Electrogenic activity and hexavalent chromium reduction by Aeromonas hydrophila CrMF5

Abstract

One of the main challenges for today’s science is to decrease the dependence on the use of nonrenewable energy and the decontamination of natural environments. In this regard, microorganisms could play an important role, since their great capacity for metabolic adaptation, high phenotypic plasticity and rapid reproduction, are key elements in the search of solutions of the previously mentioned problems. The current study evaluated the potential for the reduction of hexavalent chromium associated to biological activity and enzyme activity of chromate reductases using cell-free extract of the electrogenic and chromium resistant strain Aeromonas hydrophila CrMF5. Analysis of the 16S rRNA gene placed the isolate close to the species A. hydrophila. Strain CrMF5 tolerates a chromium concentration of approximately 360mg/L; in the microbial fuel cells, this organism showed electrogeneric activity by registering a maximum power density of 7.1mW/m². 100% reduction of Cr(VI) in 10mg/L bioassays that was reached at 10 hours in LB medium and at 38 hours in industrial wastewater. The cell-free extract showed Cr(VI) reduction activity with optimum values with a temperature of 32°C and pH of 6.0, in the presence of NADH. The results suggest a good potential for the production of electrical energy in microbial fuel cell and bioremediation processes of water contaminated with hexavalent chromium, particularly when concerning detoxification of industrial wastewater.

Keywords: Aeromonas, cell-free extract, Cr (VI) reduction, microbial fuel cell

Introduction

Chromium is a metal of transition with multiple states of oxidation from -II to VI, being the trivalent chromium, Cr(III) and hexavalent chromium, Cr(VI) the most common, stable and biologically important states.1 The different oxidation states of chromium confer it different chemical properties, degrees of toxicity and mobility in natural environments.2 Hexavalent chromium is one of the most hazardous metal ions that is released into the environment by electroplating, leather tanning, mining and metal finishing industries, amongst others.

Cr(VI) exhibits a much greater solubility, mobility, bioavailability, and toxicity than Cr(III). The Cr(III), is stable in natural environments, presents a low solubility through biological membranes and precipitates, is quickly absorbed by suspended matter or sediments.1 In contrast, Cr(VI) is found principally soluble in aquatic environments, is extremely toxic to living organisms causing allergies, irritations and respiratory track disorders and it is considered mutagenic and carcinogenic agent.3 This toxic action is due to the fact that Cr(VI) complexes can easily penetrate cellular membranes and undergo immediate reduction reactions, leading to the formation of various reactive intermediates that cause damages to DNA and alterations to biological functions.3,5,6

A wide variety of Chromium resistant bacteria have been reported for reducing Cr(VI) to Cr(III).3,7,8 It has also been demonstrated, that the prokaryotes possess high metabolic plasticity, rapid adaptation and mechanisms that confer them greater resistance to the toxic effects of Cr (VI) in comparison to eukaryotes.9 The use of Cr(VI)-resistant bacteria for the detoxification of Cr(VI) has been considered an economical, effective and safe procedure over physical and chemical conventional methods.5,10

There are few reports that indicated biological reduction of Cr(VI) by representatives of genus Aeromonas [33], some of these indicated that Aeromonas exhibited poor chromate-reducing activity.11 Representatives of the genus Aeromonas have been detected in MFCs anodes,12 showing great electrogenic capacity.13 Microbial fuel cells (MFC) are an emerging biotechnology that allows the conversion of chemical energy contained in organic wastes into electricity while providing a mechanism for simultaneously treating the wastewaters containing pollutants, using microorganisms. This treatment uses the microbologically catalyzed reduction of Cr(VI) to Cr(III) in MFCs, process which has recently gained extensive attention.14-16 By understanding the importance of the biological component in the development of this biotechnology, this study sought to contribute to the knowledge of electrogenic microorganisms with the ability to reduce chromium.

Material and methods

The bacterial isolate CrMF5 was previously isolated from the anodic biofilm placed in sediment from a microbial fuel cell operated with lake sediments.

Characterization and identification by 16S rRNA gene

The molecular characterization of bacterial strain CrMF5 was performed; using the Ultra Clean Microbial DNA Isolation Kit (MOBIO LAB), extraction genomic DNA was performed. The extracted DNA was amplified using primers: 27F 5'AGAGTTTGATCMTGGCTCAG3' and 1492R 5'TACGGYTACCTTGTTACGACTT3'. The sequence was determined by standard procedures, using the service provided by the Macrogen Company (USA). The sequence obtained was deposited in the GenBank database with the accession number: KU724074.

16S rRNA partial gene obtained was compared with sequences deposited in the GenBank using the tool BLAST available on NCBI website and Classifier tool available on the Ribosomal Database Project II website.17 Sequences were collected from the different species of the genus that were related to the 16S partial sequence obtained from the CrMF5 isolate. Nucleotide sequences were aligned using ClustalW as implemented in the BioEdit software.18 The phylogenetic tree was inferred by PAUP, version 4.0b10 (maximum likelihood method), the nucleotide substitution model was selected by Akaike criterion in the ModelTest, version 2.1.318 and statistical support was evaluated by means of the bootstrap of maximum likelihood with 1000 repetitions.
Electrogenic activity and hexavalent chromium reduction by Aeromonas hydrophila CrMFC5

Electrogenic characterization of bacterial isolated CrMFC5

The electrochemical performance of the bacterial isolated CrMFC5 was evaluated in a microbial fuel cell (MFC). Single-chamber microbial fuel cells were constructed using 100ml containers. Electrodes were constructed using graphite rods with a surface area of 11.3cm²; for the cathode, the electrode was coated with platinum-carbon catalyst with a concentration of 0.1mg/cm² prepared in a solution of 5% Nafion. The MFC was kept in operation during 15 days, time in which the electrochemical performance of the microorganism was monitored. Voltage and current were taken using digital multimeters (EX505 EXTECH). The data that was obtained was used to construct power density curves versus time.

Minimum inhibitory concentration assay

To determine the minimum inhibitory concentration (MIC) the microtiter plate-based microsasay method was employed, using resazurin as an indicator of viability. Resazurin is an oxidation-reduction indicator that changes from blue in its oxidized state (resazurin) to pink (resorufin) in its partially reduced state. This change of color is caused by the presence of a reducing environment, generated during cell metabolism by the action of oxidoreductases enzymes that are present only in living cells. Different concentrations of Cr(VI) were tested in each well of the plate, while incubating the CrMFC5 isolated bacteria at 30ªC for 24 hours. To confirm microorganism growth resazurin was added to a final concentration of 0.05%

Bioassay of hexavalent chromium reduction

The potential of Cr(VI) reduction and the growth kinetics of the Aeromonas CrMFC5 was evaluated in Luria Bertani broth (LB) and industrial wastewater (IW), adjusting the concentration of Cr (VI) to 10mg/L in both cases. The growth kinetics was estimated by optical density (OD) at 550nm. Cells were harvested (8000rpm for 5min) and the obtained supernatant was used for chromium estimation. Hexavalent chromium was determined colorimetrically using the 1,5-diphenylcarbazide (DPC) method. Cr(VI) in the sample was assayed by adding 200µl of DPC reagent, 40µl of H2SO4 (5M) and 500µl of chromium samples to 9.26ml of distilled water, mixed gently and kept at room temperature for five minutes. The absorbance was measured at 540nm. The Cr(VI) concentration in the samples were calculated from a standard curve using K2Cr2O7 as standard.

The IW was obtained from an electroplating company, using the wastewater produced after passing through the retention tanks and before being discharged as waste. The waste had a pH of 5.5 and contained nickel (14.11mg/L), chromium (10.45mg/L) and zinc (0.67mg/L). The IW was supplemented with glucose (5mM) and sterilized by filtration.

Cell-free extract preparation

The CrMFC5 isolate was grown overnight in LB medium, with 20mg/L of Cr(VI). The cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C, washed twice and re-suspended in phosphate buffer (0.1M; pH 6.5) in a volume equivalent to 10% of the original culture and kept in an ice bath. Cells were lysed with an ultrasonicator (five cycles of 59s on and 59s off at 130watt) (Sonics, Vibra Cell Ultrasonic Processor VCX-750). After sonication, the suspension was centrifuged at 12000g for 10min at 4°C. The supernatant that contained cell-free extract (CFE) was collected and used as crude chromate reductase enzyme.

Chromate reductase assay

The reaction mixture (1ml) contained Cr(VI) (5mg/L), 0.1mM of NADH as an electron donor in 800µl of phosphate buffer (0.1M; pH 6.5) and 200µl of CFE. A mixture with similar composition without enzyme was used as control sample. The reduction of Cr(VI) was measured by estimating the decrease of Cr(VI) in the reaction mixture after 30min of incubation and colorimetrically quantified using DPC as the complexing reagent.

Effect of pH and temperature on chromate reductase activity

The optimum pH and temperature for the activity of the chromate reductase present in the CFE was determined by incubating the reaction mixture at different pHs using various phosphate buffers (0.1mM, pH 5.5, 6.0, 6.5, 7.0 and 7.5) at 32°C and different temperatures ranging from 28 to 40°C at a pH of 6.5. The enzyme activity was determined after 30 minutes of incubation by the DPC method. Relative activity was expressed as a percentage of maximum activity taken as 100%.

Results

Strain CrMFC5 is a facultative and Gram-negative bacilli microorganism that formed circular cream colored and flat colonies; presented smooth surface creamy consistency; bright colony. It showed resistance to Cr(VI) with a MIC value of 350mg/L.

Molecular characterization

The naive Bayesian classifier tool was used to compare the sequence obtained to the sequences deposited in Ribosomal Database Project, indicating that said sequence belongs to the genus Aeromonas with a significance value of 100%. Figure 1 shows the phylogenetic analysis of the sequence of the isolated CrMFC5 with different sequences of species from the genus Aeromonas represented by Maximum likelihood tree based on the GTR (1+G) model. This model was used with estimated values for the following parameters: base frequencies (A=0.2465, C=0.2276, G=0.3257, T=0.2002), the proportion of invariable sites (0.4820), and the gamma-shaped parameter (0.4040). On the phylogenetic reconstruction based on the 16S rRNA gene sequence, strain CrMFC5 was assigned to the species Aeromonas hydrophila, which is included in the group Proteobacteria, subgroup Gamma, and in the family of Aeromonadace.

Figure 1 Maximum likelihood tree of the genus Aeromonas based on the 16S rRNA gene, representing the phylogenetic inference of the sequences. Numbers at nodes represent bootstrap values.

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Electrogenic activity of *Aeromonas* CrMFCS

The electrogenic activity of *Aeromonas hydrophila* CrMFCS is shown in Figure 2. The phase of latency or colonization of the anode took place before day two, showing low values of power density (0.026mW/m²). Subsequently, an increase of values was recorded, up to a maximum value of power density of 7.1mW/m². The behavior of the data and the registered power density values indicate that *A. hydrophila* CrMFCS is an electrogenic microorganism.

**Figure 2** Electrogenic activity of *A. hydrophila* CrMFCS.

**Biological reduction of hexavalent chromium**

Chromium reduction and the kinetics of the bacterial growth of *A. hydrophila* CrMF5 in LB broth and industrial wastewater are shown in Figure 3. 100% reduction of Cr(VI) was registered in the LB broth in 10 hours, with an average rate of reduction of 1mg/L per hour. The bacterial growth in LB medium kept increasing constantly, showing a maximum value of absorbance of 0.856 at the end of the test. In contrast, 100% reduction of Cr(VI) in the IW was registered in 38 hours, with an average rate of reduction of 0.31mg/L, indicating a 68% decrease in the average rate of Cr(VI) reduction in contrast to that in the LB medium. The kinetics of growth in the IW were different from those in the LB medium, showing a slight increase in the first 10 hours of the test with a maximum absorbance of 0.495 at the 10th hour.

**Figure 3** Chromium reduction and kinetics of bacterial growth. (A) Luria Bertani broth. (B) Industrial wastewater.

**Chromate reductase activity of cell-free extract**

The cell-free extract of *A. hydrophila* CrMF5 showed Cr(VI) reductase activity. The optimal temperature of the CFE Cr(VI) reductase activity by CFE was determined by using different incubation temperatures between 28 to 40°C. Maximum Cr(VI) reductase activity was observed at 32°C (Figure 4). Activity at 30, 34, 36 and 38°C was about 54% of the optimal activity. Activity at 28°C was 64% of the optimal activity and at 40°C, the activity was at 45% of the optimum.

The effect of pH on reductase activity of CFE was assessed at a pH range from 5.5 to 7.5, using phosphate buffer. Significant values of Cr(VI) reduction activity were observed over a wide pH range. At pH 6 and 7, the Cr(VI) reduction activity shown was of 95 and 84% respectively, with an optimum at pH 6.0 (Figure 5). An observed decrease in chromate reducing activity was similar towards both alkaline and acidic conditions. The chromate reducing activity diminished rapidly prior to a pH of 6.0 and after a pH of 7.0, showing a 17 and 13% of the optimal activity, respectively.

**Figure 4** Effect of pH on Cr(VI) reduction activity by CFE of the bacterial strain *A. hydrophila* CrMFCS. The reductase activity was determined after 30min of incubation at 32°C.

**Figure 5** Effect of temperature on Cr(VI) reduction activity by CFE of the bacterial strain *A. hydrophila* CrMFCS. The reductase activity was determined after 30 min of incubation at pH 6.5.

**Discussion**

Phylogenetic analysis revealed that the strain CrMF5 is included in a group comprehended by *Aeromonas hydrophila*. In this document, we described a new Cr(VI) resistant and Cr(VI) reducing strain, *A. hydrophila* CrMF5. The genus *Aeromonas* is a group of ubiquitous microorganisms found in a wide range of aquatic environments and according to scientific literature, these species of bacteria are rarely known by their electrogenic capacity and Cr(VI) reduction.

The power density data registered by *A. hydrophila* CrMF5 (7.1mW/m²) is low in comparison to other authors. Notwithstanding, these low values reported were a product of the materials used in the construction of the electrodes and the small available surface area for biofilm formation. The obtained results allow for the classification of this microbial isolate as an electrogenic organism, showing great electrogenic capacity.

The rate of chromium reduction and microbial growth was completely different when *Aeromonas CrMF5* was evaluated in LB and IW. In the LB broth assay, the Cr(VI) reduction rate didn’t seem
Electrogenic activity and hexavalent chromium reduction by Aeromonas hydrophila CrMFC5

Conclusion

The isolated bacterial strain from the genus *Aeromonas* tolerated Cr(VI) and reduced Cr(VI). In addition, the CFE has enzymes with chromate reductase activity. The ability of the isolated strain to reduce Cr(VI) in industrial wastewater provides the opportunity for bioremediation of industrial water containing heavy metals. Moreover, the use of CFE could represent a further opportunity for Cr(VI) reduction. The electrogenic strain *A. hydrophila* CrMFC5 would provide the opportunity to treat Cr(VI) from wastewater and to simultaneously generate electricity. We suggest the further exploration of the potential of the microorganisms belonging to the genus *Aeromonas* and use of the new isolate in bioremediation applications.

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Conflict of interest

There is no conflict of interest to declare regarding the publication of this paper.

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