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Research article

Bioremediation of chromium contaminated water by diatoms with concomitant lipid accumulation for biofuel production

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ABSTRACT

Hexavalent chromium compounds such as chromate and dichromate, commonly designated as Cr (VI) compounds, are widely used heavy metals in different industries and are considered highly toxic to most life forms. Unfortunately, they have become a major pollutant of groundwater and rivers around dichromate using industries. Bioremediation is widely used to decrease the amount of dichromate in wastewater but requires large amounts of precious fresh water. Here we tested two marine micro-algal species, *Phaeodactylum tricornutum* strain CCY0033 and *Navicula pelliculosa* strain CCMP543, for their ability of dichromate bioremediation and concomitantly producing lipids that can serve as biofuel. Dichromate tolerance of the strains was investigated under different growth conditions in order to obtain high biomass yields, high lipid accumulation and high dichromate removal from the medium. Both algal strains grew well and produced high biomass in media containing up to 1 mg of dichromate per liter. Variations in growth conditions revealed that dichromate removal from the medium correlated positively with biomass yield. Dichromate removal using living cells was in the same order of magnitude as with autoclaved dead cells or when using extracted extracellular polymeric substances (EPS). This suggests biosorption of dichromate to cell-associated polymeric substances as the major mechanism of the bioremediation process. For both strains, optimal dichromate removal and lipid production were achieved at a light intensity of 55 μmol $m^{-2}s^{-1}$ and at a sodium nitrate concentration of 3 mM. The optimal temperature for dichromate removal and lipid production was 23 °C for *P. tricornutum* and 27 °C for *N. pelliculosa*. Compared to *P. tricornutum* strain CCY0033, *N. pelliculosa* strain CCMP543 produced an overall higher lipid yield under these conditions.

1. Introduction

Chromium exists in three major forms in nature: the uncharged metallic form (Cr), a trivalent form (Cr (III)) and a hexavalent form (Cr (VI)) (Greenwood and Earnshaw, 1997). Chromium has several important industrial applications such as in the leather industry, chrome plating, textile manufacturing, and steel industry (U.S. Department of Health and Human Services, 2008). Cr and Cr (III) are considered nontoxic and non-carcinogenic or possess only low toxicity. Moreover, in trace concentrations, Cr (III) is even an essential element for life (Straif et al., 2009; U.S. Department of Health and Human Services, 2016). Cr (VI), however, is classified as a human carcinogen (Straif et al., 2009) and is toxic to animals (Velma et al., 2009), plants (Shanker et al., 2005) and microorganisms (Agency for Toxic Substances and Disease Registry, 2012; Petrilli and De Flora, 1977; Wong and Trevors, 1988; Yao et al., 2008). An estimated 50–80% of all plant and algal species are negatively affected by Cr concentrations exceeding 100 μg/L (Federal Environmental Quality Guidelines, 2017). Toxic concentrations of Cr (VI) for microalgae varied from 1 μg/L for the diatom *Thalassiosira pseudonana* to up to 10 mg/L for a *Chlorella* sp. (Wong and Trevors, 1988). Cr (VI) toxicity affects microorganisms in pure cultures (Petrilli and De Flora, 1977) as well as in natural microbial communities. Increasing the concentration of Cr (VI) resulted in decreased microbial activity (Yao et al., 2008).

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Many ecosystems and surface- and groundwaters are polluted by heavy metals including Cr (VI), mostly in the form of chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) (Agency for Toxic Substances and Disease Registry, 2012), in which Cr (VI) concentrations range from 0.5 mg/L to up to 20 mg/L (Agency for Toxic Substances and Disease Registry, 2012; Zhitkovich, 2011). In 1991, the United States Environmental Protection Agency (US-EPA) has set the maximum allowable contamination level for total chromium at 100 ppb (100 μg/L). In a 2014 revision, the acceptable contamination level was decreased to 10 ppb (10 μg/L) and is aiming at a public health goal to reach 0.02 ppb (20 ng/L) (Federal Environmental Quality Guidelines, 2017; Agency for Toxic Substances and Disease Registry, 2012; U.S. Department of Health and Human Services, 2016). This has led to the development of various abiotic and biological approaches for the treatment of wastewater or ecosystems in order to eliminate Cr (IV) contamination (Barrera-Díaz et al., 2012). Bioremediation uses autochthonous or introduced living organisms, often microorganisms, algae, or plants in order to remove or detoxify contaminants. While plants, heterotrophic bacteria, and fungi have been widely used in bioremediation of Cr (VI) contaminated sites, the potential of applying phototrophic microorganisms has received much less attention (Barrera-Díaz et al., 2012). This is surprising because just as plants, microalgae and cyanobacteria are photoautotrophic and harvest sunlight energy, which would decrease process costs and could therefore be commercially attractive. In addition, microalgae and some cyanobacteria are known to accumulate lipids and hence their biomass can be used for the development of so-called third-generation biofuels. These organisms, in addition to their bioremediation capacities, can be grown on non-arable land and when choosing marine or salt-adapted organisms, competition with food production and the use of precious freshwater could be avoided (Sharma et al., 2012). Furthermore, biosorption using extracellular polymeric substances (EPS) is one of the major mechanisms of heavy metal bioremediation by microalgae and cyanobacteria, and it would therefore be possible to recover metals such as Cr (IV) from these biosorbents (Sen and Dastidar, 2010).

Bacillariophyta (diatoms) comprise a large and diverse group of microalgae that are widespread in aquatic ecosystems. They are often the dominant group of eukaryotic phytoplankton and are responsible for 40–45% of the primary production in the ocean (Sarthou et al., 2005; Smetacek, 1999). Moreover, diatoms are key to a worldwide algae-based bioeconomy that is used for food, feedstock, and biofilm production (Laurens et al., 2017). There have been many applications using diatoms including bioremediation of heavy metals (Bozarth et al., 2009; Pereira et al., 2011). Moreover, several strains of diatoms are known to possess natural high contents of neutral lipids that can be converted into biodiesel (Zhu et al., 2016).

This study aimed at the use of diatoms to combine the sequestration of toxic chromium (VI) and the optimization of lipid accumulation for biofuel production. For this, we have used two species, *Phaeodactylum tricornutum* and *Navicula pelliculosa*. These benthic diatoms have been shown to be oleaginous (produce lipid) and produce high amounts of extracellular polymeric substances (EPS) while growing at low silicate concentration (Coombs et al., 1967; Kaur, 2014; Lewin, 1955). By varying the culturing parameters, we optimized growth yield, chromium removal, and lipid production.

2. Materials and methods

2.1. Organisms and culture conditions

The diatoms, *P. tricornutum* CCY0033, isolated from an intertidal sediment from the North Sea beach of the Dutch barrier island Schiermonnikoog, the Netherlands, and *N. pelliculosa* CCMP543/CCY0399, originally isolated from Oyster Pond, Martha's Vineyard, Massachusetts USA, were obtained from the Culture Collection Yerseke (CCY), Royal Netherlands Institute of Sea Research. Cultures were grown in MDV medium (Supplementary material) containing 6 mM nitrate and 150 μM silicate in 250 mL polystyrene tissue culture flask (TTP, 90026, Switzerland). To 92 mL of fresh medium, 8 mL (8% v/v) of an actively growing, 3 weeks old pre-culture was added. The cultures were incubated at 14 °C and 55 μmol $m^{-2}s^{-1}$ light (photon flux density) at a 16-8 h light-dark regime. The pH of the media was adjusted at 7 ± 0.2. These conditions are hereafter called "standard growth conditions". Culturing was always performed in triplicate and the growth was followed by measuring the optical density at 600 nm (OD_{600}) using sterile MDV medium as a blank.

2.2. Chromium (VI) tolerance

To determine Cr (VI) tolerance, both diatoms were grown as described above in MDV medium containing Cr (VI) in the form of potassium dichromate $K_2Cr_2O_7$ at final concentrations ranging from 0 to 10 mg/L. Growth was assessed by end-point measuring the optical density at 600 nm (OD_{600}) after 15 days of incubation.

2.3. Analysis of dichromate concentration

Residual Cr (VI) in the medium after biosorption was assayed using a colorimetric test upon reaction with diphenyl carbazide (DPC) according to recommendations by the American Public Health Association (APHA Method 3500-Cr: Standard Methods for the Examination of Water and Wastewater (chromium), 1989 and 1996) and the microalgae samples were prepared as described by Dönmez and Aksu (2002). The samples were centrifuged for 15 min at 5000 rcf before adding DPC to the supernatant. Hexavalent chromium concentration was determined by measuring the colorimetric at 540 nm using various concentration of Cr (VI) (0, 0.25, 0.5, 0.75, and 1 mg/L) in sterile MDV medium as calibration curve.

2.4. Evaluation of heat-killed P. tricornutum and N. pelliculosa for chromium removal

To establish potential modes of chromium removal (enzymatic versus passive biosorption), chromium removal by living cells were compared with removal with heat-killed diatom cells. Diatoms were grown for 15 days in Cr (VI)-free MDV medium (100 mL) and killed by autoclaving for 20 min at 121 °C. After cooling down, the 100 mL of heat-killed cells were transferred to a dialysis tube (3 kDa cut-off) and placed in a sterile flask (Blue Cap Screw Cap bottle, 500 mL, DURAN) containing 100 mL sterile MDV. Filter sterilized Cr (VI) was added to the medium at a final concentration of 1 mg/L and incubated under standard growth conditions while stirring with a magnetic bar at 150 rpm. Incubation was maintained for 3 days to establish an equilibrium in Cr (VI) concentration and the residual hexavalent chromium concentration in the external medium was determined as described above.

2.5. Potential role of extracellular polymeric substances in chromium (VI) biosorption

EPS is abundantly produced by both diatom species and can be present as colloidal molecules suspended in the medium or attached to the cells (Staats et al., 1999). Extraction of both EPS fractions was carried out according to Staats et al. (1999) with slight modifications. Non-attached EPS was separated from the cells in both diatom cultures (100 mL) by centrifugation at 20,000 rcf (15 min and 10 °C). The loosely bound attached-EPS fraction was extracted from the cell pellet by resuspending it in 5 mL of sterile MDV medium and incubated for 1 h at 30 °C in a shaking water bath. Subsequently, the suspension was centrifuged at room temperature for 30 min at 20,000 rcf and the supernatant was combined with the non-attached EPS-containing supernatant from the previous step. Instead of precipitating the EPS, we