (**b**), and *P. stutzeri* strain Hiro-3 (**c**)

isolated from diseased watermelon, and *P. alcaligenes*, a human opportunistic pathogen, are both reported to have translucent consistency (Monias 1928; Ralston-Barrett et al. 1976; Schaad et al. 1978) which is consistent with our isolate. *P. stutzeri* isolated as an opportunistic human pathogen has wrinkled and hard/dry characteristics (Lalucat et al. 2006) which is also observed in our isolate.

Our new isolates from deep marine sediment have also shown extreme resistance and reduction activity in very high concentrations (above 1 mM) of tellurite ions. This extreme resistance could be attributed to the species-specific reduction mechanisms, differential cell physiology, or genetic adaptation mechanisms in extremely toxic environments.

Species-specific biomineralization has been observed in several bacterial strains. Common tellurium morphologies vary from spheres to nanospheres and from rods to nanorods (Turner et al. 2012). Spatial distribution of nanoparticles also shows species-specific variation. The most common localization of metal biodeposition is at the cell surface or in the cytoplasm (Turner et al. 2012). The new isolates showed intracellular mineralization of metallic tellurium nanorods. This was also evident from the blackening of bacterial colonies, while surrounding tellurite ions present in the agar media were unaffected. Intracellular crystal formation denotes influx of metal ions into the cell cytoplasm, and thus unknown ion transporters or channels might be involved in this phenomenon. Once inside the cell, enzyme-catalyzed reduction of tellurite ions into elemental tellurium precedes crystal nucleation and growth. Tellurium crystals showed minimum unit size of 60 nm which falls under the category of nanoparticles (<100 nm) and therefore has a potential use for nano-technology applications (Arenas-Salinas et al. 2016).

Together, these results show that the three new isolates identified in this study effectively reduce tellurite even at high concentrations. This reduction occurs within the cell, but determining the exact mechanisms will require further study. These strains may be useful for bioremediation, as well as for recovery of this valuable metalloid for use in manufacturing.

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