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Optimization of cultural conditions of *Arthrobacter* sp. Sphe3 for growth-associated chromate(VI) reduction in free and immobilized cell systems

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HIGHLIGHTS

- *Arthrobacter* sp. Sphe3 is used for a first time in the bioreduction of Cr(VI) to Cr(III).
- *Arthrobacter* sp. Sphe3 was able to tolerate extreme high Cr(VI) concentrations.
- Glucose improved significantly the reduction ability of the cells.
- Additives and agitation speed improved the efficiency and stability of beads.

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ABSTRACT

The current study aimed to characterize *Arthrobacter* sp. Sphe3 ability to reduce Cr(VI) in suspended cell cultures as well as in immobilized form using Ca-alginate beads. Adaptation studies in the presence of 5 mg L^{-1} Cr(VI) showed a significant increase in specific growth rate from 0.25 to 0.3 h^{-1} and bioremoval percentage from 64% to 94% ($p < 0.05$), whereas *Arthrobacter* sp. Sphe3 could tolerate up to 50 mg L^{-1} Cr(VI). Optimization of culture conditions resulted in complete reduction of 45 mg L^{-1} Cr(VI) at 30 °C, pH 8 and 10 g L^{-1} of glucose. High glucose concentrations helped at reducing (80 ± 2.4)% of initial 100 mg L^{-1} Cr(VI), whereas the bacterial strain could tolerate 850 mg L^{-1} Cr(VI). Cr(III) formation was first evidenced by the appearance of a green insoluble precipitate in the medium. Cell biomass was successfully immobilized in Ca-alginate beads that were evaluated for their stability. Cell release was sharply decreased when 4% Na-alginate was used under non-shaking conditions. Biotransformation efficiency was enhanced when 25–50 mg cells mL^{-1} Na-alginate from the exponential growth phase were collected and co-encapsulated with either 1% glucose and 0.5% $(NH_4)_2SO_4$, or 1% LB medium. Immobilized biocatalyst could be reused up to 6 continuous cycles in the presence of 10 mg L^{-1} Cr(VI), but its performance was lowered at higher metal concentrations comparing with free cells that significantly maintained their reducing ability up to 300 mg L^{-1} Cr(VI).

1. Introduction

Chromium is one of the most widely used metals in industry such as leather tanning, electroplating and paints manufacturing resulting in large quantities being discharged into the environment (Bhattacharya and Gupta, 2013). Cr(VI) exists in solution as CrO_4^{-2} and due to its structural similarity with SO_4^{-2} it can easily penetrate the cell membrane causing various acute and chronic diseases. In contrast Cr(III) cannot readily enter the cell and therefore is not considered as carcinogenic (Field et al., 2013).

Biological reduction of Cr(VI) using indigenous microorganisms offer a new cost-effective and environmentally compatible technology. A wide variety of bacteria in diverse locations, including natural ecosystems, industrial and municipal waste are capable of reducing Cr(VI) (Bachate et al., 2013; Bennett et al., 2013). Immobilized and suspended cell systems are frequently used for the bioremediation of wastewater (Chatterjee et al., 2011; Tekerlekopoulou et al., 2013). Each system offers several advantages. The advantage of free cells systems is their simple operation and control. On the other hand, use of immobilized cells is also attractive mainly due to resistance to toxic shock loading, no necessity of sludge separation unit and high biomass loading (Kosseva, 2011; Shetty et al., 2012).

The objective of the present study was to determine the ability of *Arthrobacter* sp. Sphe3 to reduce high concentration of Cr(VI) to Cr(III) in free and immobilized cell systems in Ca-alginate matrix. Therefore, a strategy was developed in which the growth and

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Cr(VI)-reducing ability of the bacterium was optimized, by selecting the proper culture conditions such as carbon source, time course, pH and temperature. In an attempt to explore biomass reusability as a biocatalyst, additional studies with encapsulated cells in Ca-alginate matrix were carried out. Parameters such as bacteria physiological state, the weight of biomass entrapped, capsule rigidity as well as the effect of some additives were also examined for optimization of the immobilized system.

2. Materials and methods

2.1. Microorganism and growth medium

The bacterial strain *Arthrobacter* sp. Sphe3 used in this study was isolated from a creosote polluted site (Kallimanis et al., 2009). Bioreduction experiments were carried out in shaking conical flasks (150 rpm) in minimal salts medium M9 (6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl, 1 mL L^{-1} of 1 M $MgSO_4{\cdot}7H_2O$, 1 mL L^{-1} of 0.1 M $CaCl_2$) in the presence of 1 g L^{-1} glucose as a carbon source at 30 °C.

2.2. Acclimation and bioreduction experiments in suspended cell cultures

Cells were grown in LB media (1% tryptone, 0.5% yeast extract and 0.5% NaCl) at 30 °C. Chromium(VI) was periodically added in increments of 5 mg L^{-1} in a series of 250 mL flasks.

After acclimation, bioreduction experiments were carried out in the presence of various Cr(VI) concentrations from 5 to 500 mg L^{-1}. The specific growth rates were estimated by the slope of the exponential phase of each growth curve in every concentration of Cr(VI) investigated in this work. Control experiments without cells, as well as in the presence of dead cells were also carried out to examine if any abiotic transformation of Cr(VI) or interaction with cell surface components occur.

2.3. Optimization of Cr(VI) bioreduction in suspended cell cultures

The effect of pH (4–8), temperature (25–40 °C) and various organic compounds at 1 g L^{-1} (glucose, sucrose, lactose, maltose, peptone, tryptone and glycine) on Cr(VI) bioreduction at 20 mg L^{-1} using *Arthrobacter* sp. Sphe3 was investigated. The experiments were conducted by keeping all parameters constant, except the one that had to be examined at each experiment.

2.4. Entrapment of bacterial cells in calcium alginate beads

An exponentially-grown culture of 100 mL in M9 medium was collected and centrifuged. The cell pellet was washed 3 times with 0.9% sterile NaCl solution and resuspended in 10 mL of sodium alginate solution. The mixture was then dropped with an insulin syringe into a gently stirred 3.5% solution of $CaCl_2$ at a volumetric ratio of 1:5. A spontaneous cross-linking reaction produced spherical hydrogel beads of calcium alginate, which remained overnight at 30 °C. After stabilization, the beads were washed with 0.9% v/v NaCl (twice) and transferred to Tris–HCl buffer (pH 7.0) containing 10 mg L^{-1} Cr(VI).

2.5. Optimization of immobilization technique

Beads rigidity was examined using various concentrations of Na-alginate from 1–4%, whereas the conical flasks were either non-shaking or in circular motion in the incubator.

In order to overcome immobilization stress, various additives were also incorporated within the capsule. For this, the cell pellet was mixed with (i) sodium alginate solution only (control), (ii) sodium alginate solution with 1% glucose, (iii) sodium alginate solution, 1% glucose and 0.5% $(NH_4)_2SO_4$ and (iv) sodium alginate solution with 1% Luria Bertani medium before addition in $CaCl_2$ solution.

The influence of bacteria growing state was also examined using either cells collected in exponential (6 h) or stationary phase (15 h). The effect of biomass loading inside the beads at 10, 25 and 50 mg of cells L^{-1} Na-alginate was also examined.

The recycling stability of the beads formed was tested by collecting the coated cells by filtration at the end of each batch, rinsing with Tris–HCl buffer and then added to the next reaction cycle.

2.6. Reagents

Stock Cr(VI) solution (500 mg L^{-1}) was prepared by dissolving 141.4 mg of 99.5% $K_2Cr_2O_7$ (Sigma Chemical Co.), in 100 ml of deionized water. 1,5-Diphenylcarbazide solution (Fluka Chemicals) was prepared by dissolving 250 mg in 50 mL of HPLC-grade acetone and stored in a brown bottle.

2.7. Analytical methods

Cr(VI) concentration was determined by the 1,5-diphenylcarbazide colorimetric method (Shimadzu UV–Vis spectrophotometer, Bestech, Irvine, CA) at 540 nm (Snell and Snell, 1959). Cr(VI) standard curve was prepared in the range 0.3–2 mg L^{-1}. The detection limit was found to be 0.05 mg L^{-1}. Total chromium was determined by oxidizing Cr(III) with $KMnO_4$, followed by Cr(VI) analysis. Cr(III) content in liquid medium was obtained by subtracting the content of Cr(VI) from that of total chromium and expressed as:

$$\%\ \mathrm{Cr(III)} = \frac{\text{Concentration of Cr(III) formed in the supernatant}}{\text{Initial concentration of Cr(VI) added in the medium}} \cdot 100\% \quad (1)$$

The cell density of liquid culture was determined as the optical density (OD) at 600 nm.

2.8. Statistical analysis

Descriptive statistics was used to quantitatively describe all results in this study. For every data group, mean, standard error and standard deviation values were calculated. Statistical significance of the data was evaluated by ANOVA test ($p \leqslant 0.05$). All results were processed using Microsoft Excel, 2003.

3. Results and discussion

Our results showed no abiotic physicochemical Cr(VI) removal since its concentration remained constant in the absence of cells. In addition, at pH 6.5 (pH of the medium) no significant sorption on dead cells surface could be observed due to the divalent form of chromate anions ($HCrO_4^-$, $Cr_2O_7^{-2}$) and the negative surface charge of the biosorbent (Pehlivan et al., 2012).

3.1. Acclimation of Arthrobacter sp. Sphe3 cells

Continuous cultures with subsequent increments of Cr(VI) concentration showed that *Arthrobacter* sp. Sphe3 could tolerate hexavalent chromium up to 50 mg L^{-1} in the presence of 1 g L^{-1} glucose. The growth profile of *Arthrobacter* sp. Sphe3 sequentially changed, no lag phase was evidenced and an increase in specific growth rate from 0.25 ± 0.02 to 0.3 ± 0.03 h^{-1} and Cr(VI) bioremoval from 64 ± 3.4% to 94 ± 12% at 5 mg L^{-1} was observed ($p < 0.05$).