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Reduction of Hexavalent Chromium by *Escherichia coli* ATCC 33456 in Batch and Continuous Cultures

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Toxic hexavalent chromium, Cr(VI), was reduced to a less toxic trivalent chromium form by *E. coli* ATCC 33456. The suitable electron donor for Cr(VI) reduction was glucose. *E. coli* ATCC 33456 was more resistant to metal cations than other reported Cr(VI) reducing microorganisms. Cell growth was inhibited by the presence of Cr(VI) in a liquid medium and Cr(VI) reduction accompanied cell growth. With a hydraulic retention time of 20 h, Cr(VI) reducing efficiency was 100% to 84% when Cr(VI) concentration in the influent was in the range of 10 to 40 mg L^{-1}. Specific rate of Cr(VI) reduction was 2.41 mg Cr(VI) g DCW^{-1} h^{-1} when 40 mg L^{-1} of Cr(VI) influent was used. This result suggested the potential application of *E. coli* ATCC 33456 for the detoxification of Cr(VI) in Cr(VI) contaminated wastewater.

Key words: Cr(VI), Cr(VI) reduction, batch culture, continuous culture, *Escherichia coli* ATCC 33456

Hexavalent chromium, Cr(VI), is widely used in metal finishing industries, petroleum refining, leather tanning, iron and steel industries, inorganic chemical production, textile manufacturing and pulp production (19). Cr(VI) compounds are highly water-soluble and toxic, thus necessitating the treatment of wastewater, soils, and sediments. However, trivalent chromium, Cr(III), compounds are less toxic, and at neutral pH, they readily form insoluble chromium hydroxides that can be easily removed from environments (23). Recently, many researchers discovered the potential of using Cr(VI) reducing strains for detoxifying Cr(VI) contaminated environments. Results which indicate biological reduction of Cr(VI) occurs near neutral pH and over a moderate temperature range imply that no costly chemical reagents and extensive energy inputs are required for the biological reduction of Cr(VI) (3, 4, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22). However, the requirements for suitable electron donors, and inhibition by heavy metals, which occur as co-contaminants, may render the biological treatment of industrial effluents less efficient (17).

Here, we report the ability of *E. coli* ATCC 33456 to reduce Cr(VI) in batch culture and continuous culture, and factors that may be important in the treatment of Cr(VI) waste with this organism.

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Materials and Methods

Bacterial strain and culture conditions

E. coli ATCC 33456 was purchased from the American Type Culture Collection (20). Cells were grown in an appropriate culture medium at 37°C and agitated at 200 rpm. After centrifugation at 6,000 *g* at 4°C for 20 min, the resulting cell pellet was washed and resuspended in 50 mM phosphate buffer (pH 7.0) for Cr(VI) reduction study. For the selection of culture medium ATCC 57 (K_2HPO_4 7.0 g, KH_2PO_4 3.0 g, glycerol 5.0 g, $(NH_4)_2SO_4$ 1.5 g, L-lysine 0.1 g, $MgSO_4$ 0.1 g, $CaCl_2$ 0.01 g, and $FeSO_4$ $7H_2O$ 0.5 mg in 1 l of distilled water), ATCC 60 (K_2HPO_4 7.0 g, KH_2PO_4 3.0 g, glycerol 2 g, $(NH_4)_2SO_4$ 0.1 g, casein hydrolysate 2.0 g, sodium citrate 0.4 g, and $MgSO_4 \cdot 7H_2O$ 0.1 g in 1 l of distilled water), nutrient broth, and Luria-Bertani broth were compared. In order to understand the effect of added Cr(VI) on growth, ATCC 57 medium containing 0~50 mg Cr(VI) l^{-1} was inoculated with a culture (5%) of *E. coli* ATCC 33456 which was grown overnight.

Continuous culture

Continuous culture experiments were conducted using a 3 l jar fermenter (Korea Fermenter Co.) with a working volume of 1.44 l. The agitation speed was set at 400 rpm, and pH was controlled to 7.0 using 1 N NaOH and 1 N HCl. Filtered compressed air was supplied at a fixed rate of 1 vvm and dissolved oxygen of the culture was monitored without a control. After overnight batch culture in ATCC

57 medium, the appropriate medium containing various Cr(VI) concentrations (10, 20, 40 mg l^{-1}), and glycerol concentration (10 and 20 g l^{-1}) was fed from 20 l carboy reservoirs using a peristaltic pump (Chemap, Switzerland) at a dilution rate of 0.05 h^{-1}. The overflow from the reactor was collected and assayed for the remaining Cr(VI), glycerol concentration, and cell mass.

Chromium reduction tests

The reaction mixture containing Cr(VI) solution (5 mg Cr l^{-1} in 50 mM KH_2PO_4, pH 7.0), electron donor, and late exponential phase cells grown in ATCC 57 medium was incubated on a continuous shaker at 37°C and 200 rpm for 1 to 4 h. The compounds tested for their effect on Cr(VI) reduction of pre-grown cells include 13 possible electron donors and 8 metal cations. All tests were duplicated and conducted with a cell density of approximately 1.0 OD ml^{-1} at 600 nm. For all reactions, except electron donor usability, 100 mM glucose was added as an electron donor.

Analytical method

Samples were centrifuged at 6000 *g* at 4°C for 20 min to remove cells. The supernatant was analyzed to detect any remaining chromium. Cr(VI) concentration was determined colorimetrically using a spectrophotometer (Ultrospec III, Pharmacia, USA) at 540 nm by reaction with 1,5-diphenylcarbazide in acid solution (1). The optical density of the bacterial cell suspension was determined by turbidometric measurement in a spectrophotometer at 600 nm and correlated to dry cell weight (1).

Glycerol concentration was determined by reaction with periodate (65 mg of $NaIO_4$, 7.7 g of ammonium acetate, and 10 ml of glacial acetic acid in 100 ml of distilled water) and acetylacetate (247.5 ml of isopropanol, and 2.5 ml of acetylacetate). The original cell free samples were diluted to give a final concentration of glycerol in the 0-100 mg l^{-1} range. 0.5 ml of the diluted sample, 1 ml of periodate reagent, and 2.5 ml of acetylacetate reagent were pipetted into dry test tubes. After mixing thoroughly, the solution was heated in a 45°C water bath for 20 min. The absorbance of each sample was measured at 410 nm.

Results

Effect of culture media

Cr(VI) reduction by *E. coli* ATCC 33456 was evaluated within a pH range of 3-8 and at a temperature range of 40-60°C. The optimum pH was 7.0 and the optimum temperature was 37°C (data not shown). To determine a suitable culture medium that supports rapid cell growth and good Cr(VI) reduction, 4 different media such as ATCC 57, ATCC 60, nutrient broth, and LB were compared. As shown in Table 1, ATCC 57 medium showed the highest growth rate and specific Cr(VI) reducing activity.

Table 1. Growth parameters of *E. coli* ATCC 33456

Medium	Specific growth rate (h^{-1})	Specific rate of Cr(VI) reduction (mg g^{-1} h^{-1})
ATCC 60	0.37	0.31
ATCC 57	1.15	0.70
NB	0.69	0.33
LB	0.68	0.66

Electron donor usability and metal ion effect

Reduction of toxic Cr(VI) to less toxic Cr(III) is an electron consuming process. To determine the effective electron donor, 100 mM of various organic substances was added to the reaction mixture. The cells showed some Cr(VI) reducing activity even in the absence of any added electron donor. We presumed that endogenous electron sources served as electron donors. Glucose was the best electron donor for Cr(VI) reducing activity. Except for succinate and casitone, most organic substrates slightly stimulated the rate of Cr(VI) reducing activity (Table 2). There was no measurable increase in cell density. No measurable reduction of Cr(VI) was detected with either the cell-free or heat-killed cell control. This result suggested that it was an enzymatic reaction and that there was no significant chemical Cr(VI) reduction or non-specific adsorption onto cells.

However, several metals can inhibit enzyme reaction. To determine the effect of metal ions on the reduction of Cr(VI) by this strain, metal cations, which could be found together with Cr(VI), were tested. The degree of inhibition caused by the tested metal cations was Hg^{2+}>Ag^{2+} >Mn^{2+}>Zn^{2+}>Pb^{2+}>Cd^{2}>Ca^{2+}>Mg^{2+}. Though Mg^{2+} increased

Table 2. Effects of organic substrate addition on Cr(VI) reduction by *E. coli* ATCC 33456

Organic substrate	Specific rate of Cr(VI) reduction (mg g^{-1} h^{-1})
Acetate	0.35
Citrate	0.40
Lactate	0.36
Oxalate	0.40
Succinate	0.20
Tartrate	0.33
Glucose	0.62
Glycerol	0.39
NADH	0.36
NADPH	0.24
Casamino acid	0.36
Casitone	0.20
Tryptone	0.48
No substrate	0.22

Table 3. Effects of metal cations on Cr(VI) reduction by *E. coli* ATCC 33456

Metal (1 mM)	Relative activity (%)
None	100
Ag^{2+}	27.3
Ca^{2+}	99.3
Cd^{2+}	90.6
Hg^{2+}	0.7
Mg^{2+}	113.8
Mn^{2+}	59.9
Pb^{2+}	79.5
Zn^{2+}	68.0

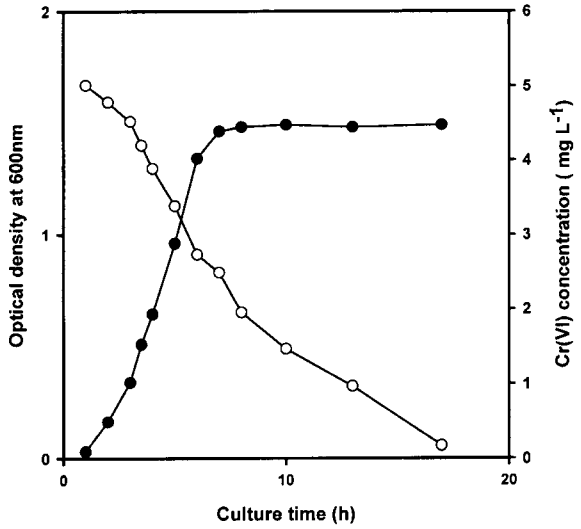

Fig. 1. Growth and reduction of Cr(VI) by *E. coli* ATCC 33456. Cell growth (●), Cr(VI) concentration (○)

Cr(VI) reducing activity, Hg^{2+} completely inhibited Cr (VI) reduction at a concentration of 1 mM (Table 3).

Cr(VI) reduction during batch culture and continuous culture

At a moderate concentration of Cr(VI) (5 mg l^{-1}), *E. coli* ATCC 33456 actively reduced Cr(VI) to Cr(III) while growing under aerobic growth conditions (Fig. 1). Since the Cr(VI) solution is yellow, a decrease in the color of the culture was used as indication of Cr(VI) reduction. As Cr(VI) concentration in the culture medium increased, the maximum specific growth rate decreased while the lag period increased (Table 4). Cells cultivated in Cr(VI) supplemented culture medium did not show any increase in Cr(VI) reducing activity.

The results of Cr(VI) reduction in continuous cultures using *E. coli* ATCC 33456 are summarized in Table 5. When 10 g l^{-1} of glycerol was used as a carbon source, Cr(VI) reduction efficiency decreased from 99.3% to 84.3% as Cr(VI) concentration of the influent increased from 10 to 40 mg l^{-1}. However the specific Cr(VI) reduction rate increased from 0.72 to 2.41 as the Cr(VI) concentration increased.

Table 4. Effect of Cr(VI) concentration on growth parameters of *E. coli* ATCC 33456 in batch culture

Cr(VI) concentration (mg l^{-1})	Specific growth rate (h^{-1})	Lag period (h)
0	1.15	0
5	0.52	2
10	0.46	8
15	0.41	15
20	0.31	24
30	0.28	32
50	0.15	41

Discussion

Cr(VI) reducing strains can utilize a variety of organic compounds as electron donors in the process of Cr(VI) reduction. The majority of known electron donors are low molecular weight carbohydrates, amino acids, and fatty acids (23). Glucose was used as an electron donor in most Cr(VI) reducing strains (23) and tryptone is known to be an effective electron donor in *Enterobacter cloacae* (16). By choosing an appropriate electron donor, it is conceivable that a new microbial method may be developed for the detoxification of Cr(VI) in contaminated wastewater. *E. coli* ATCC 33456 showed high activity when glucose and tryptone were used as electron donors (Table 2).

In developing a biological treatment, however, the fact that Cr(VI) bearing wastewater from industrial processes generally contains a variety of heavy metal cations must be considered. Fortunately, when metal cations were tested at a concentration of 1 mM, they only moderately inhibited Cr(VI) reduction by *E. coli* ATCC 33456 except Ag^{2+} and Hg^{2+} (Table 3). This result is similar to the strong inhibition of Cr(VI) reduction by Ag^{2+} and Hg^{2+} in *Pseudomonas putida* (8), and 11 metals (0.1 mM) possessed slight effects on Cr(VI) reduction by *Desulfovibrio vulgaris* (14). *E. coli* ATCC 33456 might be more useful than *Enterobacter cloacae* in that the latter was completely inhibited by low concentrations of Hg^{2+}(1 μM), Cu^{2+}(390 μM), Mn^{2+}(480 μM), and Zn^{2+}(0.3 μM) (5).

Highly soluble Cr(VI) compounds have been shown to induce frame-shift mutation and, to a great extent, base-pair substitutions both at G-C and A-T base pairs in bacterial cells. It has also been proposed that bacterial SOS functions can repair DNA damage produced by Cr(VI) (2). A decrease in cell growth and increase in the lag period by Cr(VI) addition (Table 4) may be due to an increased time for adaptation of DNA repair during exposure to Cr(VI) in the medium.

Using a continuous culture, the specific rate of Cr(VI) reduction of 2.41 mg Cr(VI)/g DCW/h was obtained when 40 mg l^{-1} of Cr(VI) influent was used (Table 5). This value is relatively higher than previously reported results. Microorganism of leachate reduced 1.476 mg g^{-1} h^{-1} when 50 mg l^{-1} of Cr(VI) influent was used (9), and

Table 5. Results of continuous culture using ATCC 57 medium

Cr(VI) (mg l^{-1})		Cr(VI) reduction (%)	Glycerol (g l^{-1})		Glycerol consumption (%)	Dry cell weight (g l^{-1})	Specific rate of Cr(VI) reduction (mg g^{-1} h^{-1})
Input	Output		Input	Output			
10	0.07	99.3	10	0.03	99.7	0.69	0.72
20	0.45	97.7	10	0.02	99.8	0.73	1.35
40	6.27	84.3	10	0.04	99.6	0.70	2.41
10	0.00	100	20	2.17	89.2	0.67	0.74
20	0.01	100	20	0.97	95.2	0.72	1.39
40	4.03	89.9	20	0.86	95.7	0.78	2.31

Pseudomonas sp. reduced 0.82 mg g^{-1} h^{-1} when 34 mg l^{-1} of Cr(VI) influent was fed (3). In summary, the results of higher Cr(VI) reduction rates at higher Cr(VI) concentrations (Table 5) indicates the possible use of *E. coli* ATCC 33456 for aerobic reduction of Cr(VI) found in industrial wastewater.

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