Arsenic and Cadmium Bioremediation by Antarctic Bacteria Capable of Biosynthesizing CdS Fluorescent Nanoparticles

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Abstract: Use of microorganisms in contaminated water remediation is one of the most studied processes of recent years. The recovery of metal contaminants by converting them into high-value nanomaterials represents a scarcely explored topic with high potential economic impact. In this work, the authors determine the capacity to remove As and Cd from aqueous solutions by Antarctic bacteria previously reported as capable of biosynthesizing CdS fluorescent nanoparticles (NPs) at low temperatures. Bacterial characteristics favoring metal bioremediation, such as As and Cd resistance as well as high biofilm formation and metal removal (kinetic/sorption tests), were determined in Antarctic strains. In addition, the effect of As on the biosynthesis of CdS fluorescent NPs [quantum dots (QDs)] was evaluated. The presence of As inhibits the biosynthesis of CdS QDs by Antarctic bacteria. Arsenic inhibition does not involve the disruption of the Cd nanostructure or a decrease in H_2S levels produced by cells, suggesting that As inhibits CdS biosynthesis by avoiding the interaction of Cd^{2+} with S^{2-} required to produce the nanocrystal. Obtained results have significant consequences for the development of metal bioremediation strategies aimed at removing environmental heavy metals through the generation of NPs. **DOI: 10.1061/(ASCE)EE.1943-7870.0001293.** © *2017 American Society of Civil Engineers.*

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Introduction

Toxic compounds find their way into the environment through natural and anthropogenic processes. In particular, heavy metals and metalloids cause major impacts on human health because of their persistence, toxicity, and accumulation through the trophic chain, with arsenic (As) and cadmium (Cd) being two of the usual harmful compounds present in groundwater and soil (Ott 2007). Arsenic is naturally found in soils at a mean concentration of 1.5–2.0 mg/kg in the Earth's crust, although some rocks contain higher concentrations.

High levels of arsenic have been reported in surface waters in the north of Argentina and Chile and are associated with either geological origin or anthropogenic activities such as mining (Ferreccio and Sancha 2006; Avigliano et al. 2015). On the other hand, cadmium exposure could be related to natural causes such as volcanic fumes, but most emissions are anthropogenic, mainly related to the extraction process of this metal, its use as a fungicide or insecticide, and from poorly managed landfills (Aucott 2006; USGS 2010).

Arsenic and cadmium removal processes and technologies are varied and range from conventional treatment plants for ex situ methods to most recent in situ technologies. Remediation processes involve oxidation, precipitation, coagulation, reverse osmosis, microfiltration and nanofiltration, solvent extraction, adsorption, bioremediation and phytoremediation, electrodialysis, and electrokinesis (Hu et al. 2006; Litter et al. 2010; Carro Perez and Francisca 2013a; Khairy et al. 2014; Francisca and Glatstein 2016).

Use of microorganisms to remediate heavy metals represents a green and sustainable alternative for environmental decontamination. This biological process is mostly based on some physiological characteristics of bacterial cells. These characteristics include (1) the ability to incorporate significant amounts of metals; (2) bacterial resistance to high concentrations of these poisonous compounds (metal tolerance mechanisms); (3) movement across

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metal gradients in order to improve metal accessibility (motility and quorum sensing); (4) biofilm formation that allow bacterial populations to establish in contaminated zones, thereby increasing resistance and remediation rates; and (5) intracellular and extracellular reduction of metal cations rendering elemental nontoxic compounds (metal reduction mediated by cellular reductases), among many others (Bansal et al. 2005; Perez et al. 2008).

Recent studies have reported that bacteria have the ability to use some metals for the production of nanoparticles (NPs) (Órdenes-Aenishanslins et al. 2014). In particular, Cd and As can be used to produce nanoparticles with interesting technological properties because of their unique semiconductor and spectroscopic properties.

In recent decades, nanotechnology has acquired great importance because it serves as the base of a number of technological applications. At the nanometric scale, matter changes its chemical, physical, and electrochemical properties. This has allowed the development of new low-cost materials and systems with unique properties that have been used in several technological applications in the areas of electronic/optoelectronics, biomedicine, and energy (Xue et al. 2011).

Using NP biosynthesis for the bioremediation of metal-contaminated environments offers tremendous ecological and economical potential because the process could allow for the transformation of toxic metal contaminants into nanostructures of great economic value and technological potential.

The variety of applications associated with NPs has generated a growing interest in developing new processes for nanomaterial synthesis, given that most current methods use polluting substances and conditions (Peng and Peng 2001). Different chemical and physical methods for NPs synthesis have been developed in recent years (Papp et al. 2007; Kolekar et al. 2011). However, most of these methods are expensive, energy-inefficient, dangerous, and sometimes generate a significant environmental impact. This situation has generated a growing interest in developing cleaner, cheaper, more efficient, and environmentally friendly methods for the production of NPs (Díaz et al. 2012; Phuoc 2014).

Based on this premise, the use of microorganisms for the production of different nanomaterials has been proposed. It has been determined that bacteria and fungi are able to generate metal NPs, such as Fe_3O_4, Cu_2O, TiO_2, ZnO, SiO_2, CdS, PbS, FeS, and AuS (Narayanan and Sakthivel 2010; Jha and Prasad 2010; Thakkar et al. 2010; Li et al. 2011a; Gade et al. 2014; Vala 2015).

Bacterial synthesis of nanoparticles potentially offers other advantages, including low cost, aqueous solubility, improved biocompatibility, and the production of nanoparticles with novel properties (Li et al. 2011b; Mala and Rose 2014; Zhou et al. 2015; Kominkova et al. 2017). In this context, Ulloa et al. (2016) recently reported the biosynthesis of acid-stable quantum dots (QDs) by extremophile bacteria. The molecular mechanisms of microbial NPs biosynthesis are still unknown, and only the importance of some extracellular enzymes and antioxidant defenses, particularly biological thiols, has been established (Ramesh et al. 2015; Velusamy et al. 2016). The enzymatic production of H_2S has been reported as a fundamental step in CdS biosynthesis, particularly because the process favors the extracellular production and consequent purification of QDs (Plaza et al. 2016; Yang et al. 2016).

The main purpose of this paper is to evaluate the capacity to produce CdS QDs during arsenic and cadmium bioremediation of four *Pseudomonas* sp strains isolated from Antarctica (Gallardo et al. 2014). Bacterial strains can grow at low temperatures (15°C) and in the presence of high concentrations of cadmium (Gallardo et al. 2014), two conditions that favor a Cd bioremediation process. In addition, the effect of As on the biosynthesis of CdS QDs is determined.

Materials and Methods

Solutions

The cadmium solution was prepared at a metal concentration of 1,000 mg/L by dissolving cadmium nitrate tetrahydrate [$Cd(NO_3)_2 \cdot 4H_2O$, Anedra (Buenos Aires, Argentina)] in distilled water. All reagents were analytical grade and used without further purification. Depending on the tests, different dilutions between 0.1 and 100 mg/L were prepared with distilled water.

Solutions containing different As concentrations were prepared from the dissolution of arsenic trioxide [As_2O_3, Anedra (Buenos Aires, Argentina)] in an alkaline medium obtained with sodium hydroxide (NaOH) and distilled water. Arsenic concentrations ranged between 0.1 and 100 mg/L. The pH and reduction potential (Eh) of the obtained solutions were pH = 7.3–7.9 and Eh = 175–230 mV. Thus, the main expected arsenic speciation was $HAsO_4^{2-}$ (Masscheleyn et al. 1991).

Bacterial Selection

Sample Collection

Samples were provided by the Bionanotechnology and Microbiology Lab (Andres Bello University, Santiago, Chile) and correspond to bacterial strains isolated from Antarctic soil, sediments, and water collected during the 48th Chilean Antarctic Expedition (ECA) organized by the Chilean Antarctic Institute (INACH). These specimens were collected at Deception Island (named Teylya in Russia) and King George Island (known as 25 de Mayo in Argentina and Vaterloo in Russia), at the Southern Shetland Islands.

Psychrotolerant bacteria capable of biosynthesizing CdS fluorescent nanoparticles were isolated by the research group from Antarctic samples (Gallardo et al. 2014). Four *Pseudomonas* strains were used for this research, which were arbitrarily identified as 37, 197, 198, and 212.

Biofilm Assay

Bacterial strains were grown on sterile Luria Broth (LB) medium at 15°C for 72 h on plastic microplates. Biofilm was measured by discarding the medium, rinsing the wells with water three times, and staining bound bacteria with 1× crystal violet for 1 min. Then, the dye was solubilized with ethanol and absorbance at 540 nm was determined using a Synergy H1 Microplate Reader (BioTek Instruments, Winooski, Vermont) following the procedure recommended by Órdenes-Aenishanslins et al. (2014).

Nanoparticle Formation

Biosynthesis of Nanoparticles

The biosynthesis of CdS nanoparticles in presence of As was determined following the protocol described by Gallardo et al. (2014). Briefly, cultures of Antarctic bacteria were grown until a stationary phase was reached, and then cells were sedimented by centrifugation (10 min at 13,000 rpm). Collected cells were resuspended in a buffer solution containing a cadmium or arsenic solution ($CdCl_2$ and $NaAsO_2$) at 10 and 2 mg/L concentrations, respectively. Additionally, to evaluate the ability to biosynthesize NPs in these strains, cysteine was incorporated into the cultures to favor QDs biosynthesis through H_2S generation. After 24 h incubation at 15°C, fluorescence of cell pellets was evaluated through exposure to Ultraviolet (UV) light (360 nm). The fluorescence of cell pellets under these conditions is evidence of the generation of quantum dots (Plaza et al. 2016).

Effect of As on CdS Nanoparticles

To evaluate the effect of arsenic on the fluorescence of CdS nanoparticles, different concentrations of $NaAsO_2$ were tested. CdS nanoparticles were synthesized using the biomimetic method previously developed by Wansapura et al. (2015). The fluorescence of NPs was determined after exposure to UV light (360 nm) using a Synergy H1 Microplate Reader (BioTek Instruments, Winooski, Vermont). The concentrations of $NaAsO_2$ used were 10, 30, 50, 100, and 200 mg/L.

Hydrogen Sulfide Detection

The formation of hydrogen sulfide on bacterial headspaces was detected using papers soaked with a lead acetate solution (100 mg/L) as determined previously (Gallardo et al. 2014; Ulloa et al. 2016).

Remediation Tests

Selected strains were inoculated on LB medium for 24 h. After such time, optical density was measured photometrically at 600 nm (OD_{600}), and inoculums were diluted to reach an OD_{600} of 0.3. Inoculums of each bacteria studied (0.3 mL each) were added to Cd and As solutions for both kinetic and sorption isotherm tests.

Kinetic tests were carried on LB medium, amended with As and Cd concentrations of 100 mg/L, in orbitally shaken Erlenmeyer flasks. Aliquots were taken at 0, 4, 8, 24, 48, and 72 h, and OD_{600} and metal concentrations were determined. Prior to As and Cd determinations, aliquots were centrifuged at 10,000 rpm for 10 min to avoid errors caused by the reaction of suspended bacteria with test kits. Initial concentrations of 100 mg/L were considered for this test, being slightly lower than the minimum tolerance of the four selected strains.

Isotherm tests were performed considering kinetic results. For this reason, arsenic sorption tests were carried out for 48 h, whereas cadmium tests lasted 72 h. Initial concentrations of 0.5, 1, 10, 50, and 100 mg/L were used for As, but cadmium initial concentrations were 1, 5, 15, 25, 25, 40, 80, and 100 mg/L. Sorption capacity tests were carried isothermally at 20°C, in 50-mL acrylic Falcon tubes. When the reaction time was reached, aliquots were taken and centrifuged for 10 min at 10,000 rpm. Removal tests were carried in inoculated and sterile LB medium with different As and Cd concentrations to account for the change in contaminants' concentration caused by the culture media.

Arsenic concentrations were determined by means of arsenic test strips (Quantofix Arsen 10 and Arsen 50, Macharey-Nagel, Düren, Germany). This colorimetric method was improved by a digital image analysis technique to obtain quantitative results, enhance the accuracy of the measurements, and eliminate operator influence on results (Carro Perez and Francisca 2013b). Limit of detection (LOD) using this technique was 6.5 μg/L. Cadmium concentrations were determined with a Thermo Fischer Aquamate visible spectrophotometer (Waltham, Massachusetts), using commercially available test kits (Cd Spectroquant, Merck, Whitehouse Station, New Jersey), having a detection limit of 10 μg/L.

Results and Discussion

In recent years, the authors participated in different expeditions to Antarctica that allowed them to isolate more than 500 different bacterial strains with novel properties (Plaza et al. 2016). In the present work, four Antarctic *Pseudomonas* strains were selected from all these isolates because of their capacity to grow at temperatures between 15 and 28°C, their high tolerance to cadmium salts (up to 300 mg/L), and their capability to synthesize CdS quantum dots at low temperatures. In addition, selected strains display high tolerance to oxidative stress and UV radiation (Gallardo et al. 2014). Based on these properties, these strains constitute excellent candidates for the bioremediation of metals through the generation of NPs, particularly cadmium and arsenic, two elements commonly present in metal-contaminated environments.

As Resistance of Antarctic Strains

With the aim of using these Cd-tolerant Antarctic *Pseudomonas* strains in the bioremediation of groundwater and soils contaminated with As, the resistance of selected strains to $NaAsO_2$ was determined. Obtained results indicated that *Pseudomonas* strains 197 and 212 grew in As concentrations up to 125 mg/L, whereas strain 198 grew in As concentrations up to 250 mg/L. *Pseudomonas* strain 38 displayed the highest tolerance to As, growing at concentrations up to 500 mg/L.

Biofilm Formation

The capacity of selected strains to produce biofilms was determined given its importance on metal resistance and colonization of natural environments (Fig. 1). All Antarctic strains tested displayed a significant capacity to form biofilms, higher than that observed in the control strain *E. coli* K-12. In addition, strain 212 displayed the highest biofilm production, approximately 3–4 times higher than the other strains evaluated. Interestingly, the capacity to produce biofilms in all selected strains is in agreement with the increased metal resistance, supporting its application in a bioremediation system.

Kinetic Test

Kinetic tests were carried for individual contaminants (As and Cd) with a mix of the selected strains. For both contaminants, the bacterial mix showed an adaptation period of 8 h during which no removal of cadmium was observed, although a decrease of arsenic concentration was determined (Fig. 2).

After the lag period, bacterial growth increased rapidly until 48 h, and then maintained a nearly constant OD (<0.5 OD variation) for the last 24 h of the test. The OD increased 20 and 6 times its initial value for arsenic and cadmium tests, respectively. As expected, arsenic and cadmium removal also increased during the exponential growth period. Between 48 and 72 h, arsenic removal decreased from 22 to 15% (−32% change in removal for a 3% decrease in OD), whereas cadmium removal doubled for a 22%

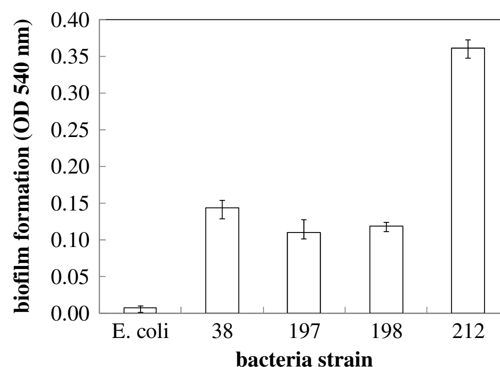


Fig. 1. Biofilm formation by Antarctic strains

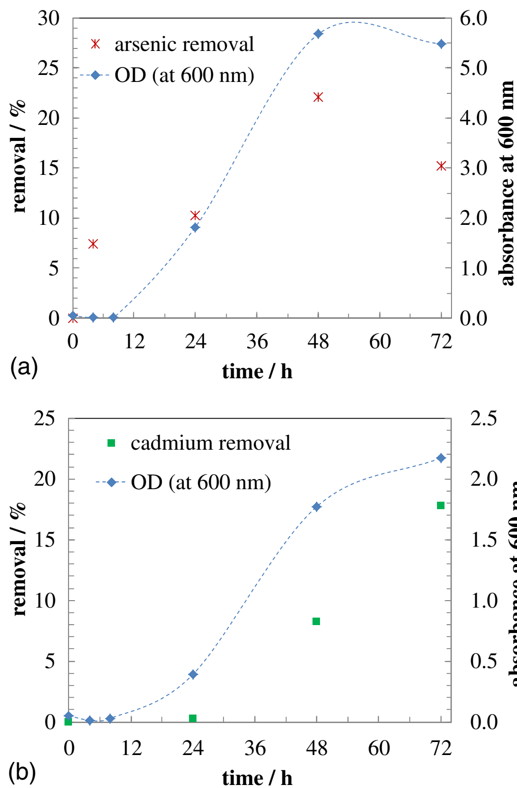


Fig. 2. Evolution of contaminant removal and bacterial growth with time: (a) As removal, (b) Cd removal; initial As and Cd concentrations = 100 mg/L; initial optical density = 0.3 (zeroed)

increase in OD. OD determinations suggest that the decrease in As removal could be related to bacterial cell lysis.

Removal Capacity

Sorption tests were carried for 48 h (As) and 72 h (Cd), considering the maximum sorption time determined in kinetic tests of Fig. 2. Arsenic and cadmium removal percentages are relatively high and similar for low concentrations (<20 mg/L). However, at higher concentrations, cadmium removal decreases abruptly, whereas arsenic remediation decreases more slowly (Fig. 3).

Different models can be used to analyze the results of isotherm tests to resemble bacterial remediation with a sorption process. The most commonly used isotherms models are the Linear, Freundlich, and Langmuir models, with one or two fitting parameters. Under this assumption, removed contaminants are adsorbed on the surface of bacteria and biofilms, and the concentration of the adsorbed contaminant (C^*, mg/g) is related to the dissolved equilibrium concentration (C, mg/L).

The linear model assumes a linear relation between the amounts of sorbate removed from solution with the amount of sorbent and, like the Freundlich model, it contemplates no maximum sorption capacity. The Freundlich model presents a logarithmic relation between the dissolved and sorbed sorbates, and despite its failure to explain the behavior at higher concentrations, is very useful for low concentrations of contaminants. The Langmuir model considers a maximum amount of contaminant removed, and despite the fact that the model considers a homogeneous material and no interactions between adsorbed compounds, it presents a simpler and more realistic model. Eqs. (1)–(3) present the linearized models

$$\text{Linear model: } C^* = k_d \cdot C \quad (1)$$

$$\text{Freundlich model: } \log C^* = \log k_F + n^{-1} \cdot \log C \quad (2)$$

$$\text{Langmuir model: } C \cdot C^{*-1} = (\alpha \cdot \beta)^{-1} + C \cdot \alpha^{-1} \quad (3)$$

where k_d (L/g) = partition constant; k_F (mg/g) and n = fitting parameters of the Freundlich model related to adsorption capacity and the adsorption intensity, respectively; α (mg/g) = maximum coverage capacity of a monolayer in Langmuir model; and β = fitting parameter of this model.

Fig. 3 presents the removal results represented by the three models. Because the equations are linearized, a high linear correlation (R^2) represents a good agreement with the model. In the case of arsenic, the Freundlich model represents the process with high accuracy, but no model is indicative for the removal behavior of cadmium.

The cadmium removal process can be divided in two series, one for low concentrations and the other for the higher ones, and it is possible to observe an increasing removal for the first series and a decreasing tendency for the second. This is in agreement with previous results, possibly indicating a high toxicity of the metal or even cellular lysis resulting in Cd liberation.

Effect of As on the Biosynthesis of CdS Nanoparticles

The capacity to biosynthesize cadmium fluorescent NPs in presence of As was evaluated in *Pseudomonas* Antarctic strains 38, 197, 198, and 212. QDs biosynthesis involves the incubation of bacterial cells with cysteine and Cd^{2+}, and the evaluation of the fluorescence of bacterial cell pellets after exposure to UV light (Fig. 4).

Under the conditions tested, fluorescent pellets evidencing QDs biosynthesis were observed only in the presence of cysteine. No fluorescence was observed in the absence of As, Cd, or cysteine, or in the presence of 2 mg/L As. Both controls discard the production of fluorescent pigments under these conditions. No fluorescence was observed in the presence of 2 mg/L As and cysteine, a result that discards the generation of fluorescent As-composed NPs under these conditions.

Finally, the effect of As on the biosynthesis of CdS was evaluated in all selected strains. As indicated at the bottom of Fig. 4, incorporation of As (2 or 4 mg/L) avoids the generation of CdS fluorescent NPs in all strains because bacterial cell pellets do not display fluorescence.

The As-mediated inhibition of Cd-QDs biosynthesis can be associated with destabilization of NPs or can be a consequence of As-mediated subtraction of CdS substrates (i.e., Cd^{2+} or S^{2-}). Two independent experiments were performed to evaluate these possibilities. For a first approach, the authors exposed cadmium fluorescent nanoparticles synthesized by a chemical biomimetic method to increasing concentrations of $NaAsO_2$ (0, 10, 30, 50, 100, and 200 mg/L) and evaluated their fluorescent properties after 24 h exposure (Fig. 5). Fig. 5(a) shows obtained results for red QDs and Fig. 5(b) the same information for green QDs. It has been previously shown that biomimetic CdS NPs are affected by different metal ions that can disrupt the nanostructure or quench its fluorescence (Plaza et al. 2016).

As shown in Fig. 5, no effect on QDs fluorescence was determined in all As concentrations tested. Qualitative analyses of tubes containing the fluorescent solutions exposed to UV light (360 nm) do not show any effect on fluorescence intensity (Fig. S1). Accordingly, fluorescence spectra of all experiments confirm that As does not generate any quenching of fluorescence, confirming that NPs

Fig. 3. Sorption isotherm models; linear model of (a) arsenic and (b) cadmium; Freundlich model of (c) arsenic and (d) cadmium; and Langmuir model of (e) arsenic and (f) cadmium

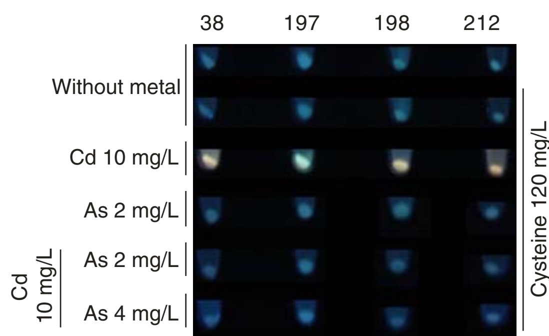
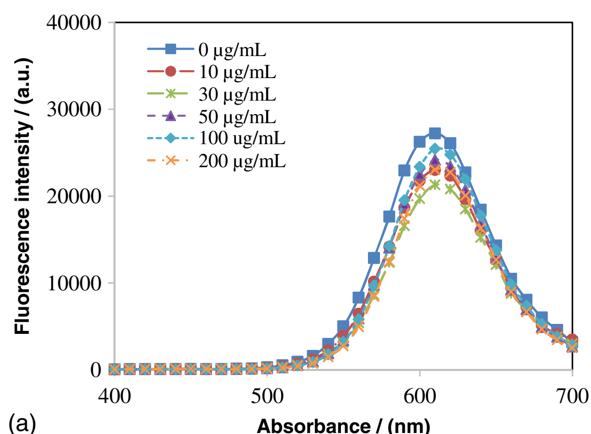


Fig. 4. Effect of As and cysteine on the biosynthesis of CdS nanoparticles

are not affected (or dismantled) by As (Fig. 5). No effect of As on fluorescence was observed even at 120 h exposure.

Gallardo et al. (2014) demonstrated that sulfide generation by the Antarctic strains used in this work is required for CdS biosynthesis because S^{2-} interaction with Cd^{2+} is necessary to produce CdS nanocrystals. Based on this, a second approach was addressed to evaluate the effect of As over sulfide levels in cells biosynthesizing CdS QDs. Cells exposed to cysteine increase production of H_2S as a consequence of the enzymatic reaction of cysteine desulfurase enzymes with cysteine. This is reflected as a darkening on lead acetate papers (compare the effect of cysteine on S^{2-} production in Fig. 6). When cells are grown in the presence of cysteine and cadmium (biosynthesis conditions), there is a decrease in H_2S levels as a consequence of the interaction of Cd^{2+} with S^{2-} generated by cysteine desulfurases (Fig. 6).



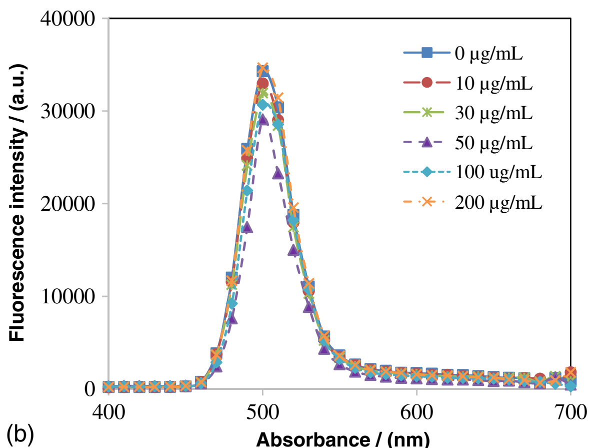


Fig. 5. Effect of arsenic on the fluorescence of biomimetic Cd-based nanoparticles

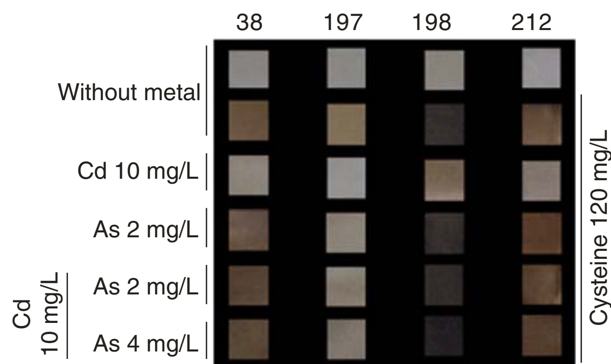


Fig. 6. Effect of As on H_2S levels produced by Antarctic strains during CdS biosynthesis; level of H_2S is observed as a dark precipitate on white papers treated with a lead acetate solution

In the presence of arsenic, no decrease in H_2S level was observed, indicating that As is not reacting with the volatile sulfur compound under these conditions. When As (2 and 4 mg/L) was incorporated into cells treated with Cd and cysteine (biosynthesis conditions), no Cd-mediated decrease on H_2S levels was observed (Fig. 6 bottom). This result explains the absence of fluorescence of cell pellets under biosynthesis conditions of Fig. 4; no CdS QDs are produced in the presence of As, likely because this element avoids the interaction of Cd^{2+} and S^{2-} required to produce the nanocrystals.

Taken together, the characteristics identified for the selected Antarctic bacterial strains in terms of metal resistance, growth temperature, biofilm formation, QDs biosynthesis, and increased generation of H_2S in cells exposed to As, validates the use of these four Antarctic strains in Cd and As bioremediation assays. However, obtained results indicate that the production of CdS QDs in cells exposed to the mixture of Cd and As is impaired, a result that constitutes a limitation of the proposed approach that must be considered when planning in situ bioremediation strategies based on the production of CdS NPs.

Conclusions

This paper presented results and analysis on the bioremediation of arsenic and cadmium by Antarctic bacteria capable of producing nanoparticles. Minimal inhibitory concentration of the contaminants, and kinetic and remediation capacity were addressed. Formation of nanoparticles was also analyzed.

Kinetic tests indicated that As remediation reaches an equilibrium point at 48 h, with a small decrease in optical density and a redissolution of the metalloid after this time. Conversely, cadmium removal kinetics is slower, increasing for the duration of the test (72 h). For this reason, the time considered for the isotherm test is the time at which the exponential growth phase ended. Arsenic and cadmium removal percentages are relatively high and similar for low concentrations (<20 mg/L), but for higher concentrations, cadmium removal decreases abruptly whereas arsenic remediation decreases more slowly.

The removal process of arsenic is well predicted by the Freundlich model, but cadmium removal, if considering the whole range of analyzed concentrations, cannot be explained by any of the considered models. However, if only low Cd concentrations (<20 mg/L) are considered, the removal can be properly explained by a linear sorption model, indicating a high toxicity of the metal and a possible cellular lysis, which may consequently lead to the release of the adsorbed metal at higher concentrations.

The high concentrations of cadmium and arsenic that bacterial strains can tolerate and their relevant metal uptake capacity favors the use of these Antarctic strains for bioremediation of contaminated soils. Additionally, the capacity of these strains to synthesize CdS quantum dots could allow use of bacteria to provide new uses for these metals. This would permit the conversion of an environmental problem that can cause damage to the health of the population into a solution based on the extraction and reuse of these or other metals of high economic value. Obtained results support the use of selected Antarctic strains for the bioremediation of arsenic and cadmium contaminated water in a process that generates CdS nanoparticles as a by-product.

Future research should focus on identifying or constructing new bacterial strains capable of synthesizing CdS QDs in the presence of As and other contaminants that may inhibit the formation of QDs. The discovery and manipulation of new genetic determinants of QDs biosynthesis may significantly contribute to finding novel applications in the bioremediation field. In addition, given the potential for bioremediation of the analyzed strains, technological aspects should be addressed in order to identify the limitations of the proposed techniques because in situ methods with immobilized cells may lead to results different from those obtained in laboratory tests.

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Supplemental Data

Fig. S1 is available online in the ASCE Library (www.ascelibrary.org)

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