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An extracellular polymeric substance quickly chelates mercury(II) with N-heterocyclic groups

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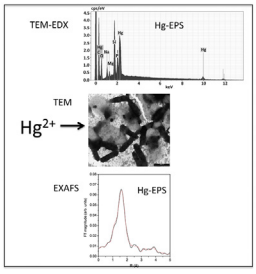
HIGHLIGHTS

- A strain of *Klebsiella oxytoca* tolerates the Hg(II) toxicity by producing a specific EPS.
- The EPS contains a specific exopolysaccharide and outer membrane proteins.
- The protein fraction of EPS is rich of histidine which binds quickly Hg^{2+}.
- Cyclic Voltammetry analysis indicates the Hg^{2+} complexing properties of EPS.
- The EXAFS model indicates imidazole rings as the major ligands for Hg(II).

GRAPHICAL ABSTRACT



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ABSTRACT

A strain of *Klebsiella oxytoca* DSM 29614 is grown on sodium citrate in the presence of 50 mg l^{-1} of Hg as $Hg(NO_3)_2$. During growth, the strain produces an extracellular polymeric substance (EPS), constituted by a mixture of proteins and a specific exopolysaccharide. The protein components, derived from the outer membrane of cells, are co-extracted with the extracellular exopolysaccharide using ethanol. The extracted EPS contains 7.5% of Hg (total amount). This indicates that EPS is an excellent material for the biosorption of Hg^{2+}, through chemical complexation with the EPS components. The binding capacity of these species towards Hg^{2+} is studied by cyclic voltammetry, and Hg L_3-edge XANES and EXAFS spectroscopy. The results found indicate that Hg^{2+} is mainly bound to the nitrogen of the imidazole ring or other N-heterocycle compounds. The hydroxyl moities of sugars and/or the carboxyl groups of two glucuronic acids in the polysaccharide can also play an important role in sequestring Hg^{2+} ions. However, N-heterocyclic groups of proteins bind Hg^{2+} faster than hydroxyl and carboxyl groups of the polysaccharide.

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1. Introduction

A number of poly-anionic polymers, by either algae,fungi, and bacteria, exhibit the ability of generating stable interactions with cations, basically due to electrostatic attraction between the positively charged ions and the negative charges, at specific sites. In particular Gram positive and Gram negative bacteria produce and secret different polymers such as peptidoglycan (PG), capsular polysaccharides (CPS), extracellular polysaccharides (EP), extracellular polymeric substances (EPS), lipopolysaccharides (LPS) and teichoic acids (TA). These often provide efficient sites for the chelation of metals, due to the usual presence, within their structures of a variety of functional groups including hydroxy, carboxyl, carbonyl (chetones), amine, imidazolic and phosphonate groups (Arundhati and Paul, 2008; Zhang et al., 2010). Due to this great potential, it is evident that bacterial biopolymers can be used for heavy metal biosorption either for reducing direct health threat or for metal recovery for commercial purposes (Volesky, 2007). Bacteria, cultivated for biotechnological applications in bioreactors, produce a variety of extracellular polymers, such as exopolysaccharides and proteins, often as a consequence of their adaptation to extreme environmental conditions. The metal binding ability by these extracellular heteropolymers provide organo-metal complexes, which enable the organisms to tolerate toxicity of heavy metals (Beech and Sunner, 2004). Biosorption of Hg^{2+} is one of the few strategies allowing removing their threat from the environment (Volesky, 2007). Studies related to Hg^{2+} complexation by microbial exudates have mainly regarded materials coming from bacterial biofilms (Zhang et al., 2010), active sludges (Guibaud et al., 2005), humic substances (Provenzano et al., 2004) and even marine mucillages (Mecozzi et al., 2001).

In this study, a strain of *K. oxytoca* DSMN 29614 is considered and the EPS produced during bacteria growth is able to sequester Hg^{2+} and to reduce its environmental impact. The strain is known to produce a specific exopolysaccharide in the presence of high concentrations of Na-citrate and/or Fe(III)-citrate as sole energy and carbon source (Baldi et al., 2010). Compositional analysis of purified polysaccharide revealed the presence of L-Rhamnose (Rha), D-Glucuronic Acid (GlcA) and D-Galactose (Gal) in the relative ratio 4:2:1. Methylation analysis for detection of the glycosylation sites revealed the occurrence of 2-substituted-Rha (2-Rha), 3-substituted-Rha (3-Rha), 3,4-disubstituted-Rha (3,4-Rha), terminal-GlcA (t-GlcA), 4-substituted-GlcA (4-GlcA) and 3-substituted-Gal (3-Gal), in the relative ratio 2:1:1:1:1:1 (Leone et al., 2007). Moreover, the exopolysaccharide production under aerobic and anaerobic conditions also lead to the formation of metal nanoparticles with catalytic properties (Baldi et al., 2010; Paganelli et al., 2013; Battistel et al., 2015) and antimicrobial activities (Battistel et al., 2015; Baldi et al., 2016).

This study is devoted to characterize the exopolymeric substance (EPS) derived from the *K. oxytoca* DSMN 29614 culture exposed to Hg^{2+}, for mercury removal from contaminated waters. The intrinsic characteristics for the mercury binding polymer were studied by using different techniques including electron microscopies fluorescence and Fourier transform infrared spectroscopies and voltammetry. In order to identify the binding moieties of the EPS, most probably responsible for the Hg^{2+} binding, Hg L3-edge extended X-ray absorption fine structure spectroscopy (EXAFS) was employed.

2. Material and methods

2.1. Klebsiella oxytoca DSM 29614 cultivations

Cells of *K. oxytoca* DSM 29614 were stored in cryovials at −80 °C in 25% glycerol and were retrieved in Nutrient Broth (Difco). The EPS were obtained in a NAC medium, which is constituted per liter by 2.5 g sodium hydrogen carbonate, 1.2 g ammonium nitrate, 1.5 g magnesium sulphate heptahydrate, 0.6 g l^{-1}sodium dihydrogen phosphate, 0.132 g l^{-1}potassium acetate and 14.7 g l^{-1}sodium citrate. The medium was buffered at pH 7.6 with NaOH. An inoculum of culture of DSM 29614 strain was made in 1 L of NAC medium (1:100, v/v). The cultures were incubated at 30 °C for 6 days in static mode until cell flocculation occurred in the bottom of flask.

2.2. Hg-EPS purification

Cultures of *K. oxytoca* in NAC medium were harvested by centrifuging 1 L at 4000 *g* for 25 min. The EPS was separated by centrifugation as much as possible from cells. The polymeric fraction was suspended again in phosphate buffer (PBS, pH 7.4) and treated with cooled 95% ethanol (4 °C), in order to reach a 70% alcohol final concentration to precipitate the EPS by maintaining the extract at 4 °C overnight. The precipitate was dried out under vacuum to obtain a solid material, which was pulverized in a mortar and then stored at 4 °C until it was used. The Hg-EPS was prepared by modifying the previous medium, which was spiked with 50 mg l^{-1} of Hg^{2+} as $Hg(NO_3)_2$, (i.e. 250 μM Hg^{2+}). The total carbohydrate content in EPS was determined with the phenol/sulphuric acid method of Dubois et al. (1956) after an extraction with 72% H_2SO_4 at 30 °C for 1 h against a calibration curve of glucose. Total proteins in EPS were determined according to Bradford (1976) micro-method recommended by Bio-Rad reagent.

2.3. Transmission electron microscopy

The strain DSM 29614 exposed to $Hg(NO_3)_2$ were observed after 24 h of incubation with transmission electron microscopy (TEM). The cells were fixed in 3% glutaraldehyde for 30 min and 1% osmium tetroxide for the same time, then the sample was dehydrated using increasing concentrations of ethanol (40%, 60%, 80%, 100%) at room temperature and gradually infiltrated in Spurr's low viscosity embedding resin and polymerized at 70 °C for 7 h. After polymerization, specimens were sectioned with an LKB III ultra microtome using a diamond knife. The thin sections were collected on copper grids, stained for 3 min in 2% uranyl acetate and 2% lead citrate and then they were observed by TEM (model 100 b JEOL JEM) (Baldi et al., 2016).

An aliquot of 10 μg of dry Hg-EPS was resuspended in 1 ml milliQ, and previously treated for 10 min in ultrasonic bath, 10 μL of suspension was mounted on a platinum grid treated with Formvar resin biofilm to determine the structures of electron-dense metal particles. The liquid was evaporated at room temperature and the sample was observed with TEM 100 operating under standard conditions (Baldi et al., 2016).

2.4. SEM-EDX microanalysis

The microanalysis determination of Hg-EPS powder was performed with scanning electron microscopy (SEM) Jeol-JSM5600 equipped with Oxford-Isis 300 series EDX system. Specimens were prepared depositing the powder of Hg-EPS on metal grid and EDX determination were performed via multipoint acquisition on different spots of the specimens.

2.5. Determination of total Hg in Hg-EPS

Total Hg in 10 mg of solid Hg-EPS from DSM 29614 culture was determined in triplicates. The sample was mineralized with aqua-regia for 4 h at 70 °C. The total Hg was determined by flameless AAS (Varian model AA250).