Process Biochemistry 48 (2013) 496–509

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

Co-remediation of pentachlorophenol and Cr^{6+} by free and immobilized cells of native *Bacillus cereus* isolate: Spectrometric characterization of PCP dechlorination products, bioreactor trial and chromate reductase activity

Manikant Tripathi, Satyendra Kumar Garg*

Centre of Excellence, Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad 224001, India

ARTICLE INFO

Article history:
Received 17 August 2012
Received in revised form 4 February 2013
Accepted 7 February 2013
Available online 15 February 2013

Keywords:
Bioremediation
Chromate reductase
GC–MS
Immobilization
Pentachlorophenol
Tannery effluent

ABSTRACT

In this study, *Bacillus cereus* isolate efficiently remediated 57% PCP and 74% Cr^{6+} simultaneously with uptake rate of 0.65 mg Cr^{6+} g^{-1} biomass h^{-1} at initial 200 mg Cr^{6+} and 500 mg PCP l^{-1} concentration under optimized 0.4% glucose, 0.2% NH_4Cl, pH 7.0, 35 °C, 1.0% inoculum during 60 h incubation. Optimization of agitation (100 rpm) and aeration (0.6 vvm) in bioreactor further enhanced PCP dechlorination by ~5.0% and Cr^{6+} removal 7.5%. Presence of other heavy metals variedly affected bioremediation of both the toxicants. Maximum and minimum inhibition was exhibited by mercury and lead, respectively. Out of 74% Cr^{6+} remediated, 90% reduced to Cr^{3+}, of which 52.8% was associated with cell biomass and 37.2% with culture supernatant. Maximum chromate reductase activity was evident in culture supernatant followed by cytosolic fraction and cell debris. A direct correlation existed between chromate reductase activity and reduced Cr^{3+} in different cell fractions. Among matrices, alginate was most suitable for biomass immobilization, which enhanced Cr^{6+} removal by 20.2% compared to free cells at 36 h. Gas chromatography and mass spectrometry detected 2,3,4,6-tetrachlorophenol, 2,4,6-trichlorophenol, 2,6-dichlorophenol and 6-chlorohydroxyquinol as PCP dechlorination products. Our promising strain can be efficiently employed for simultaneous bioremediation of PCP and Cr^{6+} under wide environmental conditions.

1. Introduction

Tannery effluent contains chromium along with other heavy metals and pentachlorophenol co-contaminants, which are detrimental to a variety of living species, including humans. Poopal and Laxman [1] have indicated < 1.0 mg l^{-1} as permissible limit for toxic chromate discharged in water bodies. Chromium occurs in oxidation states Cr^{2-} to Cr^{6+}, but only Cr^{3+} and Cr^{6+} are biologically important. Chromium sulfate (Cr^{3+}) is used as tanning agent during leather processing. It is transformed into Cr^{6+} which is most toxic, mutagenic, carcinogenic and teratogenic [2] species of chromium due to its strong oxidizing nature [3]. Contrary to that, Cr^{3+} is an essential micronutrient for humans, and is relatively insoluble. It is required for normal sugar and lipid metabolism [4], and its deficiency leads to increased risk associated with diabetes and cardiovascular diseases. Therefore, reduction of hazardous Cr^{6+} to immobile Cr^{3+} would constitute a potential detoxification process that could be achieved either through chemical or biological methods. While chemical reduction is an energy intensive process, the biological reduction could serve as an alternative economical, effective and safe procedure without environmental implications. Bioremediation of chromium through reduction can be achieved by augmentation of bacteria in wastewater. Chromate reducing bacteria could be a viable solution for immobilization of highly hazardous soluble Cr^{6+} to less toxic/insoluble Cr^{3+} in the environment.

Microbial cell immobilization is a better option for cleaning toxic heavy metals from the environment. Several researchers have employed immobilization technique for bioremediation of Cr^{6+} [1,5,6]. Many processing steps become simple in the immobilization process. For instance, it is easier to (i) separate biosorbent from the effluent, (ii) repeat biosorption cycles, and (iii) elute (desorb) metals from the biomass [7,8].

Pentachlorophenol (PCP) and related chlorinated compounds are primarily used as biocides in the leather tanning processes. PCP is a highly toxic and recalcitrant compound, capable of being biodegraded by only a limited number of bacteria. Aerobic biodegradation of polychlorinated phenols has been extensively studied during last couple of years [9]. Several PCP degrading bacterial strains, viz., *Acinetobacter* sp., *Bacillus cereus*, *Serratia marcescens*, *Kocuria* sp., etc., have been reported from sites contaminated with PCP [10–12]. The United State's Environmental Protection Agency has listed PCP as a priority pollutant, and considers 0.1 mg l^{-1} hazardous for land disposal [13]. The accumulation of PCP through the food chain has been established, and it is

* Corresponding author. Tel.: +91 5278 247350; fax: +91 5278 246330.
E-mail addresses: sk_garg001@yahoo.com, skgarg86@yahoo.com (S.K. Garg).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.02.009

considered mutagenic or at least, co-mutagenic to humans. Thus, PCP exposure in environment poses significant health hazards [11]. Extensive exposure to PCP could lead to severe ailments such as cancer, acute pancreatitis, immunodeficiency and neurological problems [14]. PCP is toxic to aquatic life also at a concentration as low as 0.5 mg l^{-1} [15].

An extensive literature is available on individual bioreduction of Cr^{6+} and biodegradation of PCP either by single organism or consortium. However, very limited research has been carried out on simultaneous bioremediation of dual or multiple contaminants coexisting in the environment, particularly by indigenous bacterial isolates. Industrial effluents containing toxic organic compounds and other heavy metals generally create hindrance in the growth and bioremediation of chromium by the microorganisms.

This state of affair prompted us to search for an efficient bacterial species from the native niche which has the ability to simultaneously bioremediate both the highly toxic contaminants. The bacterial strain isolated from tannery effluent and characterized as *B. cereus* RMLAU1 exhibiting tolerance to 200 mg Cr^{6+} l^{-1} and 500 mg PCP l^{-1} was employed in this study. We report here the optimization of conditions for bioreduction of highly toxic soluble Cr^{6+} to insoluble Cr^{3+} and simultaneous dechlorination of PCP at flask scale to bioreactor level. Chromate reductase activity was also investigated to establish the enzymatic reduction of Cr^{6+}. The relative location of reduced chromium in cell biomass and/or culture supernatant is being elucidated. The Cr^{6+} removal ability of immobilized biomass of *B. cereus* was also attempted along with the stability of matrix during different operational cycles. The gas chromatography and mass spectrometry (GC–MS) was performed to characterize the PCP dechlorination products during the bioremediation process.

2. Materials and methods

2.1. Bacterial culture

The strain of *B. cereus* RMLAU1 (MTCC 9777; GenBank accession number FJ959366), isolated previously in our laboratory from treated tannery effluent, was employed in the present study. The bacterium was sub-cultured and maintained on minimal salt agar medium slants containing PCP 500 mg l^{-1} and Cr^{6+} 200 mg l^{-1} (pH 7.0) and stored at 4 °C for further use.

2.2. Culture conditions

The bacterial isolate was grown in minimal salt medium (MSM) containing (g l^{-1}): KH_2PO_4, 6.0; Na_2HPO_4, 7.0; $MgSO_4{\cdot}7H_2O$, 0.2 with carbon and nitrogen source as per the treatment and amended with 500 mg PCP l^{-1} and 200 mg Cr^{6+} l^{-1}. The medium was sterilized at 10 psi for 20 min. Except stated otherwise, the sterilized medium (pH 7.0) was inoculated with exponentially growing culture (at 1.0%, v/v) of 0.86 absorbance having cell density 3.0×10^6 cfu ml^{-1} and incubated at 35 °C in an incubator shaker (150 rpm). Samples withdrawn periodically at 12 h intervals up to 60 h were centrifuged at 10 000 rpm for 10 min at 4 °C in refrigerated centrifuge. The chloride ions released from PCP were analyzed in culture supernatant. The total chromium and residual Cr^{6+} were analyzed in culture supernatant as well as in bacterial biomass as per the analytical determinations. The Cr^{3+} concentration was derived from the difference of total chromium and Cr^{6+} levels. The MSM broth without bacterial culture, but with PCP and Cr^{6+} as per experimental conditions served as a control.

2.3. Effect of carbon and nitrogen sources

The effect of nutritional parameters such as carbon (viz., glucose, maltose, sucrose) at 0.2, 0.4 and 0.6% (w/v) and nitrogen sources (viz., ammonium chloride, ammonium nitrate, urea) at 0.1 and 0.2% (w/v) was studied for simultaneous bioremediation of PCP and Cr^{6+} in the above medium.

2.4. Effect of initial pH and temperature

The pH of above medium was adjusted in the range of 6.5–8.0 using 0.1 N HCl or 0.1 N NaOH, prior to sterilization. The sterilized culture flasks were inoculated as per the culture conditions and incubated for 60 h at different temperatures in the range of 25–40 °C in an incubator shaker at 150 rpm so as to determine the combined effect of pH and temperature on simultaneous PCP dechlorination and Cr^{6+} reduction.

2.5. Effect of inoculum size

The above MSM medium containing optimized glucose (at 0.4%, w/v) and ammonium chloride (at 0.2%, w/v) adjusted to optimized pH 7.0, sterilized and inoculated with 0.5–2.5% (v/v) exponentially growing culture, was incubated at optimized 35 °C in an incubator shaker at 150 rpm.

2.6. Bench-scale bioreactor level bioremediation of PCP and Cr^{6+}

2.6.1. Effect of aeration

Bioremediation of PCP and Cr^{6+} were performed in a stirred tank bioreactor (Bioflo 110, New Brunswick Scientific Co. Inc., Edison, NJ, USA) of 3 l capacity. The fermentor was equipped with direct drive dual Rushton style impeller, PID temperature, agitation control, probes and controller of pH and DO. The bioremediation study was carried out in 1.0 l working volume of MSM broth amended with PCP (500 mg l^{-1}) and Cr^{6+} (200 mg l^{-1}) under flask level optimized cultural conditions of pH 7.0, 35 °C and 150 rpm. The medium was inoculated (at 1.0%, v/v) with *B. cereus* RMLAU1 (OD_{600} 0.86; 1.0 cm cuvette) containing 3.0×10^6 cfu ml^{-1}. The aeration of culture broth was done at different rates (0.2–0.8 vvm), and samples (5.0 ml) were drawn periodically at 12 h intervals. The bacterial growth, PCP dechlorination and Cr^{6+} removal were assessed as per the analytical determinations.

2.6.2. Effect of agitation at optimized aeration

The bacterial growth, PCP dechlorination and Cr^{6+} removal were further studied by varying the agitation speed from 50 to 200 rpm at optimized aeration rate of 0.6 vvm. Other experimental conditions remained the same as above.

The bioreactor trial was also performed with untreated real tannery effluent (diluted 1:1 with distilled water) supplemented with glucose cosubstrate (0.4%, w/v) and ammonium chloride (0.2%, w/v) at pH 7.0, 35 °C and augmented with 1.0% (v/v) inoculum dose of *B. cereus* under above optimized aeration rate of 0.6 vvm and agitation speed of 100 rpm for Cr^{6+} bioremediation and dechlorination of chloroorganics.

2.7. Effect of other heavy metals

In order to determine the maximum tolerable concentration of other heavy metals, the exponential phase culture of bacterial isolate was inoculated aseptically in MSM broth supplemented individually with salts of other heavy metals (at 25–200 mg l^{-1}) such as lead acetate (Pb), sodium arsenate (As), zinc chloride (Zn), cobalt nitrate (Co), nickel chloride (Ni) and mercuric chloride (Hg) in addition to PCP (500 mg l^{-1}) and Cr^{6+} (200 mg l^{-1}), and growth was determined spectrophotometrically at 600 nm after 48 h incubation.

For studying the effect of heavy metals on Cr^{6+} remediation and PCP dechlorination, the MSM broth (pH 7.0) containing PCP (500 mg l^{-1}) and Cr^{6+} (200 mg l^{-1}) was amended with maximum tolerable concentration of other individual heavy metals (mg l^{-1}): Pb (175), As (105), Hg (25), Zn (60), Co (80) and Ni (105). The MSM broth amended with PCP (500 mg l^{-1}) and Cr^{6+} (200 mg l^{-1}) without any other heavy metal served as control (reference). The sterilized medium was inoculated with optimized 1.0% (v/v) *B. cereus* inoculum and incubated at 35 °C.

2.8. Chromate reductase activity

Preparation of cell-free extract and chromate reductase assay were performed as per the slightly modified method of Ilias et al. [16]. The bacterial cells grown in MSM broth (100 ml) were harvested during the exponential growth phase (36 h) and centrifuged at 10 000 rpm (4 °C) for 10 min. The culture supernatant was collected and assayed for soluble protein and chromate reductase activity. The cells' pellet was suspended in 5.0 ml phosphate buffer (50 mM, pH 7.0), kept in an ice bath (4 °C) and disrupted with an ultrasonicator at 30 s pulses. Further treatment was as per Ilias et al. [16]. Boiled culture supernatant, cell debris and cytosolic fraction served as controls. The chromate reductase activity was assayed in reaction mixture containing 0.5 ml enzyme solution (culture supernatant, cell debris and cytosolic fraction), 0.5 ml of 50 mM phosphate buffer and $K_2Cr_2O_7$ as Cr^{6+} at 3.4 μM final concentration. The reaction mixture was incubated at 30 °C for 30 min. The reaction was stopped by adding 0.2 ml of 20% trichloroactetic acid [17]. Cr^{6+} reduction was measured by estimating the decrease in Cr^{6+} concentration in the reaction mixture using 1,5 diphenyl carbazide method. Protein was estimated as per Bradford method [18]. One unit of enzyme activity was defined as 1.0 μM of Cr^{6+} reduced min^{-1}.

2.9. PCP dechlorination studies

The PCP dechlorination products were analyzed by GC equipped with FID detector (Agilent 7890A) and MS (Jeol AccuTOF GCV). The experiment was performed in MSM containing 500 mg PCP and 200 mg Cr^{6+} l^{-1} broth. Control (0 h) and experimental (48 h) cultured broth (100 ml) were taken and centrifuged (4 °C) at 10 000 rpm for 10 min. In cell-free supernatant fractions, the dechlorination metabolites were extracted using equal volume of n-hexane and acetone mixture (1:1 ratio). The organic layer was dried with anhydrous sodium sulfate and solvent evaporated till dryness. The sample residue was diluted with 1.0 ml n-hexane and analyzed immediately on GC–MS. In GC, the column and injector temperature was maintained at 240 °C and all injections (5.0 μl each for control and dechlorinated samples)