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A bioprocessing strategy that allows for the selection of Cr(VI)-reducing bacteria from soils

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Anaerobic bacteria that reduce hexavalent chromium [Cr(VI)] to trivalent [Cr(III)] are common in soils and were used to develop a bioprocess employing a selection strategy. Indigenous Cr(VI)-reducers were enriched from Cr(VI)-contaminated soil under anaerobic conditions. The mixed culture was then tested for Cr(VI)-reducing activity in a chemostat, followed by transfer to a 1-L packed-bed bioreactor operated at 30°C for additional study. The support material used in the reactor consisted of 6-mm porcelain saddles. Cr(VI) concentrations in the liquid ranged from 140–750 mg L^{-1}. Cr(VI)-reducing bacteria were the dominant population with Cr(VI)-reduction rates of approximately 0.71 mg g^{-1} dry cells h^{-1} achieved at Cr(VI) concentrations of 750 mg L^{-1}. These results indicate a potential for selecting and maintaining indigenous Cr(VI)-reducers in a bioreactor for Cr(VI)-remediation of groundwater or soil wash effluents.

Keywords: Cr(VI)-reducing bacteria; bioprocess; anaerobic; soil; selection strategy

Introduction

Hexavalent chromium [Cr(VI)], in the forms of chromate (CrO_4^{-2}) and dichromate ($Cr_2O_7^{-2}$) often enters the environment through anthropogenic activity and is regarded as a highly toxic pollutant. The common use of Cr(VI) in such industrial applications as anticorrosive agents, rust proofing, metal plating, and the manufacture of dyes and inks has contributed to its wide distribution in the environment [15].

As an environmental pollutant, Cr(VI) represents a considerable health risk [20]. Its toxicity has been well established in man as well as in animals and plants [10,12,13,18]. Human exposure to Cr(VI) can result in ulceration of skin, eyes and mucus membranes, as well as mutagenic and carcinogenic effects [20]. Cr(VI) has been projected to continue to be an environmental problem in the future if remediation action is not addressed [21].

Upon reduction of Cr(VI) to trivalent chromium [Cr(III)], the toxic potential is significantly decreased for humans, animals and plants due to a decrease in the solubility and bioavailability of Cr(III). Discoveries of bacteria capable of the direct reduction of Cr(VI) to Cr(III) [8,9,16,19] have suggested the possibility of utilizing a bioprocess employing Cr(VI)-reducing bacteria for the remediation of Cr(VI)-contaminated sites. Treatment of the Cr(VI)-contaminated effluents from soil washing and pump-and-treat technologies may be possible with a bioprocess. Aerobic bioprocesses incorporating pure cultures of Cr(VI)-reducing bacteria have been developed for some industrial and soil wash effluents [1,2,5,7]. *Enterobacter cloacae* HO1 has been reported to use Cr(VI) as a terminal electron acceptor during anaerobic growth [14], and has been incorporated into a bioreactor for Cr(VI)-reduction of industrial effluents [6].

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A bioprocess for the reduction of Cr(VI) from contaminated soils and/or ground water could not rely on sterilization of the influent to be treated due to economic constraints. Application of a pure culture for Cr(VI)-reduction in a nonsterile environment may lead to the establishment of contaminant organisms as a significant portion of the bioreactor population or even the dominant population. The consequence of this scenario may result in a Cr(VI)-bioreactor with drastically decreased efficiency due to a non-optimized population of Cr(VI)-resistant soil bacteria with minimal or no Cr(VI)-reducing ability.

A strategy can be developed in which the growth and Cr(VI)-reducing ability of indigenous bacteria are selected and optimized. It has been postulated that aerobic, bacterial Cr(VI)-reduction occurs to provide a less toxic environment more suitable for microbial growth [4], however Cr(VI)-reduction and resistance are separate genetic characteristics that need not exist simultaneously in the same bacterium [3]. As an example, 9 out of 20 aerobic soil isolates, resistant to Cr(VI), were capable of greater than 30% Cr(VI)-reduction [11]. In contrast, anaerobic Cr(VI)-reducing bacteria from soils constituted 92% of bacteria capable of greater than 30% Cr(VI)-reduction [17]. Anaerobic Cr(VI)-reducing bacteria may have a growth advantage over non-reducing anaerobes due to a selection advantage afforded by Cr(VI)-utilization resulting in anaerobic respiration, Cr(VI)-resistance, or both. This paper describes an anaerobic bioprocess which incorporates and maintains a mixed culture of Cr(VI)-reducing bacteria selected from soil by creating an environment in the bioreactor that optimizes growth conditions for anaerobic Cr(VI)-reducing bacteria and establishes them as the dominant population.

Materials and methods

Bacterial enrichment

Soil from a site on the east coast of the US, contaminated with processed chromite ore tailings was collected in sterile

containers and stored at 4°C immediately after collection until needed. Cr(VI) concentration in the soil was 250 mg kg^{-1}. Soil dilutions (10^{-3} g ml^{-1}) were made using sterile phosphate buffer (19.5 ml 0.2 M $NaHPO_4$ and 30.5 ml 0.2 M Na_2HPO_4, diluted to 100 ml with deionized water) and were inoculated into sealed serum vials containing Tryptic Soy Broth, containing 2.5 g L^{-1} dextrose (TSB, Difco Laboratories, Detroit, MI, USA) and nitrogen gas (N_2) in the headspace. K_2CrO_4 was added to a final concentration of 20 mg L^{-1} of Cr(VI). Cultures were incubated at 30°C, on a gyratory shaker at 100 rpm. Samples were withdrawn periodically and analyzed for Cr(VI) concentration and cell density.

Chromium analyses

Cr(VI) concentrations in the samples were measured by clarifying via 5 min of centrifugation at $7200 \times g$, diluting the clarified solution, adding 1,5-diphenylcarbohydrazide (ChromaVer 3 Chromium Reagent Powder, Hach Chemical Company, Loveland, CO, USA), and measuring the absorbance of the mixed solution at 542 nm.

Total Cr concentrations of bioreactor effluent were measured using inductively coupled plasma emission spectroscopy (Model 3410, ARL, Valencia, CA, USA).

Bacterial measurement

Cell densities of the bacterial suspensions in bioreactor effluent and batch studies were measured as turbidity at 600 nm and correlated to dry cell weight as previously described [1].

Cr(VI)-reduction rates

Batch experiments were used to determine Cr(VI)-reduction rates at various initial Cr(VI) concentrations. These studies were performed in 165-ml sealed serum vials containing 50 ml TSB in a nitrogen atmosphere at 30°C and 100 rpm for 48 h. Growth and Cr(VI)-reduction rates were calculated from samples taken during log phase growth.

The Cr(VI)-reduction rate V_{red} (mg Cr(VI) reduced per h per g cell dry weight) was calculated using least squares fit of batch culture data to first order kinetics described by Equation 1, with V_m = maximum Cr(VI)-reduction rate; S = initial Cr(VI) concentration (mg L^{-1}); K_s = half saturation constant (mg L^{-1}); K_i = Cr inhibition constant (mg L^{-1}):

$$V_{red} = V_m S/(K_s + S)(1 + S/K_i) \quad (1)$$

Studies using bioreactors

The mixed culture was grown in TSB with 10–60 mg L^{-1} of Cr(VI) in a 1.4-L chemostat at 30°C with a dilution rate of 0.5 day^{-1}. After 250 h of operation of the chemostat, cells were harvested and added to a packed bed reactor with sterile, porcelain, 6-mm Berl saddles (Fisher Scientific, Pittsburgh, PA, USA) as the solid support. Liquid volume of the packed bed reactor was 1 L. Growth conditions were the same as above. Prior to each experiment the bioreactor was operated in batch mode for 48 h with initial Cr(VI) concentrations of 200 mg L^{-1} for the first two experiments and 400 mg L^{-1} for the third. During continuous operation Cr(VI) concentrations were maintained at 200–750 mg L^{-1} with a syringe pump that continually added Cr(VI) to the input medium at prescribed rates. Nutrients and Cr(VI) were circulated through the reactor with a peristaltic pump positioned downstream of the reactor.

Periodically the pH and Cr(VI) concentrations of the influent medium were recorded. Cr(VI), total Cr, pH and bacterial density in the effluent were obtained from the reactor effluent. The preweighed porcelain saddles were sampled after the first and third experiments to determine bacterial density of the reactor by drying them at 103°C until stable weights were achieved.

Bacterial analysis

Throughout the second and third bioreactor trials, bacterial diversity in the bioreactor was determined with Tryptic Soy agar by the spread plate method. Bacterial isolates were distinguished by biochemical analysis and colony characteristics. Isolates were also assayed for growth, in batch studies, in the presence and absence of 400 mg L^{-1} Cr(VI), and Cr(VI)-reducing ability was determined as previously described [17].

Results and discussion

Bacteria enriched from Cr(VI)-contaminated soil demonstrated potential for use in bioreactor studies, based on the estimates of the following kinetic parameters: $V_m = 67.5$ mg h^{-1} g^{-1} dry cells; $K_s = 158.3$ mg L^{-1}; $K_i = 168.9$ mg L^{-1} (Figure 1). These estimates indicate that this consortium appears to be able to utilize Cr(VI) for growth with minimal inhibitory effects from Cr. Abiotic reduction of various concentrations of Cr(VI) in TSB was negligible relative to the Cr(VI) concentrations used in these studies and was therefore not factored into the results.

Cr(VI) reduction occurred during each of three bioreactor studies with complete reduction observed at the lower concentration (Figure 2), in which the bioreactor received concentrations of Cr(VI) from 140–200 mg L^{-1}. Concentrations of Cr(VI) were increased for the second and third studies in order to determine the maximum rate of reduction, which was approximately 6.9 mg Cr(VI) L^{-1} h^{-1} (0.71 mg Cr(VI)

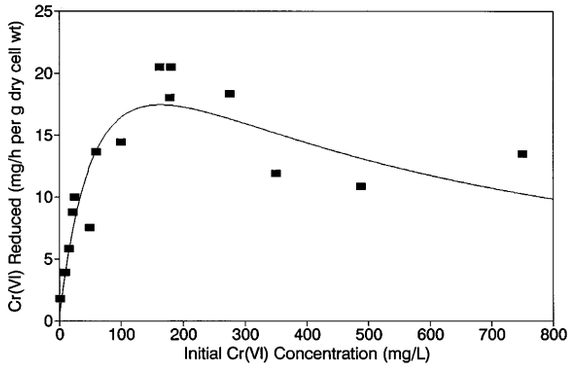

Figure 1 Cr(VI)-reduction rate of a mixed culture as a function of Cr(VI) concentration. The equation (see text) was used to estimate kinetic parameters. Data points represent rates calculated during log phase growth in batch cultures conducted with TSB at 30°C.

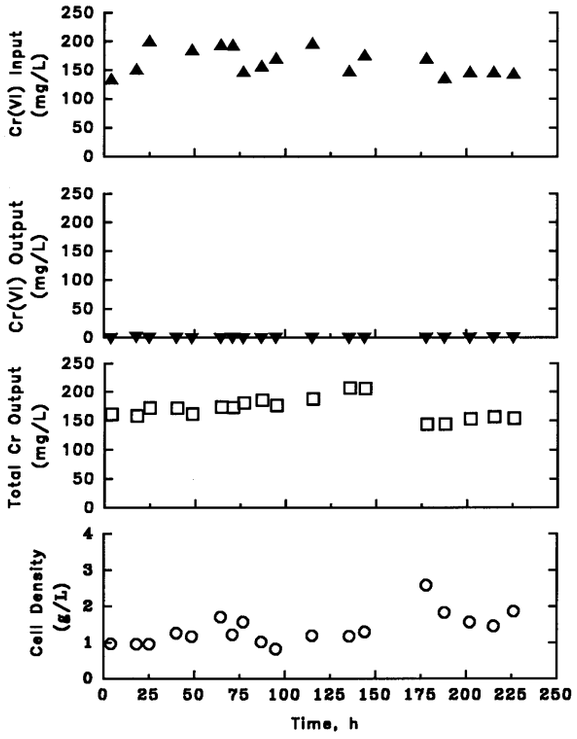

Figure 2 Operation of a continuous anaerobic bioreactor at 30°C with a retention time of 48 h for Cr(VI)-reduction incorporating Cr(VI)-reducing facultative anaerobes from Cr(VI)-contaminated soil.

g^{-1} dry cells h^{-1}) and occurred at a Cr(VI) input concentration of 750 mg L^{-1}. Bacterial growth and Cr(VI) reduction occurred at input concentrations as high as 750 mg Cr(VI) L^{-1} (data not shown), which is corroborated by values obtained from the batch kinetic study (Figure 1). Biomass density in the bioreactor was lower than anticipated, which may have been due to inhibition from prolonged exposure to Cr(III) as described previously [3]

Total chromium concentrations measured in the bioreactor effluent agreed with those of Cr(VI) concentrations in the input during the first bioreactor study (Figure 2). The absence of Cr(VI) in the effluent along with the complete recovery of total chromium indicates complete Cr(VI) reduction to Cr(III) by the bacterial consortium. The presence of Cr(III) in the reactor supernatant indicates suspension of the Cr(III), which may be due to Cr(III) complexation with the organics of the nutrient medium. With bioreactor studies at higher inputs, total chromium concentrations in the effluent were consistent with Cr(VI) concentrations in the input for the first 100 h (two retention times). Total chromium concentrations then decreased by approximately 20%. The decrease in total chromium density occurred concomitantly with decreased bacterial density in the reactor effluent (data not shown). Chromium adsorption to nonviable cells may have occurred, decreasing total chromium concentrations in the effluent.

The pH of the influent to the reactor was 7.0 throughout the study while the effluent pH ranged from 6.8–6.2 with an average of 6.45.

Evaluation of the dominant bacterial strains revealed that one isolate, LWS1, (presumptive identification as *Bacillus* sp) predominated during the early stages of continuous operation while the population shifted after 100 h operation to two other isolates, SYS1 and SWS1, (presumptive identification as *Micrococcus* sp and *Rhodococcus* sp respectively) emerging as the dominant population. Isolates SYS1 and SWS1 repeatedly emerged as the dominant organisms at the termination of each reactor experiment. Isolate LWS1 demonstrated better growth when incubated anaerobically without Cr(VI) while isolates SYS1 and SWS1 (data not shown) grew better with Cr(VI) in anaerobic TSB (Figure 3). Isolate LWS1, which exhibited better Cr(VI)-reducing ability relative to the other strains and better growth in the absence of Cr(VI) (Figure 3), emerged as the dominant organism during batch growth in the bioreactor as Cr(VI) concentrations decreased. However as Cr(VI)

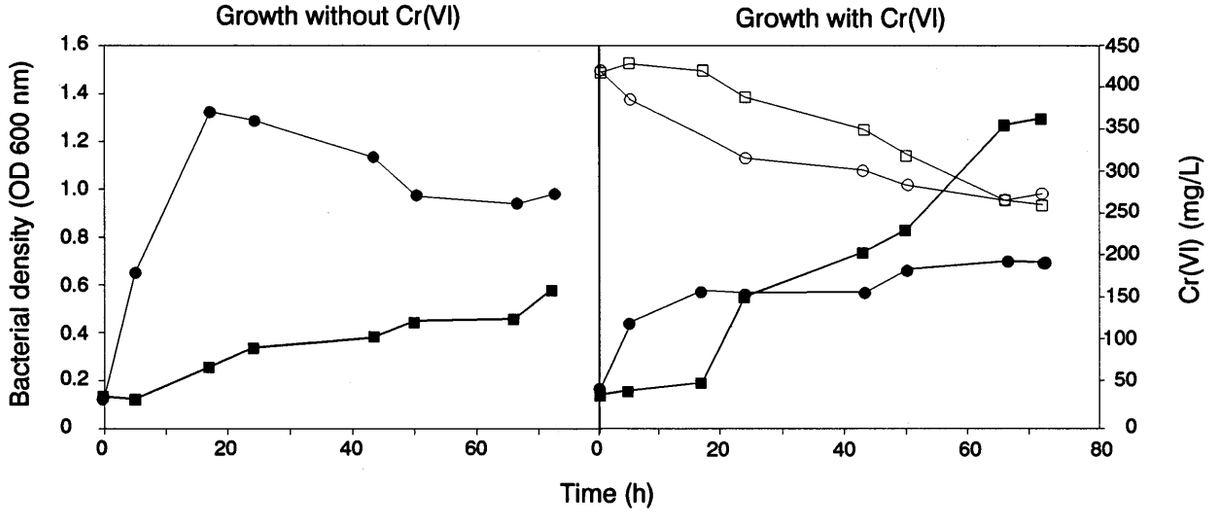

Figure 3 Anaerobic growth and Cr(VI)-reduction in TSB at 30°C of two predominant bacterial strains isolated from the anaerobic Cr(VI)-reducing bioreactor. Growth was monitored in batch as turbidity for two bioreactor isolates: isolate LWS1, ●; isolate SYS1, ■; in the presence and absence of Cr(VI). Cr(VI)-reduction occurred during growth of reactor isolates (LWS1, ○; SYS1, □).

concentrations increased when continuous operation was initiated, isolates SWS1 and SYS1 became the dominant organisms, apparently due to higher Cr(VI)-resistance and improved growth in the presence of Cr(VI), relative to strain LWS1. The bacteria demonstrating the most resistance to Cr(VI) and the ability to utilize Cr(VI) for growth were favored in this selection strategy. The increased growth rates of isolates SWS1 and SYS1 appear to have compensated for their less efficient Cr(VI)-reducing ability since no change in the Cr(VI)-reduction rates were evident during bioreactor operations even though population dominance shifted from isolate LWS1 to isolates SWS1 and SYS1 after 100 h of operation (Figure 2).

Population dominance in the bioreactor by Cr(VI)-reducing bacteria demonstrates the successful employment of a strategy to provide an environment for Cr(VI)-reducing facultative anaerobes. The absence of Cr(VI)-resistant, non-reducing anaerobes suggests that they were outcompeted by Cr(VI)-resistant strains capable of utilizing Cr(VI) and should not pose a problem of decreased bioreactor efficiency. This work should simplify the use of biological treatment of Cr(VI)-contaminated waste streams by eliminating the need to use pure cultures of bacteria. In this way bacterial contamination is not a problem, since survival in the bioreactor is in favor of the Cr(VI)-reducers. Another advantage of this selection strategy is the potential, continual improvement of Cr(VI)-reducing efficiency as bacteria that are more suited for Cr(VI)-reduction enter the bioreactor and establish dominance. In addition, another benefit of utilizing indigenous bacteria to treat Cr(VI)-contaminated groundwater or soil wash effluents would be resistance many of the bacteria may have developed to other contaminants in the environment.

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