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ORIGINAL ARTICLE

Microbial synthesis of antimony sulfide nanoparticles and their characterization

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Abstract During recent years, biological synthesis of nanoparticles by microorganisms has been receiving increasing attention. In this investigation, an antimony-transforming bacterium was isolated from the Caspian Sea in northern Iran and was used for intracellular biosynthesis of antimony sulfide nanoparticles. This isolate was identified as non-pigmented *Serratia marcescens* using conventional identification assays and the 16S rDNA fragment amplification method, and was used to prepare inorganic antimony nanoparticles. The biogenic nanoparticles were released by liquid nitrogen and extracted using two sequential solvent extraction systems. Different characterizations of the extracted inorganic nanoparticles such as particle shape, size and composition were carried out with different instruments. The energy-dispersive x-ray demonstrated that the extracted nanoparticles consisted of antimony and sulfur atoms. In addition, the transmission electron micrograph showed the small and regular non-aggregated nanoparticles ranging in size less than 35 nm. Although the chemical synthesis of antimony sulfide nanoparticles has been reported in the literature, the biological synthesis of antimony sulfide nanoparticles has not previously been published. This is the first report to demonstrate a biological method for synthesizing inorganic nanoparticles composed of antimony. A simple extraction method for isolation of antimony sulfide nanoparticles from bacterial biomass is also reported in the current investigation.

Keywords Antimony · Nanoparticles · Biosynthesis · *Serratia marcescens*

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Introduction

A new strategy in biotechnology has contributed to the biosynthesis of particular nanoparticles (NPs) by microorganisms as novel bio-factories. Biosynthesis processes for preparation of different NPs such as silver, gold, selenium, cadmium sulfide, magnetite iron oxide and other nanomaterials have been developed in recent years (Mandal et al. 2006; Jha et al. 2009c; Vaidyanathan et al. 2009; Shakibaei et al. 2010a; Thakkar et al. 2010). These bioprocesses compete with other synthetic techniques due to the method's environmental impact, such as clean, nontoxic and eco-friendly features (Mandal et al. 2006; Jha et al. 2009c; Vaidyanathan et al. 2009; Shakibaei et al. 2010a; Thakkar et al. 2010). Today, there has been increasing interest in the use of inorganic NPs in different health materials or industrial products such as antimicrobials, catalysts, lubricants and microelectronics instruments (Balaji et al. 2009; Jha et al. 2009b, c; Sathishkumar et al. 2009).

Among the inorganic compounds, antimonial preparations have many applications (Steely et al. 2007; Isago et al. 2008; Smichowski 2008). For example, they are used in electronics as poor conductors of heat and electricity. Very pure antimony is used to make certain types of semiconductor devices, such as diodes and infrared detectors (Grigorescu and Stradling 2001; Wei et al. 2006). Antimony, as a metallic form, is not soluble in body fluids and, as reported in old literature, cannot produce any effect upon the human system (Filella et al. 2002a, b). In contrast, organic or inorganic salts of antimony can be decomposed by the fluids and have been used for therapeutic purposes (Filella et

al. 2002a, b; Johnson et al. 2005). Antimony salts are currently chosen for the treatment of leishmaniasis, a disease that affects 12 million people annually around the world (Berman 1997; Haldar et al. 2011). Moreover, trivalent antimony compounds have been used in treating bilharzia, trypanosomiasis and kala-azar for more than a century (Berman 1997; Isago et al. 2008).

Recently, many reports have been published in the literature on the synthesis of different NPs by biological methods (Mandal et al. 2006; Thakkar et al. 2010). However, the synthesis of antimony sulfide NPs by microorganisms has not yet been investigated and should be performed. In this study, we screened different samples from the Caspian Sea to isolate an antimony sulfide NPs-producing microorganism. The isolated strain was identified and employed for preparing antimony sulfide NPs. Biogenic NPs were extracted and purified from cell debris using a liquid–liquid phase-partitioning method and characterized by different methods such as UV-visible (UV-vis) spectroscopy, transmission electron microscopy (TEM), and energy dispersive spectroscopy (EDS).

Materials and methods

Screening procedure

Fresh seawater samples from the Caspian Sea in northern Iran were collected in sterile bottles during the summer of 2008. Portions of the samples were aseptically transferred to fresh nutrient agar (NA) culture media (Merck, Darmstadt, Germany) supplemented with antimony ions. For this purpose, nutrient agar (NA) medium (1,000 ml) (Merck) was prepared and sterilized at 121°C for 20 min. The sterile NA medium was then allowed to cool to 45°C. Sterile antimony chloride (Merck) solution (10 g/l) was prepared and this antimony salt was added to the above NA medium. The prepared antimony supplemented agar media (10 mg/ml) was dispensed in sterile Petri dishes. To isolate the antimony sulfide NPs-producing bacteria, prepared NA plates containing antimony ions (10 mg/ml) were inoculated with diluted seawater samples.

All plates were incubated aerobically for 72 h at 30°C. Conversion of metal ions to elemental form or inorganic salts usually leads to a change in the color of the colonies to various other colors, and this provisional marker can be preliminarily used for screening of metal-transforming bacteria (Hayat et al. 2010; Li and Wong 2010). In the next step, a single yellow colony was selected and re-cultivated in the $SbCl_3$-supplemented NA plates (10 mg/ml) at the described conditions to obtain a pure culture (72 h, 30°C). The organism was maintained on the $SbCl_3$-supplemented NA medium plates (10 mg/ml) at 4°C for further experiments.

Identification of bacterial isolate

The following phenotypic and physiological characterizations of the isolate were carried out according to the methods described in determinative bacteriology textbooks (Bergey et al. 1994; Weissfeld et al. 1998): Gram-staining, cell morphology, pigmentation, oxygen requirements by fluid thioglycollate medium, motility, oxidase test, lactose fermentation, indole test, urease test, lysine decarboxylase test, H_2S production, and growth at 4 and 40°C.

In addition, the identification procedures were further confirmed by 16S rDNA fragment amplification method using polymerase chain reaction (PCR). A single colony was picked up and suspended in 50 µl of distilled water and lysed by heating at 95°C for 10 min. Cell lysates, after centrifugation, were used for PCR amplification. Amplification of 16S rDNA was performed in two different steps of identification using two sets of forward and reverse primers as follow:

1) Forward 5′ CAAgTCgAgCggTAACACAg 3′
1) Reverse 5′ gTgTCTCAgTTCCAgTgTggC 3′
2) Forward 5′ TCTgAgACAggTgCTgCATg 3′
2) Reverse 5′ CgTATTCACCgTAgCATTCTg 3′

The reaction mixture consisted of 1.5 mM $MgCl_2$, 10 mM PCR buffer, 200 µM dNTP (deoxynucleotide), 20 pmol of each forward and reverse primers, 2 µl of lysed cell suspension, and 1.25 U Super *Taq* DNA polymerase and distilled water up to 25 µl. All the PCR reagents were purchased from Gen Fanavaran (Tehran, Iran). The mixtures were incubated at 94°C for 2 min and then cycled 30 times using the following profile: 94°C for 20 s, 56°C for 30 s, 72°C for 1.5 min, and then incubated for 7 min at 72°C. The amplified DNA fragments were purified from agarose 1% gel using the QIAquick gel extraction kit (Qiagen, USA) according to the supplier's instructions and were sent for automated sequencing using the above primers (Takapouzist, Iran). The obtained 16S rDNA sequence was aligned with nucleotide databases using BLAST program (National Center for Biotechnology Information) and the sequence was submitted to GenBank.

Synthesis of antimony inorganic-based NPs using bacterium

Antimony sulfide NPs were prepared from antimony chloride using the bacterial isolate from the Caspian Sea with solid state fermentation. In the first step, antimony-supplemented NA plates ($SbCl_3$, 1% w/v) were inoculated with the bacterial isolate. These inoculated NA media were

incubated aerobically at 30°C. After 72 h, bacterial cells were harvested from the surface of culture media by a sterile loop. Harvested biomass was suspended in normal saline and washed with distilled water by centrifugation process (5,000 *g*, 10 min). Cell pellets were transferred to a mortar, and by adding some liquid nitrogen, the pellets were frozen and then were disrupted with a pestle. The resulting slurry containing antimony sulfide NPs and cell debris were suspended in a two-phase partitioning system of 1:2 n-octyl alcohol and distilled water. Then, the mixture was shaken vigorously. The two mixed phases were completely separated by centrifuging at 5,000 *g* for 15 min. After this process, the biogenic NPs were observed at the bottom of the tubes. The supernatants were discarded, and the settled NPs were resuspended in mixed solvent system composed of chloroform, ethyl alcohol and water (3:1:4). Subsequently, the organic and aqueous phases were separated by centrifuging at 5,000 *g* for 15 min. The upper phase containing antimony NPs was gently collected and subjected to further characterization assays.

Characterization of inorganic antimony NPs

The UV-visible spectra of the purified NPs suspension in the wavelength range 220–400 nm (Niedzielski and Siepak 2003; González et al. 2005) were measured on a Labomed Model UVD-2950 UV-VIS Double Beam PC Scanning spectrophotometer, operated at a resolution of 1 nm. These NPs were also characterized with transmission electron microscopy (TEM) and energy-dispersive spectroscopy (EDS) (model EM 208 Philips). Before characterization, aqueous suspension containing the inorganic antimony NPs was passed through the ultrasonication process, and a few drops of suspension were deposited on carbon-coated copper TEM grids and dried at room temperature. Micrographs were achieved using a TEM (model EM 208 Philips) operated at an accelerating voltage at 100 kV.

Results and discussion

Identification of the microorganism

A bacterium with the intercellular ability to convert antimony ions to insoluble compounds was isolated from seawater samples collected from the Caspian Sea. Figure 1 shows the cultures of this isolate on the plain NA medium (Plate A) and antimony supplemented NA medium (Plate B). Also, two captured images in Fig. 1 demonstrate the colonies of this isolate on the above culture media with higher magnification. As can be observed in these close-up images, the colonies appear in yellow on the surface of Sb^{+3}-supplemented NA medium. In microbiological examinations, the strain had Gram-negative rods with no pigmentation, and showed good growth on sheep blood agar, MacConkey (MAC) agar and Eosin Methylene Blue (EMB) agar media. The isolate was facultative and grown in a temperature range of 25–40°C. In addition, this strain was negative in the oxidase, lactose and urease tests. Furthermore, no H_2S production and no indole fermentation were observed in the biochemical diagnostic tests for this isolate. In contrast, this isolate was motile and halotolerant up to 7% NaCl, and the lysine decarboxylase test was positive.

After the amplification of bacterial 16S rDNA by PCR, two fragments of 232 and 283 bp were developed. They were aligned to sequences in GenBank and the alignment was further confirmed that the isolated bacterium was representative of *Serratia marcescens*. The sequences were submitted to GenBank and assigned the following accession numbers, respectively: JF682843 and JF682844. Alignment results revealed 99% identity with the *S. marcescens* bacterium.

Antimony sulfide NPs' preparation and characterization

Figure 2 shows the test tube containing the selenium-enriched *S. marcescens* cells which were disrupted by liquid

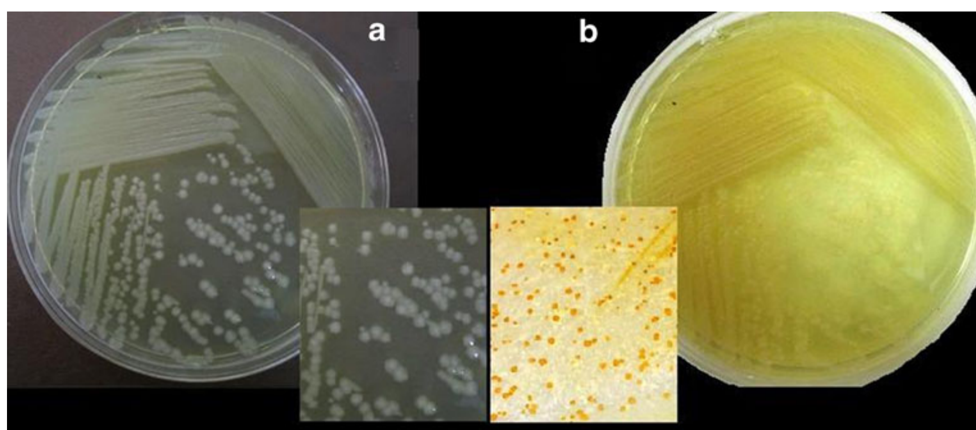


Fig. 1 A photograph from nutrient agar with (plate *B*) and without (plate *A*) antimony chloride which streaked with isolated *Serratia marcescens*. This isolate transform antimony ions to antimony sulfide and form yellowish colonies (plate *B*)

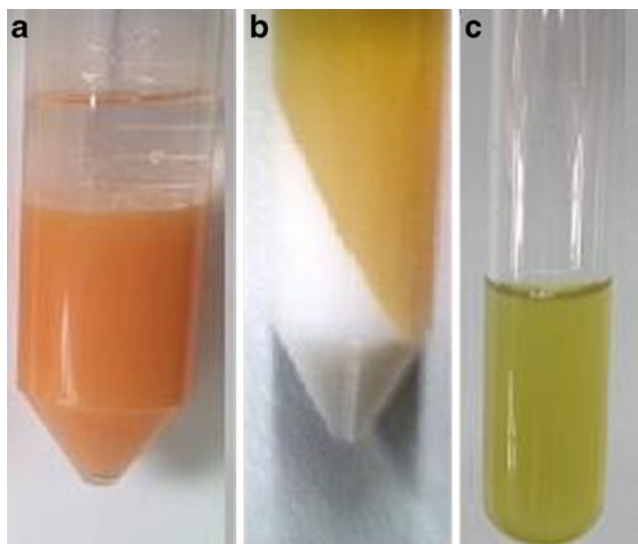


Fig. 2 The left tube (tube *A*) shows a container containing selenium-enriched *Serratia marcescens* cells which were disrupted by liquid nitrogen and extracted with n-octyl alcohol. Also, tubes *B* and *C* show this extracted material before (tube *B*) and after (tube *C*) additional extraction with mixed solvents of chloroform, ethyl alcohol and distilled water (3:1:4)

nitrogen and extracted with n-octyl alcohol (tube A). Also, tubes B and C show this slurry before (tube B) and after (tube C) additional extraction with a mixed solvents of chloroform, ethyl alcohol and distilled water (3:1:4). Cell debris materials were dissolved in the n-octyl alcohol-water system (tube A) or coagulated by mixed chloroform and ethanol solvents during additional solvent extraction process (bottom organic phase in tube B). This extraction was repeated three times. Finally, aqueous phase (tube B), was gently aspirated by Pasteur pipette and transferred to a new tube (tube C). The UV-vis spectrum of the extracted NPs is shown in Fig. 3. The spectra obtained show an absorption peak at 258 nm, which is supported by previous work reported in the literature (Niedzielski and Siepak 2003; González et al. 2005)

The left-hand illustration in Fig. 4 shows a representative TEM image recorded from the drop-coated film of the extracted NPs prepared by a biological method using *S. marcescens* bacterium. The particle size histogram of the extracted NPs (right-hand illustration in Fig. 4) shows that the particles range in size from 10 to 35 nm and possess an average size of 22.5 nm. In the analysis of the extracted NPs by energy dispersive spectroscopy (EDS), the presence of signals contributed to sulfur and antimony elements were confirmed (Fig. 5). The sulfur displays an optical absorption band peaking at ~2.2 keV. Also, optical absorption bands corresponding to the antimony element were observed at ~3.5 and 3.7 keV (Fig. 5) confirming the composition of extracted NPs as antimony sulfide (Ozimina 2002). Further information obtained from the EDS experiment

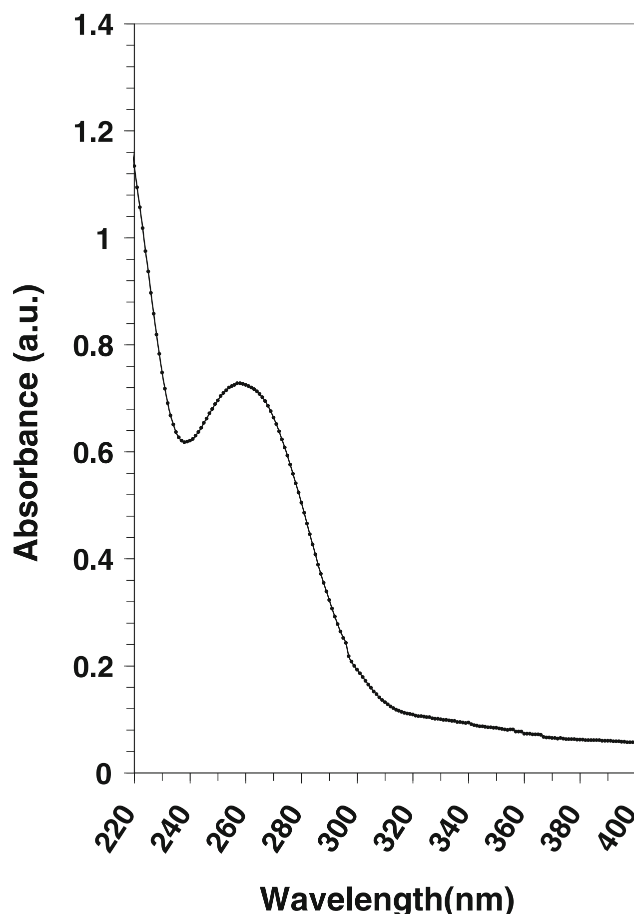


Fig. 3 UV-vis absorption spectra of biogenic antimony sulfide colloids measured between 220 and 400 nm

reveals that the extracted NPs were composed of sulfur and antimony atoms at ratio of about 84/16. The atomic ratio of S/Sb in Sb_2S_3 equals 1.5. Therefore, the atomic ratio (5.25) obtained for the extracted inorganic antimony NPs shows that these NPs may contain free sulfur (S_8) in combination with Sb_2S_3. Both Sb_2S_3 and free sulfur can be generated by a spontaneous chemical reaction from Sb_2S_5 according to the following reaction which has been described previously (Svehla 1996).

$$4\,Sb_2S_5 \rightarrow 4\,Sb_2S_3 + S_8 \qquad (1)$$

The ratio of total sulfur to antimony atoms for the products of the above reaction (Sb_2S_3 and S_8) can be simply calculated and is about 5.5 (S/Sb:11/2). The calculated ratio approximately corresponds to the ratio obtained by the EDS experiment and demonstrates that the isolated *S. marcescens* may deposit the antimony element as Sb_2S_5 in their cytoplasm or other internal bacterial spaces. This compound may be spontaneously decomposed according the chemical reaction (Eq. 1) during the incubation period or NPs extraction process.

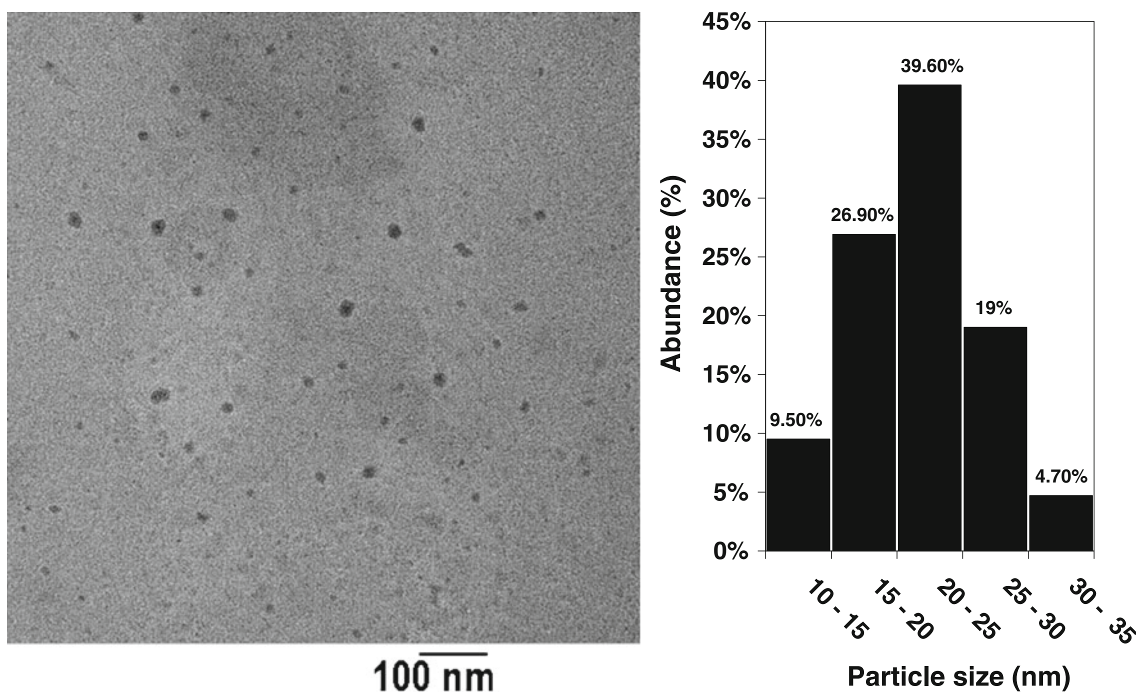
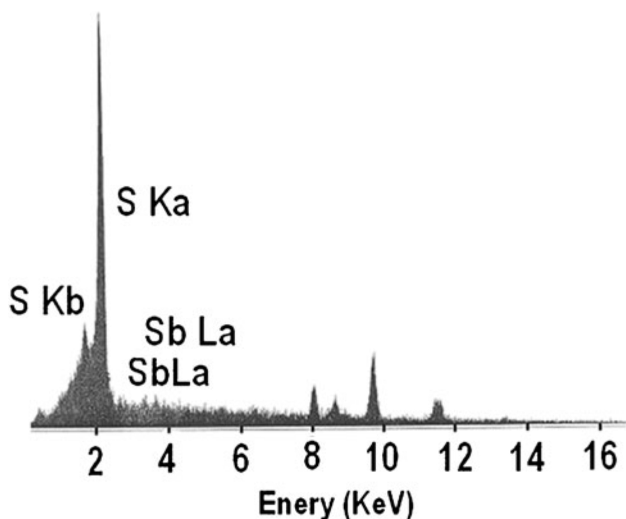


Fig. 4 EDS spectrum of biogenic antimony sulfide prepared by *Serratia marcescens*



Element nanosized	Atomic %
S K	84.11
SbL	15.89
Total	100.00

Fig. 5 Transmission electron micrograph of extracted antimony sulfide nanoparticles and its particle size distribution histogram

In this study, the ability of a marine isolated bacterium identified as *S. marcescens* to form antimony sulfide NPs was investigated. Many reports have been published in the literature on the bacterial synthesis of metal-based NPs (Mandal et al. 2006; Thakkar et al. 2010). The bacterial synthesis of metal-based NPs is considered a green technology and can also be performed by other organisms such as fungi or plants (Jha et al. 2009a, b; Arwidsson and Allard 2010; Thakkar et al. 2010). For example, research on the biosynthesis of silver NPs (Shahverdi et al. 2007; Balaji et al. 2009; Kathiresan et al. 2009), magnetite iron NPs (Bharde et al. 2006), selenium NPs (Oremland et al. 2004; Shakibaei et al. 2010a), and gold NPs (Husseiny et al. 2007; Shakibaei et al. 2010b) has been recently published. According to our literature survey, the biosynthesis of antimony sulfide NPs from antimony chloride has not yet been investigated. There is a single report published in the literature on the synthesis of Sb_2S_5 NPs by mechanochemical reduction of insoluble Sb_2S_3 with elemental Mg (Godosikova et al. 2008).

Different trace elements such as antimony are non-essential elements in humans, animals, bacteria and plants and have toxic effects against some species (Petit de Penã et al. 2001; Filella et al. 2002a; Lecureur et al. 2002). The detoxification potential of different bacterial strains for metal oxyanions has been previously reported (Oremland et al. 2004). Antimony as a toxic metal can also be accumulated by microorganisms in their cytoplasm or in other intracellular compartments such as the cell wall (Feng et al. 2011). In

this investigation, *S. marcescens* was observed to deposit antimony ions as intracellular antimony sulfide NPs. In the current study, based on the lipophilic nature of the cell debris and insolubility of antimony sulfide NPs in the aqueous or organic phases, we used organic–aqueous partitioning systems. According to the EDS results (Fig. 5), the separated biogenic NPs comprised the antimony atoms and sulfur. A TEM image of extracted NPs showed small and regular-shaped NPs ranging in size from 10 to 35 nm with no aggregation (Fig. 4). As mentioned in the "Introduction", various antimony compounds are currently used in the treatment of different parasitic diseases such as filariasis, leishmaniasis, lymphogranuloma, schistosomiasis, and trypanosomiasis (Berman 1997; Isago et al. 2008). However, no study has been performed on the anti-parasitic effects of antimony sulfide NPs, and these NPs can be considered in future for in vitro and in vivo evaluation against these parasitic diseases, especially in the case of leishmaniasis.

Conclusion

We screened and employed *S. marcescens* for biosynthesis of antimony sulfide NPs as a green synthetic method. Prepared NPs were released and extracted using two- solvent phase partitioning systems. TEM images showed small NPs ranging in sizes less than 35 nm. EDS spectrum analysis of the extracted sample indicated that Sb_2S_5 NPs may be prepared using *S. marcescens*, and this compound may be decomposed to Sb_2S_3 and free sulfur during incubation time or extraction procedure. To the best of our knowledge, based on a literature survey, this is the first report on the biosynthesis of antimony sulfide NPs using a microorganism, and its characterization with different instrumental devices.

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