Ecotoxicology and Environmental Safety 106 (2014) 46–53

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Cadmium-induced oxidative stress tolerance in cadmium resistant *Aspergillus foetidus*: its possible role in cadmium bioremediation

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ARTICLE INFO

Article history:
Received 6 August 2013
Received in revised form
19 March 2014
Accepted 4 April 2014
Available online 14 May 2014

Keywords:
Aspergillus foetidus
Bioremediation
Oxidative stress
Antioxidative enzymes
Lipid peroxidation
Thiol

ABSTRACT

Toxic effects of cadmium (Cd) were examined on a cadmium-resistant strain of *Aspergillus foetidus* isolated from wastewater. The Cd removal potential was analyzed. The results indicated that the strain could tolerate up to 25 mM and 63 mM Cd in liquid and solid Czapek-Dox media, respectively. It efficiently removed Cd from liquid growth media and industrial wastewater by mycelial biosorption. The strain produced oxalic acid for the purpose of Cd bioleaching as confirmed by the presence of cadmium oxalate crystals on the mycelial surface. Intracellular proline contents and the antioxidative enzyme activities increased up to a certain level to detoxify the overproduced free radicals. These data indicate that the strain has inherent mechanisms to grow in Cd contaminated environment, tolerate high Cd doses and high Cd uptake potential which are pre-requisite for acting as a suitable candidate for Cd bioremediation.

1. Introduction

Cadmium (Cd) is one of the most deleterious trace heavy metals to plants and animals (Dong et al., 2007). It is used in rechargeable batteries, electronic equipments, bearing alloys, pigments for ceramic glazes, paints and plastics (Adamis et al., 2003). Cd is also present in phosphate fertilizers (Perez and Anderson, 2009). Cd has been accepted as a category 1 (human) carcinogen by the International Agency for Research on Cancer (Hossain and Huq, 2002).

The environmental Cd pollution occurs due to its continuous release from the industrial and agricultural sources (Jarup and Akesson, 2009). Cd easily translocates from plant roots to above ground tissues (Zhou and Qiu, 2005) and interferes with physiological processes (Maksymiec et al., 2007; Li et al., 2008). Cd enters the food chain through plants and therefore induces its adverse effects on human and other organisms.

The mechanism of Cd toxicity is of prime interest and it is important to see how the toxic effects are counterbalanced by the living cells. The thiol compounds, including reduced glutathione, phytochelatins (PCs), and metallothioneins, are essential components of Cd detoxification pathways in various organisms (Hall, 2002; Brunetti et al., 2011).

Cd induces oxidative stress by forming reactive oxygen species (ROS) in the living cells (Schutzendubel et al., 2001). The interaction of Cd with the antioxidative systems has been studied in several plants and animals (Vitoria et al., 2001; Fornazier et al., 2002). Cd^{2+} inhibits nitrate reductase activity in *Aspergillus niger* (Aiken et al., 2003). The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) have been reported to increase as a result of excess amount of ROS formation induced by the Cd^{2+} toxicity (Guelfi et al., 2003). Cd^{2+} induces an increase in SOD activity in *A. niger* B 77 strain; however CAT activity decreases significantly with the increased Cd^{2+} stress (Todorova et al., 2008).

Cd-induced lipid peroxidation has also been reported (Howlett and Avery, 1997; Tao et al., 2007). Lipid peroxidation occurs via peroxidation of unsaturated fatty acids. Free radical damage to phospholipids is an important factor in developing toxic conditions. The free radical scavengers and antioxidants are shown to be useful in protection against Cd toxicity (Sarkar et al., 1998; Ognjanovic et al., 2003).

Many plants and algae reduce heavy metal toxicity by the production of proline. Increased proline level in response to Cd toxicity in plants has been reported previously (Balestrasse et al., 2005; Dinakar et al., 2008). Proline may reduce hazardous effects of ROS by acting as an inhibitor of lipid peroxidation (Mehta and Gaur, 1999), a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989) and a singlet oxygen scavenger (Alia and Matysik, 2001).

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http://dx.doi.org/10.1016/j.ecoenv.2014.04.007
0147-6513/

Traditional methods like chemical precipitation, filtration, electrochemical treatments, reverse osmosis, ion exchange, adsorption etc. are expensive and inadequate for the removal of heavy metals from water. In this regard, microorganisms may be considered as a biological tool for metal processing as they can concentrate, remove and recover heavy metals from contaminated aquatic environments (Riggle and Kumamoto, 2000). In recent years, the filamentous fungi are gaining more importance as they are capable of removing heavy metals by biosorption as well as by intracellular uptake (Kapoor and Viraraghavan, 1995).

Some Cd-resistant organisms have been studied and are of considerable values in the remediation of soils and aquatic systems heavily contaminated with Cd (Zhu et al., 1999). Several *Aspergillus* species have also been found to be efficient in bioleaching of Cd and other heavy metals (Aung and Ting, 2005; Santhiya and Ting, 2006).

A. niger biomass pretreated by boiling in NaOH solution exhibits high Cd removal capacity (Kapoor et al., 1999). *A. niger* has been successfully used to remove Cd from oil field water (Barros Jnior et al., 2003). Dried, non-living and granulated biomass of *Aspergillus fumigatus* can remove Cd^{2+} from solutions efficiently (Rama Rao et al., 2005). *Aspergillus clavatus* has been reported to immobilize high amount of Cd^{2+} from aqueous solution (Cernansky et al., 2008). *Aspergillus foetidus*, the strain under the current report, can reduce chromium(VI) to chromium(III) by complexation of chromium(VI) with the organic compounds released by the fungi due to their metabolic activity and can take up chromium(VI) from solution (Prasenjit and Sumathi, 2005). Multimetal tolerant *A. foetidus* has been found to be effective in the bioleaching of nickel laterite ores (Le et al., 2006).

In this work, we studied the mechanism of the Cd toxicity in a Cd-tolerant strain of *A. foetidus*. We also assessed the Cd tolerance mechanism of the strain by analyzing certain cellular responses evoked in respect of certain enzymes and biomolecules to counter the toxicity. The strain was used to quantify the Cd biosorption capacity from the liquid growth media and from experimental wastewater samples. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopic (EDS) studies were performed to characterize the biomass in order to determine the possible Cd binding mechanisms.

2. Materials and methods

2.1. Samples

The sewage sediment sample was collected from the water treatment center, Kalyani, India. The sample was diluted serially with a sterile 145 mM NaCl solution and thoroughly shaken (10 times). Czapek Dox (CD) media were used for all the growth experiments. The CD media contained (per liter): $NaNO_3$ (2.0 g), $MgSO_4$ (0.5 g), KCl (0.5 g), $FeSO_4$ (0.01 g), $ZnSO_4$ (0.01 g), glucose (40.0 g). KH_2PO_4 was used as a phosphate source. The properly diluted (100 times) sample was used to spread onto solid Czapek Dox (CD) plates consisting of 4.95 ml CD media with 50 μl of 1 M cadmium chloride ($CdCl_2$) solution to reach a final concentration of $CdCl_2$ to 10 mM.

2.2. Isolation of microorganisms

The isolation and enumeration of microorganisms were carried out in solid CD media, as described earlier (Raper and Thom, 1949). The pH of the media was maintained at 5.0. The media were solidified with 2 percent agar as solid CD medium (CDA). Streptomycin was added to the media to arrest bacterial growth. The sewage sample was plated on CDA and the plates were incubated at 32 °C for 96 h. The best grown fungal colony with black conidia was primarily identified as a high Cd tolerant strain and was preserved in the CDA slants containing 10 mM $CdCl_2$.

2.3. Preparation of pure culture and its maintenance

The conidia of the preserved strain were taken in sterile water and shaken vigorously. The properly diluted conidial suspension was spread onto CDA supplemented with $CdCl_2$ and allowed to grow at 32 °C for 96 h. The best grown colony was preserved in CDA slants. The slant cultures were routinely sub-cultured every one month prior to the experimental use; 8-day old spores were used as inoculums.

2.4. Preparation of fungal biomass

The liquid CD broth with added streptomycin, (pH 5.0) was used for the growth of the fungus. Conical flasks containing CD media were inoculated with the spores (10^{10} conidia L^{-1}) of the strain and shaken at 175 rpm at 32 °C. Ten different Cd concentrations including 10 μM, 50 μM, 100 μM, 500 μM, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM and 25 mM were added separately to the growth media. For the enzymatic and biomolecule studies, the biomass grown at four different Cd doses (5, 10, 15 and 20 mM) along with a control (no Cd in growth media) were used. To study the effect of chloride ions (Cl^-), the fungus was grown at different Cl^- doses (5, 10, 15, 20 mM). NaCl was used as the source of Cl^- ions. The biomass was harvested after 96 h, filtered and washed with de-ionized water and kept at −20 °C for the biochemical analyses.

100 mg of the fungal biomass was introduced into wastewater samples collected from River Damodar near Asansol Industrial area, West Bengal and electroplating industry effluent at Kolkata, West Bengal, India. The biomass was allowed to stand for 96 h in the wastewater samples at room temperature with occasional shaking.

2.5. Estimation of Cd by atomic absorption spectroscopy

The fungal mycelia, the spent media and the wastewater samples were analyzed by atomic absorption spectrometer (AAS) for Cd estimation. Approximately 300 mg of dried mycelia and 2 ml of spent media or wastewater sample were digested in 4 ml of concentrated HNO_3 and 1 ml of 30 percent H_2O_2 in closed PTFE vessels in a digestion block at 90 °C for 3 h. The digest was diluted with MilliQ water up to a volume of 25 ml (Maihara et al., 2008). A Perkin Elmer AAnalyst 400 atomic absorption spectrometer with Zeeman background correction at 228.8 nm for Cd was used.

2.6. SEM and EDS analysis

The samples were prepared for SEM according to Gharieb et al. (1995). The samples were analyzed by a field emission scanning electron microscope Jeol JSM 6700F with an accelerating voltage of 20 kV.

2.7. Changes in pH of the spent media

The initial pH of the CD broth was maintained at 5.0 after autoclaving, followed by the addition of Cd for different treatment groups. The media were inoculated with the fungal spores and the biomass was harvested after a 96 h growth period. The pH of the spent media was measured for each treatment group.

2.8. Assay of total thiol (–SH) contents

For total thiol assay, a modified Ellman (1959) method was followed. The fungal mycelia were ground with alumina and extracted with 50 mM phosphate buffer (pH 7.0) with and without 50 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 2000*g* for 20 min at 4 °C. The supernatant was mixed with phosphate buffer (pH 7.0) and distilled water (3:2:5). 20 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) solution was added to 3 ml of the reaction mixture, shaken well and absorbance was recorded at 412 nm. The thiol contents were calculated using the molar extinction coefficient value of 13600 M^{-1} cm^{-1} for DTNB at 412 nm.

2.9. Assay of CAT, GR and peroxidase activities

Fresh mycelia were freeze-dried with liquid nitrogen, ground with alumina and extracted with an ice-cold 50 mM phosphate buffer (pH 7.0) containing 1 percent polyvinylpyrrolidone. The homogenate was centrifuged at 15,000*g* for 20 min at 4 °C. The supernatant was used for the assay of enzyme activities and the extent of lipid peroxidation.

The CAT activity was measured according to Chance and Maehly (1955). One unit CAT activity was defined as the absorbance change of 0.01 unit per min.

The GR activity was determined spectrophotometrically according to Carlberg and Mannervik (1985). One enzyme unit was defined as the oxidation of 1 μmol NADPH per min.

The activity of peroxidase towards syringaldazine (SPX) was assayed according to Imberty et al. (1985). The peroxidase activity towards guaiacol (GPX) was assayed according to Maehly and Chance (1954). One unit peroxidase activity was defined as the oxidation of 1 μmol substrate per min.