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Full Length Article

Improvement of simultaneous Cr(VI) and phenol removal by an immobilised bacterial consortium and characterisation of biodegradation products

Ornella M. Ontañon[a,*], Paola S. González[a], Germán G. Barros[b], Elizabeth Agostini[a]

[a] Departamento de Biología Molecular, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Argentina
[b] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Argentina

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ABSTRACT

Microbial bioremediation emerged some decades ago as an eco-friendly technology to restore polluted sites. Traditionally, the search for microorganisms suitable for bioremediation has been based on the selection of isolated strains able to remove a specific type of pollutant. However, this strategy has now become obsolete, since co-pollution is a global reality. Thus, current studies attempt to find bacterial cultures capable of coping with a mixture of organic and inorganic compounds. In this sense, the bacterial consortium SFC 500-1 has demonstrated efficiency for Cr(VI) and phenol removal, both of which are found in many industrial wastewaters. In the present study, the ability of SFC 500-1 for simultaneous removal was improved through its entrapment in a Ca-alginate matrix. This strategy led to an increased removal of Cr(VI), which was partially reduced to Cr(III). Immobilised cells were able to tolerate and degrade phenol up to 1,500 mg/l at high rates, forming catechol and *cis,cis*-muconate as oxidation intermediates. Successful removal potential through 5 cycles of reuse, as well as after long-term storage, was another important advantage of the immobilised consortium. These characteristics make SFC 500-1 an interesting system for potential application in the biotreatment of co-polluted effluents.

Introduction

Cr(VI) and phenol are toxic chemicals released into the environment through wastewaters from a large number of industries, including tanning, oil refineries, paint manufacturing and chemical plants [1,2]. The severity of the pollution with Cr(VI) and phenol lies in their toxicity for living beings and the difficulties of their removal. For this reason, International organisations for environmental protection have set permissible limits, in the order of some micrograms per liter, for their discharge into the environment [3]. Nevertheless, phenol and its derivatives have been found at concentrations over 1,000 mg/l in diverse industrial effluents, which are also known to contain prohibited levels of Cr (VI) [4,5]. Therefore, developing efficient methods for simultaneous detoxification of these chemicals is a priority challenge in environmental sciences. Although bioremediation is a cost-effective and eco-friendly technique, finding microorganisms able to cope with mixtures of contaminants has not been an easy task. Moreover, few environmental bacteria have proven to be efficient in jointly removing Cr(VI) and phenols [3,4,6,7].

The presence of heavy metals is known to affect the biodegradation of organic compounds and *vice versa* through the impact they have on both the physiology and ecology of microorganisms [3]. The main limitations of simultaneous Cr(VI) and phenol bioremediation are related to the cellular toxicity caused by combining high concentrations of these contaminants, as well as the harmful effect of Cr(VI) on phenol oxidising enzymes [6–8]. Moreover, some bacterial strains are unable to reduce Cr(VI) to Cr(III) employing phenol as electron donor, rendering it necessary to supplement the culture media with high concentrations of nutrient sources. This last strategy often favours Cr(VI) reduction but adversely affects phenol degradation [3].

Immobilisation is a promising alternative to solve some of these bioremediation drawbacks. Bacterial entrapment protects cells from the toxic effects of hazardous compounds and increases their survival and metabolic activity in bioremediation systems [1,9,10]. In addition, among the most important advantages of using immobilised rather than free bacteria, is the avoidance of secondary pollution due to difficulties in handling and cell separation, as well as their better adaptation to diverse polluted environments and the possibility of reutilisation [11,12]. Various methods of active or passive immobilisation have been described,

* Corresponding author at: Departamento de Biología Molecular, FCEFQyN, UNRC, Ruta 36 Km 601, CP 5800 Río Cuarto (Cba) Argentina.
E-mail address: oontanon@exa.unrc.edu.ar (O.M. Ontañon).

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and active entrapment within polymeric gel matrices has often been successful for bioremediation applications [13]. Alginate is one of the most frequently used matrices for this purpose. It is a highly porous, biodegradable polymer that provides rapid and simple aqueous immobilisation [14] and for this reason alginate entrapped cells have been extensively used for bioremediation of organic and inorganic contaminants [14–16].

The bacterial consortium SFC 500-1, isolated from polluted sediments belonging to a tannery discharge channel, was able to simultaneously remove high concentrations of Cr(VI) and phenol, but this effectiveness significantly decreased in media with a low content of organic matter [17]. Based on this background, the objective of the present study was to evaluate the immobilisation of this bacterial consortium in calcium alginate (Ca-alginate) as a means of improving the simultaneous removal potential of SFC 500-1 and overcome its limitations. Cell load, tolerance and removal capabilities have been analysed and compared between free and immobilised bacteria. Products of Cr(VI) and phenol biotransformation were also explored, as well as bead stability and their reuse over time.

Materials and methods

Microorganisms and culture conditions

The consortium SFC 500-1, comprising *Acinetobacter guillouiae* SFC 500-1A and *Bacillus* sp. SFC 500-1E (GenBank accession numbers JX198426 and JQ701739, respectively), previously isolated from tannery sediments and characterised in our laboratory, was employed in the present study [7,17]. The maintenance of SFC 500-1 was carried out on agar plates with NM medium, containing (g/l): tryptone 5.0; yeast extract, 3.0; $CaCl_2$, 0.5 [18]. All plates contained Cr(VI) 20 mg/l and phenol 300 mg/l. A bacterial overnight culture grown at 28 °C in NM medium was employed to inoculate NM medium containing Cr(VI) 2.5 mg/l and phenol 100 mg/l, which was also incubated for 20–24 h in a rotary shaker (150 rpm). The pre-adapted cells were centrifuged (10,000 rpm, 10 min), washed and suspended in physiological solution (NaCl 0.9%), and used for bioremediation assays under free or immobilised conditions.

Cell immobilisation

The consortium SFC 500-1 was immobilised in Ca-alginate as previously described by Ravichandra et al. [19]. Briefly, bacterial suspensions in physiological solution containing about 6×10^9 CFU/ml were mixed with sterilised Na-alginate solution to achieve a final alginate concentration of 3%. The resulting alginate/cell mixtures were dropped into sterile 0.2 M $CaCl_2$, producing gel beads of approximately 3 mm in diameter. The beads were then hardened in fresh $CaCl_2$, washed several times with 0.9% NaCl and conserved at 4 °C until their use for removal experiments.

Determination of cellular viability under immobilised and free conditions

To count viable immobilised cells, 5 randomly selected alginate beads were suspended in 200 μl sterile 0.2 M phosphate buffer, pH 7.5 [20]. After complete dissolution of the alginate by mechanical disruption, the number of total viable cells (CFU/ml) was determined by plating serially diluted cell suspensions on NM plates. Colonies were counted on agar plates after incubation for 24 h at 28 °C. The count of viable free cells was performed with 100 μl of culture medium, following the same protocol. The colonies belonging to both strains from the consortium were distinguished on the basis of their morphological characteristics.

Cr(VI) and phenol removal employing immobilised cells

Simultaneous Cr(VI) and phenol removal by the immobilised consortium SFC 500-1 was carried out in a solution containing NaCl (0.9%) and glucose 0.3% as the reaction medium. To establish the most suitable inoculum size of immobilized bacteria, different numbers of beads were incorporated into the reaction medium, achieving three initial microorganism counts: 3.6×10^8; 7.2×10^8 and 1.5×10^9 CFU/ml. Removal of 25 mg/l Cr(VI) and 300 mg/l phenol was analysed in three successive cycles of 24 h. The most suitable concentration of cells was employed for all subsequent experiments.

The effect of increasing the initial concentration of each contaminant on simultaneous removal was analysed. Cr(VI) concentrations of 10, 25 and 50 mg/l were assayed using a fixed phenol concentration (300 mg/l). The initial concentrations of phenol were then adjusted to 100, 300, 500, 750, 1000 and 1,500 mg/l in combination with Cr(VI) 25 mg/l.

To investigate the effect of storage on the removal capability of immobilised cells, beads containing entrapped cells were stored at 4 °C. The immobilised cells were used for simultaneous removal of Cr(VI) 25 mg/l and phenol 300 mg/l after storage for 15, 30, 45, 60, 75, 90, 105 and 120 d.

The reusability of beads was also evaluated. When maximal removal was achieved, the reaction medium was replaced by a fresh medium containing the same initial concentration of both contaminants [Cr(VI) 25 mg/l and phenol 300 mg/l]. Immobilized cells were reused until a significant reduction in removal capabilities was observed.

Flasks for all the experiments were incubated in a rotary shaker (120 rpm) at 28 °C. Samples were taken from the reaction medium every 2, 3, 6, 12 or 24 h for analysis of residual contaminants (Section "Analytical methods"). The cellular count inside the beads was determined at the end of each cycle. Sterile beads and reaction medium without beads were used as controls to monitor the abiotic loss of contaminants in all the experiments performed.

Removal efficiency comparison between free and immobilised cells

Removal of Cr(VI) concentrations between 10 and 50 mg/l in the presence of phenol (300 mg/l) was evaluated and compared employing the same number of free or immobilised cells. Similarly, for solutions containing phenol (100–1,500 mg/l) plus Cr(VI) (25 mg/l), phenol degradation rates and simultaneous Cr(VI) removal were analysed using free and immobilised cells. In the latter experiment, bacterial tolerance was also compared between both conditions at the end of a removal cycle.

Analytical methods

(a) Phenol analysis through colourimetric techniques

Phenol concentrations were determined using the 4-amino-antipyrine method [21]. Samples of 100 μl were mixed with 700 μl of sodium bicarbonate (pH 8), 100 μl of 4-aminoantipyrine (20.8 mM) and 100 μl of potassium ferricyanide (83.4 mM). After 5 min, absorbance was measured at 510 nm. The absorbance data were converted to phenol concentrations using a calibration curve.

(b) Phenol analysis through high-performance liquid chromatography (HPLC)

Phenol and its oxidation intermediates were detected in the reaction medium of immobilized SFC 500-1 during simultaneous removal of Cr(VI) and phenol. The experiments were carried out using NaCl 0.9% plus phenol 300 mg/l and Cr(VI) 25 mg/l as culture