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TELLURITE RESISTANCE AND REDUCTION DURING AEROBIC AND ANAEROBIC GROWTH OF BACTERIA ISOLATED FROM SARCHESHME COPPER MINE

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ABSTRACT

Tellurium compounds can be found in high concentrations in land and water near sites of waste discharge of industrial manufacturing processes and anodic sludge of copper mine. Potassium tellurite (K_2TeO_3) is toxic to many microorganisms at concentrations >1mg/mL. In this research, some species of facultative anaerobic bacteria (*Bacillus* sp.) were isolated from Sarcheshme copper mine(Kerman, Iran) which demonstrated high-level-resistance to tellurite and accumulation of metallic tellurium crystals. High-level-resistance was observed for Bacilli and cocci grown with certain organic carbon sources, implying that tellurite reduction is not essential to confer tellurite resistance. Level of adsorption was determined by inductively coupled plasma and spectrophotometer (Diethyldithiocarbamate method). The level of tellurite concentration in the bacteria cell and the formation of tellurium nanocrystals were illuminated by transmission electron microscope and scanning electron microscope. The Te(0) crystals occur internally and each microorganism forms a distinctly different structure (for example *Bacillus selenitreducens* make tellurium nano rod). In this study it was found that microorganism can grow 3.in 1500mg/L-2000mg/L and higher tellurite concentrations. The use of microorganisms to generate Te nanomaterials may be an alternative for bench-scale syntheses. Additionally, they may also generate products with unique properties unattainable by conventional physical/chemical methods. This study is important because native bacteria from Sarcheshme (Kerman, Iran) that may show high-level-resistance to tellurite, were isolated.

Key world: Tellurium, ICP, Nanorod, Transmission electron microscope, Scanning electron microscope, High-level-resistance, Tellurite

INTRODUCTION

Tellurium is a toxic metalloid present as a trace constituent (2mg/L) in the earth's crust. It occurs in nature in four oxidation states of 6^+, 4^+, 0, and 2^-. The first two forms are the partially soluble oxyanions tellurate [TeO_4^{2-}, or Te(VI)] and tellurite [TeO_3^{2-}, or Te(IV)], and with respect to the latter two, the occurrence of native tellurium [Te(0)] is rare, while metal telluride represent the most common form found in minerals. Although some marine ferromanganese crusts are enriched in their Te content (Herbel, 2000), the concentrating mechanism from seawater is not known. Tellurium oxyanions were once examined as potential antibacterial agents (Fleming, *et al*.; Young, 1940) but more recently, the resistance of diverse bacteria to Te(VI) and Te(IV) has been studied (Moscoso, 1998, O'Gara, 1997). The mechanisms of resistance to Te oxyanions, most commonly Te (IV), involve their physical removal from the cell's immediate aqueous environment, a response similar to that for toxic selenium oxyanions. This can be achieved by either volatilization to form dimethyl telluride (Basnayake, 2001) or reductive precipitation to form insoluble Te(0). The precipitated Te(0) can occur outside (Klonowska, *et al*., 2005) or, more commonly, inside the cells (Borghese,2004), sometimes in association with the inner cell envelope (Tebo, 1998).

It is not known whether Te has a biogeochemical

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cycle; however, a number of toxic elements clearly do have such cycles. On the reductive side, uranium (Lovley, 1991), chromium (Taylor, 1994), and vanadium can all serve as electron acceptors for the anaerobic growth of diverse prokaryotes. The recent discovery of Te(VI) reduction by bacteria isolated from the surfaces of marine worms found near hydrothermal vents was suggestive of respiration.

A constitutive high-level resistance (HLR) to rare earth oxides and oxyanions at concentrations approaching 1000 mg/mL was recently described for photosynthetic purple nonsulfur bacteria of the alpha subclass of *Proteobacteria*. Obligate aerobic an oxygenic phototrophs, a new physiological group of the same subclass produce bacteriochlorophyl, but are unable to grow photosynthetically under anaerobic conditions. In this research, HLR to tellurite with bacteria isolated from Sarcheshme copper mine (located in Kerman province of Iran) were studied. These isolated species have the ability to reduce TeO_3 to metallic Te in very large amounts.

A stoichiometric growth relationship between the oxidation of the provided electron donor (Pyruvate) and the reduction of this oxyanions to Te(0) is also shown. Moreover, dissimilatory reduction of Te oxyanions by these two bacteria resulted in the formation of unusual Te(0) crystals that could have a future practical application(s) as composite or compound nanomaterials in solar cells.

MATERIALS AND METHODS

Some species of facultative anaerobic bacteria (*Bacillus* sp) were isolated from various area of Sarcheshme copper mine(Kerman, Iran). Two species (B_1:B.laterosporus and B_2:B.filicolonicus) demonstrated high-level resistance (HLR) to tellurite (2000mg/L) and accumulation of metallic tellurium crystals. These species (sample B_1 and B_2) presented HLR (with minimum inhibitory concentrations (MICs) between 500 and 2000mg/L of K_2TeO_3) in nutrient agar medium or BHI agar containing yeast extract.

Many tests were carried out for diagnosis. Morphological tests, stainig, biochemical tests such as urea Agar, Sulfate Indol Mobility Agar,Triple Suger Iron Agar, citrate test suger tests, and egg ulk,. Biochemistry test illuminated that both B_1 and B_2 belonged to *Bacillus* sp ($B_{1:}$ B.laterosporus and $B_{2.}$B.filicolonicus) and the results of biochemistry test was confirmed molecular diagnosis.(Smith, R.1985)

For this purpose,16SrDNA nucleotide test weredone.16SrDNA method has several steps as follows:

-DNA extraction with Amersham kits.

-Electrophoresis in agarose gel.

-PCR with special primer containing (Haghighat, S,2008):

Primer 5/ :TAG CTT GTT ACG ACT T

Primer 3/ :AGA GTT TGA TCA TGG C

The result was match.

The strong oxidant property of tellurite confers its toxic character for microorganisms (Herbel, *et al*., 2000). In most cases analyzed in this study, HLR to tellurite is correlated to its reduction into Te. These results imply that tellurite reduction is not essential to confer tellurite resistance and some other mechanisms, such as continuous tellurite efflux or tellurite complexing or methylation could play an important role in the resistance character. This study demonstrated that the extent of tellurite reduction in B_1 and B_2 was inversely related to the oxidation state of the carbon source present in the growth medium.

In addition to its detoxification effect, reduction of metal oxide could be a way to dispose of excess electrons by the reoxidation of NADH, FADH2, or quinones and, therefore, for maintenance of an optimal redox poise in vivo, the tellurite reduction and the oxidation state of the carbon source is not evident in the case of facultative anaerobic bacteria. Indeed this species can reduce Te(IV) to Te(0) in nutrient medium with yeast extract and other rich organic medium (RO) but not in minimal salt (MS) medium containing L-glutamine, pyruvate, or acetate as the sole organic carbon source. These results are consistent with the idea that during growth in RO medium B_1 and B_2 cells presented with an excess of reducing power, and electron carriers could be oxidized by giving electrons to tellurite.

$B_1$and B_2 were grown in anaerobic batch culture, with pyruvate as the electron donor, but with Te oxyanions serving as the electron acceptor (Oremland,1994). Growth experiments with

B_1 or B_2 were conducted by making pulsed additions of 2000 mg/L of TeO_3, respectively, to the cultures over the course of 30-day incubation. This strategy was chosen because higher starting concentrations of Te were found to inhibit growth. Pulsed 2000 ppm additions of TeO_3 were made at several intervals over the 30-day incubation. Both cultures ($B_1$and B_2) were incubated statically at 32°C and were subsampled using a sterile anaerobic technique in order to maintain the proper TeO_3 concentration in the aqueous phase. Bacterial cells were grown in 500-mL batche as described above, with nitrate as the electron acceptor for control and TeO_3 for test (no nitrate added). Second control contained 5oo-mL batches as described without nitrate or TeO_3. In each batch 5 mM pyruvate serving as the electron donor was used.

The liquid and solid fractions of the washed cells were separated by centrifugation, and the solid phase was rinsed with deionized water before being dissolved and cleaned for its Te-isotopic analysis.

Te concentrations were measured on a small weighed fraction taken from each sample solution using the ICP (Inductively coupled plasma atomic absorption). The fundamental base of this method is to produce individual gas atoms of sample and absorption of photon with special wave length that depend on sample atom. In this method standard solutions of tellurite are not needed. Tellurium isotope compositions were measured on ICP at the Research and Development Center, National Iranian Copper Industries Company in Kerman. The instrument was operated in standard-resolution mode. In diethyldilhiocarbamate (DDTC) method standard tellurite solution (different concentrations of tellurite was needed) then aliquot of tellurite containing broth was mixed with 100 µL of DDTC reagent and brought up to 500 µL with buffer.300µL tris HCL was added followed by 100micro L DDTC reagent. After mixing, the absorbance of this mixture was read at 340 nm.

Electron microscopy

Scanning electron microscopy (SEM) images were prepared by filtering the bacteria and Te(0) onto 0.1-µm Nuclepore filters, fixing them with gluteraldahyde and dehydrating them in ethanol. For transmission Electron Microscopy (TEM), thin sections were taken from cells fixed with 2% (vol/vol) glutaraldehyde in phosphate buffer, stained with 2% (wt/vol) uranyl acetate, and lid citrate. Some sections were floated on 2% uranyl acetate for 2 min and washed with water before being viewed.

RESULTS

Demonstration of respiratory growth on TeO_3

Growth of $B._1$ and $B._2$ on TeO_3 was slow (doubling time = 2.4 days; growth rate = 0.2/d) but resulted in a 10-fold increase in cell density, the near-quantitative oxidation of pyruvate, and the removal of more than 60% of the cumulatively added TeO_3. At the end of the incubation, there was a complete recovery of Te(0) from the added TeO_3 as determined by oxidation of the black precipitate back into solution with nitric acid. In contrast, there was no significant growth in live controls lacking TeO_3 and oxidation of pyruvate did not occur, while there was no loss of TeO_3 from sterile controls, which achieved a final cumulative added concentration. Similar growth phenomena were observed for B_1 and B_2 using TeO_3 as its electron acceptor. Cell densities reached 3×10^8 cells/mL (doubling time = 3.3 days; growth rate = 0.2/day), whereas controls without TeO_3 did not grow (2×10^7 cells/mL), and sterile controls did not consume TeO_3. Carbon balance was achieved between occurrences of both pyruvates as minor intermediates. Similarly, a complete reduction of 1000mg/L TeO_3 to Te(0) would require about 25mmol-eq electrons, while the observed formation of 9 mM pyruvate would generate 25-mmol-eq electrons or more from the oxidation.

Tellurite was a transient intermediate during growth of B_1 and B_2 TeO_3, with concentrations ranging between, 10-15% of the amount of TeO_3 added as pulsed additions.

Electron microscope showed that after 48 h and 72 h accumulation of metallic tellurium crystals in the bacteria cell increase so some changes in bacteria shape was seen. The amount of reduced tellurite depended upon the species and the carbon source.

Transmission electron microscopy pictures,

obtained as previously described, showed very abundant and large black crystals which sometimes occupied about 20 to 60% of the cell volume (Fig. 1A,1B).

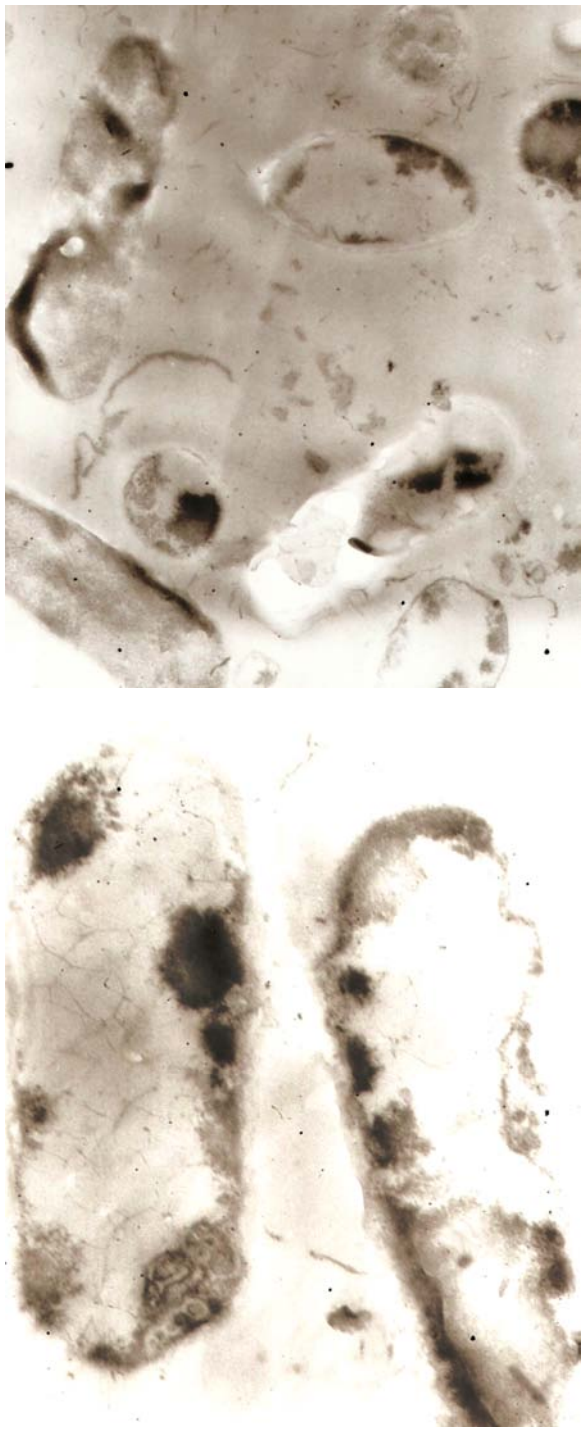
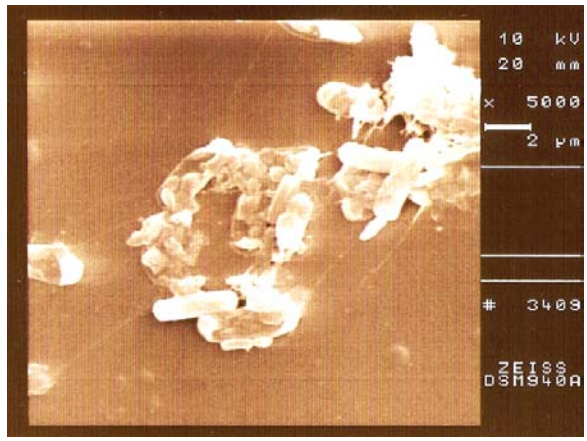


Fig.1A(x12000)- 1B(x30000)- Both washed cell suspensions and growing cultures of *B.$_1$ and B.$_2$* on TeO_3 formed abundant accumulations of te(0) on their cell surfaces

Unstained whole mounts of cell suspensions of B. laterosporus and B.filicolonicus given TeO_3 and examined by TEM revealed thick surface accumulations ("Te nanoparticle") of an electron-dense mineral, on average 32 nm in diameter, that could be seen in tight association with the cells (Figs. 2A.2B.2C). In growth experiments, these surface Te nanorods were somewhat sparser and thinner, on average 18 nm in diameter. These Te nanoparticles presumably grew from cellular nucleation points as Te was reduced. Scanning electron micrographs confirmed this and showed the presence of Te in cells and more roughness on bacteria surface of individual Te shards that sloughed off the cell surfaces. These were interpreted to be the major end product of dissimilatory TeO_3 reduction, because of their obvious abundance when viewed in a wider field (Figs.3D). These particles could be seen both on and off cells, and we suggest that these Te rosettes were composites and, once free, adhered to one another, possibly through electrostatic interaction. Surprisingly, thin sections of B_1 and B_2 revealed internal accumulations of Te(0). This occurred under growth conditions, where the Te(0) had the characteristic nanoparticle appearance (Figs. 1D.2D). These internal Te(0) accumulations often formed close to the cell's periphery and conformed to the contour of the cell envelope (Figs. 2D).



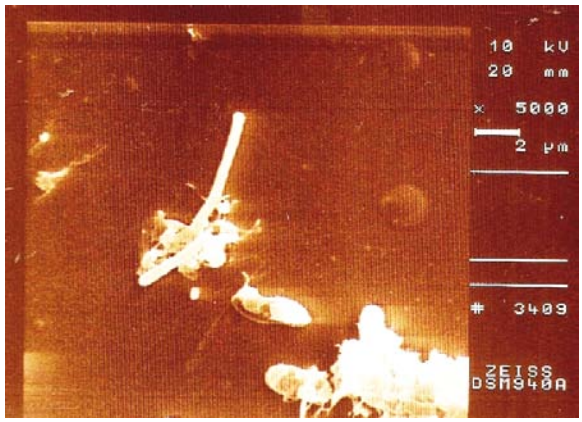


Fig.2 A-B. SEM image of B.1 and B.2.

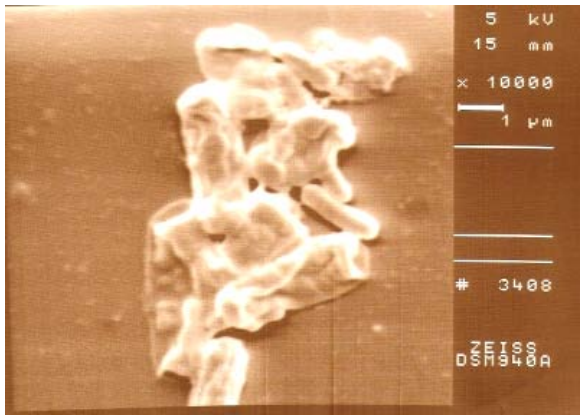


Fig.2C (Scanning electron micrographs showed the presence of Te in cells and more roughness on bacteria surface.)



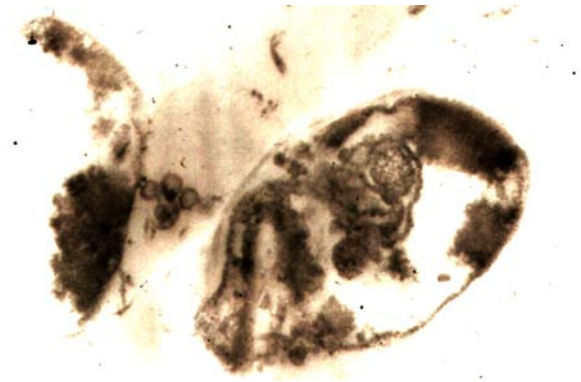


Fig.1D(x30000).2D(x30000) This occurred under growth conditions, where the Te(0) had the characteristic nanoparticle appearance.

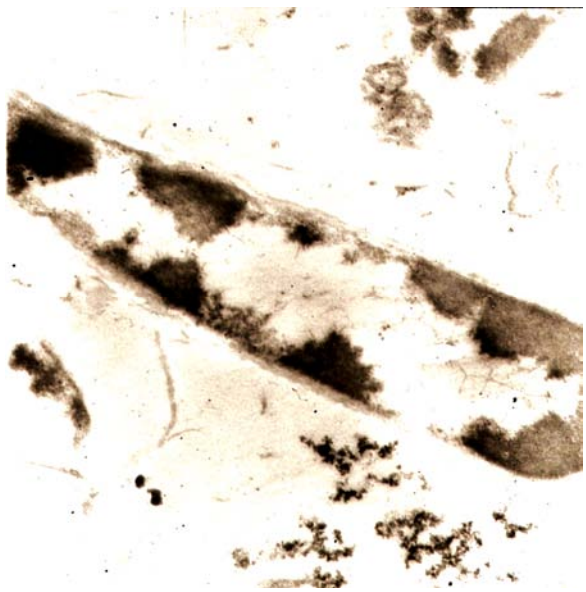


Fig.3D(x50000) It was striking to notice that the internal shards were composed of a linear arrangement of smaller nanocrystallites.

This observation suggested that Te nanoparticle were formed via metal reduction mechanisms occurring inside the cell. It was striking to notice that the internal shards were composed of a linear arrangement of smaller nanocrystallites (Figs. 1D.2D.3D), suggesting that separate nucleation points within the cytoplasm, close to the cell envelope, initiated Te reduction.

Fig.1D.2D

Fig.3D

As these small nanocrystallites grew in size, they annealed together to form the larger, shard-like nanocrystals. This method of growth helps explain the linear shard-like gross morphologies of the Te nanoparticle. After a search through

many thin sections, however, nanorods were not seen escaping from a cell. Indeed, the particulate nature of the nanorods makes it unlikely that they could escape without lysing cells; yet, few lysed cells were encountered. The similar appearances of both the external and the internal mineral phases suggested that they were identical.

Unstained whole mounts of B_1 and B_2 revealed an entirely differently shaped mineral phase. This was in the form of irregularly shaped, 20nm-sized "nanospheres" that coalesced together to form larger, 500 to 1000nm and more, (average = 430 nm) clusters, often attached to the cell surface (Figs .1D.2D)

DISCUSSION

In this study 2 native species have been found that showed high-level resistance (HLR) to TeO3 and ability to reduce TeO3 to metallic Te in very large amounts.

However, these MICs depend upon the carbon source. The highest level of resistance to tellurite (MICs between 2300 mg/L and 2700 mg/L of K_2TeO_3) in presence of pyruvate in this study is 2 to 3 times higher than the highest MICs of tellurite (800 and 1200 mg/mL) described by Moore and Kaplan for Rhodobacter capsulatus and Rhodobacter sphaeroides (Gautam, 2004).

In most cases, a black coloration appears during the growth in the presence of tellurite. This is due to the reduction of tellurite in metallic tellurium with its intracellular accumulation. Intracellular deposits appear as electron-dense crystals in electron microscopy and have been shown to consist of elemental tellurium in bacteria. Excluding B_1 and B_2 grown in the presence of L-glutamine, cycteine and pyruvate, all species examined in the present work were able to reduce TeO_3 to elemental Te in combination with HLR to this compound. Most of the tellurite was biotransformed to metallic Te in 24 h (65%).

Our results for B_1 and B_2 represent the first clear evidence that anaerobic bacterial growth can be achieved by employing tellurium oxyanions as electron acceptors. Previously, cytochromes were shown to be involved in the reduction of TeO_3 to Te(0) by some common gram negative bacteria, but no claim was made with respect to tellurium-associated energy conservation (Tebo, 1998; Shaun Baesman,2007). More recent was a growth claim for two uncharacterized marine isolates associated with a hydrothermal vent that were capable of reducing TeO_3 to Te(0). However, the evidence given for respiratory growth linked to TeO_3 was equivocal in that it was based on modest cell count increases during prolonged incubation as well protonophore inhibition of such growth (Yurkov, 1996).). No clear demonstration of energy conservation linked to TeO_3 reduction was provided. The fact that B_1 and B_2 grew in medium with TeO_3 while no growth was observed in controls lacking these oxyanions represents a clear demonstration of Te-dependent growth. The linkage of pyruvate oxidation to Te oxyanion reduction further demonstrated that these substances served as terminal electron acceptors for anaerobic respiration. Hence, the biological oxidation of organic matter using TeO_3 as an electron acceptor must be exothermic in order to support the growth of B_1 and B_2 experimental observations noted that it conformed to the following stoichiometry (Schröder, 1997):

$$\text{pyruvate} + TeO_3^{2-} + H^+ \text{ à perpionat} + Te(0)$$

Because tellurium oxyanions are relatively strong oxidants, the energy yields for equations under standard conditions are high.

The resistance of bacterial growth to Te oxyanions, primarily TeO_3, has been well studied (Borghese, 2004; Guzzo, 2000; Moscoso, 1998; Tang Z,2006). In general, MICs of 2000mg/L have been commonly reported (Stolz, 2006; Borghese, 2004). Both B_1 and B_2i were originally isolated from sarcheshme copper mine and are native and, respectively, as their respiratory electron acceptors. We undertook this current investigation to answer the simple question of whether oxyanions of tellurium, another group 16 element, could substitute for selenium oxyanions in their capacities to support the growth. While we have shown this to be true, we were surprised by the high tolerance of these bacteria to TeO_3; whether actual dissimilatory tellurite reductases that would be analogous to the substrate-specific selenate reductase of Thaurea selenatis exist (Schröder,1997). Alternatively, it could be noted that these Te reductions are achieved by enzymes

with much broader substrate affinities, such as nitrite reductase (Avarézi, 1997) or dimethyl sulfoxide reductase (Trutko,2000, Hein, 2003). The clusters of exogenous Te(0) nanospheres were clearly the major end product of dissimilatory TeO_3 reduction, as can be seen in an TEM and SEM image over a wider field.

Accordingly, electron transfer took place both inside and outside cells for reasons speculated above, but in this case, a mineral phase different from that seen with, for example *Erythromicrobium ramosum* B. selenitireducens was formed.

The size and total amount of the Te crystals in the species studied in this study were usually greater than those observed in *Escherichia* coli, or *Pseudomonas* or *Rhodobacter* species.(Zhu, 2006).

Visual observation of TEM, and SEM suggested that the inside and outside phases were identical, consisting only of Te, and were crystalline. Native Te(0) obtained from a chemical supply house did not exhibit any of these bacterial Te morphotypes, because the commercial materials either were amorphous or when structured displayed a much larger cuboidal arrangement.

Industrial activity has an enormous influence on the geochemical migration processes of some toxic heavy metals by their dispersion in water, soil, and atmosphere, or their concentration in specific areas. These processes contribute to serious pollution problems. The reduction of soluble TeO_3 to solid Te(0) could be an important mechanism for the removal of this element from polluted places. In this context, the development of microbiological methods for environmental cleaning systems for tellurium oxides is of interest.

Native facultative anaerobic bacteria isolated in this study, being able to transform TeO_3 to Te(0) in very high concentrations , may be considered as promising candidates for such a process.

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