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The use of autotrophic *Chlorella vulgaris* in chromium (VI) reduction under different reduction conditions

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ABSTRACT

Chromium (Cr) is a common heavy metal element found in the environment. Of the two predominant forms of chromium, Cr(III) has only about one-thousandth the toxicity of Cr(VI). In general, the microalgal biomass was regarded as an adsorbent during the chromium removing process. However, the results of X-ray absorption near-edge spectroscopy (XANES) supported that the more toxic Cr(VI) could be converted into the less-toxic Cr(III) by using *Chlorella vulgaris*. It was considered that Cr removal though *C. vulgaris* was occurring not only through the adsorption mechanism. The biological route of enzymatic chromium reductase is another potential way to reduce Cr(VI) in *C. vulgaris*. The reduction of Cr(VI) to Cr(III) was observed even in the batch with sterilized dead cells, thus revealing that a non-enzymatic reduction route is possibly involved in this process. Further analysis indicated that glutathione (GSH) released from the broken cells might play an auxiliary role in the Cr(VI) reduction. Conclusively, there are two probable synergistic mechanisms involved in microalgae Cr(VI) reduction: the biological mechanism (enzymatic route) and the non-biological mechanism (GSH-released). The results also indicated that the microalgal biomass density is a crucial factor in enhancing Cr(VI) reduction.

1. Introduction

The release of heavy metals to the environment is a growing concern, as they can cause significant damage to both animal life and ecosystems. The most common heavy metals found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) [1]. Among those heavy metals, chromium (Cr) is a naturally-occurring element often found in many materials, including rocks, soil and others. The oxidation states of chromium range from +2 to +6. Cr(VI) and Cr(III) are recognized as the most stable forms [2]. Cr(VI) is known to be widely used in many chemical manufacture processes, all of which generate lots of effluent-containing Cr(VI) [3]. The high solubility of Cr(VI) makes it a hazardous contaminant of water and soil, and it is also recognized as a potential carcinogen [4]. Cr(VI) can easily penetrate the cell membrane and affect its integrity, causing damage [5,6]. In contrast, the cell membrane is nearly impermeable to Cr(III), and thus the toxicity level of Cr(III) is only about one thousandth that of Cr(VI) [7,8]. Cr(VI) is estimated to be the second most common heavy metal contaminant in the natural environment, ranging in concentration from 0.008 to 173 mM in groundwater and 98 nM to 76 mM in soil and sediments [9]. Several methods of removing Cr(VI) from contaminated sites have been reported, including the potential application of constructed wetlands in the treatment of chromium-bearing wastewaters [10].

The ability of some microorganisms to remove toxic pollutants, including Cr(VI) ions, has been examined, and bacteria and fungi play central roles in this process [1,11]. Among the microorganisms that have been found to play significant roles in the bioremediation of Cr(VI) ions, bacterial chromate reductases are reported to be capable of converting soluble and toxic chromate into the insoluble and less toxic Cr(III). Chromate reductases are a group of enzymes that convert the hexavalent chromium ion Cr(VI) into the trivalent chromium ion Cr(III). The chromate reductase is optimally active at a pH of 6.3 and temperature of 65 °C and requires Ca^{2+} or Mg^{2+} for activity. The enzymatic activity of chromium reductase is also dependent on NADH or NADPH, with a preference for NADPH [12].

In addition to bacteria, several strains of microalgae such as *Scenedesmus* and *Chlorella* have been reported to be capable of removing Cr(VI) from bodies of water [13–16]. Microalgae have several advantages including economical regenerability, potential use in metal-recovery, biological sludge generation, and highly-efficient

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effluents dilution [17]. It has been reported that the maximum tolerable Cr(VI) concentrations for *Scenedesmus acutus* and *Chlorella vulgaris* are 15 and 45 mg l^{-1} respectively [13]. Microalgal biomass is regarded as the bio-adsorbent in the removal of Cr through adsorption. A maximum uptake capacity of 57.33 mg Cr(VI) g^{-1} dry biosorbent l^{-1} of solution has been achieved for an initial concentration of 100 mg l^{-1} Cr(VI) at 2.0 pH, with a 20% (w/v) biosorbent dose [15]. A microalgal strain-*Chlorella* spp. isolated from an industrial wastes disposal site had been proven to have the Cr(VI) reduction ability [18]. Both mechanisms of biosorption and bioreduction are reported to be involved in the Cr(VI) removal process, with the reduction process to Cr(III) performed after Cr(VI) adsorption [19]. Han et al further indicated that Fourier Transform Infrared Spectrometer (FTIR) analysis confirmed that the amino group on the algal biomass was the main binding site for Cr(VI) biosorption under acidic conditions, while the reduction of Cr(VI) to Cr(III) was mainly sequestered by carboxylate group [19].

Another conceptual model for Cr(VI) biosorption-mediated reduction was proposed by using a heterotrophically-grown biomass of *Chlorella vulgaris* [20]: protonated hydroxyl and amino groups can be involved in Cr(VI) sorption, hydroxyl groups can reduce sorbed Cr(VI) producing Cr(III) and new carboxyl groups. Cr(III) can be bound by labile electrostatic interactions to sulfonates. Competition with Na^+ determined the release of Cr(III) and favored further Cr(VI) reduction [20].

Due to the complex analytical process of chromium reductase activity, a novel analytical technology, called X-ray absorption near-edge spectroscopy (XANES), was adopted in this work to examine the reduction of Cr(VI) to Cr(III) by microalgae. One of the basic features of XANES is that it can show a clear shift of line shapes with a magnitude of several electrovolts measured between the hexavalent Cr(VI) ions and the trivalent Cr(III) ones. Another is the different intensities and energies of the pre-edge transitions of the hexavalent and trivalent Cr ions. According to these features, a chemical reduction of Cr ions from the hexavalent state to the trivalent one is evident in dried powders of living *Chlorella vulgaris* cells.

The purpose of this study was thus to examine whether the reduction of Cr(VI) to Cr(III) occurred in *Chlorella vulgaris* under different conditions, including with live cells, dead cells and a supernatant in which the cells were broken. The auxiliary benefits of the addition of glutathione (GSH) were also explored. The novel analytical method of XANES was adopted to detect the existence of Cr(III) during the Cr(VI) reduction process.

2. Materials and methods

2.1. Microorganisms and medium

The microalgae *Chlorella vulgaris* was generously provided by Prof. Jo-Shu Chang (National Cheng Kung University, Taiwan). A basal medium was used for autotrophic cultivation in flasks. Details of the basal medium preparation have been described in previous research [21].

2.2. Cultivation methods

The batch autotrophic cultivation was performed at 25 ± 1 °C, bubbled with 1 vvm air and supplemented with 2% CO_2 in a glass flask (containing 500-ml of medium). The bottle was agitated using a stir bar at 100 rpm, and continuously illuminated with regular fluorescent lights or white LED lights (model MR 16 with 7 Watts, Shianyih Electronic Industry Co., Taiwan) to provide sufficient light. The light intensity on the surface of the flask was measured by a light meter (LI 250, LI-COR, Inc., Lincoln, NE, USA), giving a value of 1300 µmol m^{-2} s^{-1}.

The Cr(VI) reduction trials were carried out after the cell density reached 2 g l^{-1}. 10 mg l^{-1} of Cr(VI) ($K_2Cr_2O_7$) was added to the flask after the cells. In the batch operation of the Cr(VI) reduction using sterilized dead cells, *C. vulgaris* was cultured in the glass flask according to the procedure described above. After the biomass density reached about 2 g l^{-1}, the whole flask was sterilized at 121 °C for 15 min. The 10 mg l^{-1} of Cr(VI) was added to the flask to perform the reduction. In the batch with varied addition of GSH, the Cr(VI)/GSH ratios of 1/1, 1/2 and 1/3 were adopted for the test of 10 mg l^{-1} of Cr(VI) reduction in water only (without adding *C. vulgaris*). Two chromium chemicals of $K_2Cr_2O_7$ (Cr(VI)) and Cr_2O_3 (Cr(III)) were used in this study for the chromium reduction test.

2.3. The operation of XANES

The chemical state of Cr in *Chlorella vulgaris* was investigated by X-ray absorption near-edge spectroscopy at the beamline BL16A1 in the National Synchrotron Radiation Research Center (NSRRC), Taiwan. The spectrum was recorded in the energy range between 5970 and 6055 eV in the fluorescence mode, using a Lytle detector and dried *Chlorella vulgaris* powder dispersed on a Kapton tape. Energy steps of 0.25 eV were used with a counting time of 3 s per step. The standard data processes of the XANES spectrum were carried out using the Athena 0.8.056 software program, including the background subtraction and the normalization to the main jump of absorption. The absorption edge is defined as the energy corresponding to the first inflection point. In addition, the XANES spectra of the $K_2Cr_2O_7$ (Cr(VI)) and Cr_2O_3 (Cr(III)) powders were measured as standard references. A chromium metal foil with K-edge absorption edge at 5989.0 eV was used as the calibration standard to determine the correct edge energy of each specimen.

2.4. Analytical methods

The biomass concentration was estimated by its absorbance at 560 nm, based on the relationship between the optical density (OD_{560}) and dry cell weight (DCW). The concentrations of Cr(VI) and total chromium in the supernatant were determined using a colorimetric method, as described in the Standard Methods. The absorbance of the pink-colored complex formed from reacting Cr(VI) with 1,5-diphenylcarbohydrazide was measured at 540 nm using a UV spectrophotometer (GENESYS 10 UV, Thermo, USA) [22]. The HPLC analysis of GSH was performed according to the following procedures. The standard solutions of GSH (1×10^{-3} M) were prepared in the mobile phase, which was a mixture of phosphoric acid (aqueous solution, 1 mg/ml) and acetonitrile in the ratio 64:36 (v/v). The analysis conditions were set at 1 ml/min of the flow rate and 214 nm of the detection wavelength [23].

2.5. Chromate reductase activity

Chromate reductase activity was determined by measuring the decrease of Cr(VI) concentration during enzyme assays. Before the assay, cell-free extracts of *C. vulgaris* were first prepared by the following steps. The cultivation broths were harvested at 6000 g at 4 °C for 10 min, then washed and re-suspended in 100 mM of a potassium phosphate buffer (pH 7.0). These cell suspensions were disrupted by the method of Ultrasonic Probe (Sonics Vibra Cell 500, USA). The disruption conditions were set at an amplitude of 35% at 50 W with 9 s pulses and 1 s off modes for 35 min. After that, the whole solution was then centrifuged at 32,000 g for 40 min at 4 °C to collect the supernatant. The supernatant obtained was then filtered through 0.22 mm filters (Millipore, Bedford, USA) to retrieve the cell-free extracts. The reaction solution consisted of 50 mM of Tris-HCl buffer (pH 7.0) (T50

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