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

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Highlights

- Immobilisation in Ca-alginate improved bacterial tolerance to Cr(VI) and phenol
- Efficient co-remediation of Cr(VI) and phenol by an immobilised bacterial consortium
- The entrapped bacterial consortium SFC 500-1 enzymatically reduced Cr(VI) to Cr(III)
- Catechol and *cis,cis*-muconate were the intermediary metabolites of phenol degradation
- Immobilised cells showed high removal potential after reutilisation and long storage times

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.

Keywords:

Chromium(VI), phenol, simultaneous removal, immobilisation, alginate beads

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, 7, 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, 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, 15, 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 $6X10^9$ 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, 1,000 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 to 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-aminoantipyrine 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 medium. Phenol removal was initially monitored through the 4-aminoantipyrine method. When removal was around 50, 75, 90 and 100%,

samples of the reaction medium were filtered, acidified at pH 3.5 and stored at -20°C. A control containing only phenol (300 mg/l) was also employed to evaluate the possible effect of Cr(VI) on phenol oxidation. Samples of this reaction medium were withdrawn when immobilised bacteria achieved the previously mentioned phenol removal percentages. Noninoculated medium and reaction medium of sterile beads were used as abiotic controls.

Phenol, catechol and *cis,cis*-muconate were detected and quantified in the filtrated supernatants by HPLC (Hewlett Packard model 1100 pump, Palo Alto, CA, USA). Chromatographic separation was performed on a stainless steel, C18 reversed-phase column (150 mm x 4.6 mm i.d., 5 μm particle size; Luna-Phenomenex, Torrance, CA, USA). A mixture of acetic acid and water (1:99) was used as mobile phase, which flowed at 1.0 ml/min. The wavelength of the UV detector (Hewlett Packard model 1100 programmable UV detector, Palo Alto, CA, USA) was 280 nm and a sample aliquot of 50 μl was injected. Quantification was relative to external standard curves of phenol (Merck, Darmstadt, Germany), catechol and *cis,cis*-muconate (Sigma, St. Louis, Mo.) in water. The detection limit was 0.1 mg/l for all the compounds.

(c) Cr(VI) determination

Residual Cr(VI) was measured after reaction with diphenylcarbazide (DPC) in acid solution at 540 nm, according to the technique of APHA [22] with modifications. The reaction mixture contained 500 μl sample, 500 μl 0.2N H_2SO_4 and 200 μl DPC (5 mg/l) in a final volume of 5 ml. The absorbance data were converted to Cr(VI) concentrations using a calibration curve.

Chromium speciation and accumulation

After reutilization assays, all beads were collected and digested employing phosphate buffer. Equal volumes of the supernatant obtained at the end of each removal cycle were also taken and pooled. In both fractions, Cr(VI) and Cr(III) were determined through DPC and atomic absorption spectrometry (AAS), respectively. Results were expressed as % of initial concentrations and were compared with controls containing non-inoculated beads.

Statistical analysis

All experiments were carried out in triplicate and reported data represent the average of results for each condition with their respective errors. Statistical analysis was performed using the ANOVA test, with the INFOSTAT software package (ver. 2012 E Universidad Nacional de Córdoba, Córdoba, Argentina). In all cases, $p \leq 0.05$ was regarded as statistically significant. Tukey test was used for *post-hoc* analysis.

Results and discussion

Effect of viable cells load on simultaneous Cr(VI) and phenol removal

Experiments were performed to study the effect of increasing the number of immobilised cells on simultaneous removal capabilities. Erlenmeyer flasks containing physiological solution plus glucose (0.3%), Cr(VI) 25 mg/l and phenol 300 mg/l as reaction medium were inoculated with a variable number of alginate beads to achieve initial cell concentrations of 3.6 x 10^8; 7.2 x 10^8 and 1.5 x 10^9CFU/ml. As shown in Figure 1, removal was significantly enhanced (p<0.05) when the inoculated viable cells increased. The best removal performance was obtained using an initial cell count of 1.5 x 10^9 CFU/ml. Under these conditions, phenol was completely removed throughout three reutilisation cycles of 24 h, but Cr(VI) removal diminished by about 50% between the first and third cycle.

A decrease in phenol removal over the reutilisation cycles was observed employing a smaller number of cells. Furthermore, Cr(VI) removed by 3.6 or 7.2 x 10^8 cells was significantly lower than the amount removed by 1.5 x 10^9 cells, even in the first cycle. The decreased size of the inocula reduced the potential for Cr(VI) removal almost entirely during the third cycle of reuse. There have been previous reports of a direct correlation between the number of inoculated beads and the removal of pollutants achieved by immobilised microorganisms in a polymeric matrix [23, 24]. The addition of more beads implies not only the incorporation of a higher number of viable cells but also an increasedvailability of transference sites for nutrients and pollutants into the matrix [25]. On the basis of the results and the preceding information, all subsequent experiments were performed using an initial immobilised cell concentration of 1.5 x 10^9 CFU/ml.

Effect of initial Cr(VI) concentration on simultaneous removal of both pollutants by free and immobilised cells

The effect of increasing Cr(VI) concentrations from 10 to 50 mg/l on the simultaneous removal was studied. A fixed phenol concentration of 300 mg/l was also added into the reaction medium. It was observed that Cr(VI) removal by the immobilised consortium increased when increasing the initial metal concentration, but was incomplete with any assayed condition (Figure 2). Similarly, other immobilised bacterial strains achieved better results in Cr(VI) removal upon being exposed to higher concentrations of the metal [16, 25]. This performance has been related to the diffusion of contaminants inside the matrix, which depends mostly on the concentration of the substance to be internalised. When the concentration is low, there is a limited diffusion inside the alginate beads

andhence removal is also limited. If the concentration in liquid medium is high, diffusion is favoured and the concentration inside the support increases [26].

In contrast, phenol was completely removed under all conditions assayed, although the removal rate was slower when 50 mg/l of Cr(VI) was added. These results revealed the high capacityy of this immobilised consortium to degrade a moderate phenol concentration, even in the presence of a toxic heavy metal such as Cr(VI).

Under identical conditions, free cells showed a lower remediation capability than the same number of immobilised cells, achieving a maximal Cr(VI) removal of around 8 mg/l. A similar behaviour for phenol removal was detected employing free and immobilised cells, observing a decrease in removal rate with increasing Cr(VI) concentration. However, free cells required more time than immobilised cells to achieve complete degradation. The most disruptive Cr(VI) concentration for simultaneous removal under free conditions was 50 mg/l.

Based on these results, Cr(VI) was the limiting factor for simultaneous removal. However, the immobilisation process succeeded in increasing metal removal and phenol degradation rate by SFC 500-1. Removed concentrations by alginate-immobilised bacteria of up to 19 mg/l for Cr(VI) and 300 mg/l for phenol are within the range of values detected in many industrial effluents [27, 28, 29], which widely exceed the limits set by international standards [30].

Effect of initial phenol concentration on simultaneous removal of both pollutants by immobilised and free cells

The effect of initial phenol concentration on simultaneous removal was evaluated. The immobilised consortium and free cells were exposed to phenol concentrations from 100 to 1,500 mg/l in the presence of 25 mg/l Cr(VI). All phenol concentrations assayed were completely removed and removal rates varied with changes in the initial concentration (Figure 3a). Degradation rates of alginate-trapped cells were over 35 mg/l/h for phenol concentrations up to 1,000 mg/l but decreased to 10 mg/l/h for 1,500 mg/l. Moreover, immobilised cells were able simultaneously to remove Cr(VI), achieving a maximum of 62% in the presence of phenol (300 mg/l).

Free cells completely removed phenol up to 1,000 mg/l but could not cope with 1,500 mg/l. The process catalysed by free cells was significantly slower than that carried out using immobilised cells. Free cells were similarly less efficient for Cr(VI) removal.

The better efficiency observed when using immobilised cells would correlate with their higher tolerance inside the matrix for phenol concentrations over 1,000 mg/l (Figure 3b). In this sense, the immobilisation into polymeric matrices provided a type of membrane stabilisation which is supposedly responsible for cell protection from the surrounding environment. Therefore, immobilised bacteria usually exhibit higher tolerances towards contaminants and better removal rates than free cells [31, 32]. Comparing the phenol removal capacity of SFC 500-1 with that of other immobilised bacteria allows one to understand the great potential of this entrapped consortium to degrade phenol. Although many immobilised bacterial cultures have proved to be suitable for the removal of high phenol concentrations, their degradation rates were generally lower than those achieved by SFC 500-1, even when phenol was added as the only contaminant into the culture medium [10, 33, 34].

Stability and reusability of immobilised cells

Stability during long-term storage and operation is essential for the practical application of an immobilised cell system. In this respect, immobilised cells of SFC 500-1 showed good storage stability at 4°C. Beads did not lose their removal potential after cold conservation periods of up to 45 days. After 60 days of storage, a bacterial count diminution was detected in the beads and their bioremediation efficiency decreased (Results not shown).

Reusability of the immobilised consortium was verified in repetitive batch mode. Under these conditions, 100% of phenol was removed up to the 4^{th} consecutive reuse cycle, although the degradation rate decreased significantly over time (Figure 4). In the 5^{th} cycle, phenol removal dropped to 85% after 33h. Similarly, bacteria removed 16.5 mg/l of Cr(VI) after 9h during the first cycle, but removal efficiency decreased in successive cycles.

A common characteristic of immobilised systems is that their remediation efficiency diminishes over time, mostly when they are used for the removal of metals [16, 25, 35, 36]. This behaviour has been associated with increased mechanical instability and gradual cell leakage from the beads during reutilisation cycles [37]. Cell viability loss and progressive saturation of adsorption sites could be other major causes of decreasing efficiency [35, 38]. Accordingly, the declining removal efficiency of immobilised SFC 500-1 was accompanied by progressive cell death between the 1^{st} and the 5^{th} cycles (Figure 5). In addition, towards the end of the process, modifications in the shape of the beads such as enlargement, colour change and loss of mechanic stability were observed.

Despite the decreased removal efficiency over time, the successful use of beads in up to 4-5 cycles is important since it could significantly reduce operational costs in industrial applications. Nevertheless, although the possibility of reusing immobilised cells makes them more efficient than free cells, use of immobilised bacteria for co-remediation of organic and inorganic compounds has not yet been intensively explored [2, 39].

Adsorption of both pollutants in the alginate matrix was also observed, since sterile beads showed a capacity for removing them in the first cycles (Figure 4). However, after the 3rdcycle, removal by physicochemical processes was almost negligible. On the basis of these results, Cr(VI) removal may be associated mostly with biological processes, but its interaction with the polymeric matrix should not be underestimated, as was also described by Bera et al [23].

Cr(III) was quantified after the removal process and concentrations of around 6 mg/l were detected in the reaction media collected from 5 successive cycles. In addition, approximately 2 mg/l of Cr(III) was found in the immobilisation matrix. Thus, this is evidence for a Cr(VI) reduction capability of immobilised cells. We have previously demonstrated that bacterial strains of SFC 500-1 consortium contained chromate reductases, which are the main enzymes involved in the biological mechanism for Cr(VI) detoxification through its reduction to Cr(III)[7, 17]. Cr(III) is a stable species, less soluble and less toxic than Cr(VI). For that reason, enzymatic reduction of Cr(VI) is a useful and eco-friendly process for the remediation of affected environments [40].

Detection and quantitation of phenol oxidation metabolites

According to Chung et al. [33], bacteria immobilised in polymeric matrices constitute an ideal system for the detection of intermediates formed during phenol degradation. Such matrices generate diffusion resistance and thus favour the permanence of metabolites in the reaction medium. In previous reports, it was reported that the SFC 500-1 degrades phenol through the *ortho*-oxidation of catechol [17]. Phenol and some metabolites formed through β–ketoadipate pathway were therefore analysed by HPLC. Since Cr(VI) could interfere in phenol degradation, metabolites formed over time were analysed in the presence and in the absence of Cr(VI) (Figures 6 a and b, respectively).

When 50% of phenol degradation was achieved, the highest catechol concentration in the reaction medium was detected, both in the presence and in the absence of Cr(VI). This intermediate was less stable or greatly biotransformed in media containing Cr(VI) since it was not possible to quantify it after

85% of phenol removal. Our results agree with previous reports, which indicated that catechol formed through biologic oxidation of aromatic compounds was partially released into the culture medium and subsequently re-internalised or degraded in the culture medium [33, 41, 43].

In the β–ketoadipate pathway, catechol is metabolized to *cis,cis*-muconate, which is finally transformed into succinate and acetyl-CoA and cellular biomass [42]. In our results, towards the end of the phenol removal process, *cis,cis*-muconate concentration increased and remained high even some hours after full removal. However, this behaviour was different depending on the presence or absence of Cr(VI). In media containing only phenol, *cis,cis*-muconate gradually diminished over time, but when Cr(VI) was added, this concentration was constant some hours after phenol disappearance. It has been reported that *cis,cis*-muconate formed from the oxidation of aromatic compounds could accumulate in the reaction medium and remain in a constant concentration over time [43]. Furthermore, some bacteria were unable to re-enter *cis,cis*-muconate into the cellular cytoplasm when grown under unfavourable conditions [41]. In this particular case, Cr(VI) could have affected membrane proteins involved in the transport of metabolites, causing a slowdown in the removal process [44].

In addition, two unidentified compounds were detected at the end of phenol removal with and without Cr(VI) (peaks 4 and 5 in the chromatogram, Figure 7b). They could correspond to muconolactone, ketoadipate or succinate, metabolites formed during more advanced oxidation steps.

Abiotic controls without beads did not show phenol removal over time. However, in controls performed with non-inoculated beads, phenol removal of around 14% was observed, probably due to its absorption by the matrix. Under these conditions, the formation of additional metabolites was not detected.

These results have demonstrated the ability of the consortium SFC 500-1 to efficiently degrade phenol efficientlt under immobilised conditions and in a short operational time, even in the presence of Cr(VI).

3. Conclusions:

The suitability of the Ca-alginate immobilised consortium SFC 500-1 for simultaneous removal of Cr(VI) and phenol was demonstrated. Immobilisation was a successful strategy to improve Cr(VI) removal, which was mostly associated with enzymatic reduction through the formation of Cr(III). The immobilized SFC 500-1 showed great efficiency in degrading phenol in a short time, probably due to the protective effect of the alginate matrix against the toxicity of phenol concentrations over 1,000 mg/l. Complete phenol oxidation to catechol and *cis,cis*-muconate was verified through the detection

of these intermediates in the reaction medium during the co-remediation process. The optimum removal potential, long-term storage and reusability make SFC 500-1 entrapped cells an interesting system for Cr(VI) and phenol removal in media containing low concentrations of organic matter. The low cost and high resistance of the alginate matrix are further important advantages when considering their possible application for the decontamination of real effluents.

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Figure legends

Figure 1: Effect of immobilised cells load of SFC 500-1 on Cr(VI) and phenol removal. The number of entrapped cells was expressed as colony forming units per milliliter of medium (CFU/ml). Different letters indicate statistically significant differences (SSD) in the removal of each contaminant at the end of the same removal cycle, employing a variable number of cells.

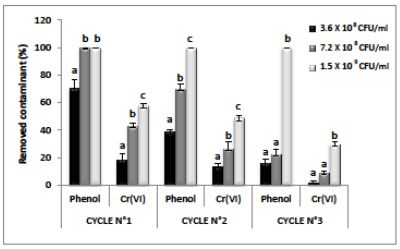


Figure 2: Cr(VI) removal (bars) and phenol degradation rates (lines) obtained using free (F) and immobilised (I) cells of SFC 500-1 exposed to increasing Cr(VI) concentrations in the presence of phenol 300 mg/l.

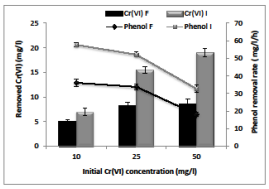


Figure 3: (a) Phenol removal rates (lines) and Cr(VI) removal percentages (bars) obtained by free (F) and immobilized (I) cells of SFC 500-1 exposed to increasing phenol concentrations in the presence of Cr(VI) 25 mg/l. (b) Counts of free and immobilised cells at inoculation time (To) and after removal of increasing phenol concentrations in the presence of Cr(VI).

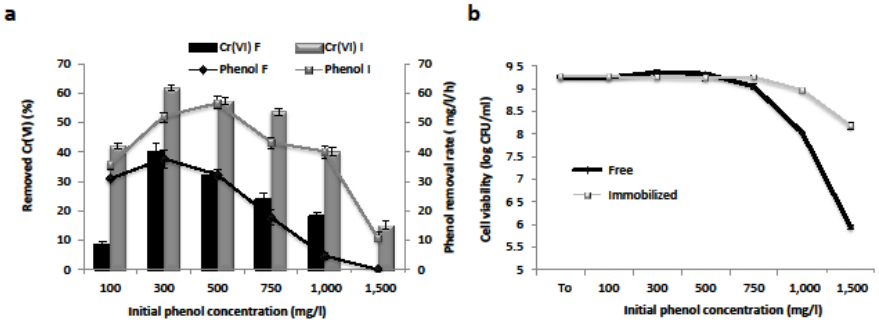


Figure 4: Cr(VI) and phenol removal by immobilised SFC 500-1 and sterile beads (control) through five subsequent utilisation cycles.

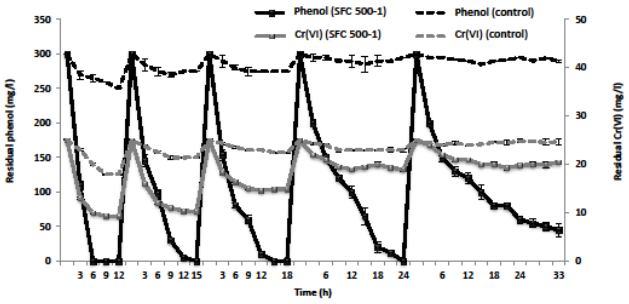


Figure 5: Cell viability of immobilised SFC 500-1 at inoculation time (To) and at the end of each cycle of simultaneous removal.

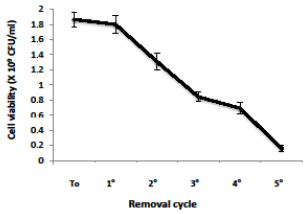


Figure 6: Time course of phenol degradation and detection of catechol and cis,cis-muconate produced by the immobilised mixed culture SFC 500-1 in media (a) non-supplemented and (b) supplemented with Cr(VI).

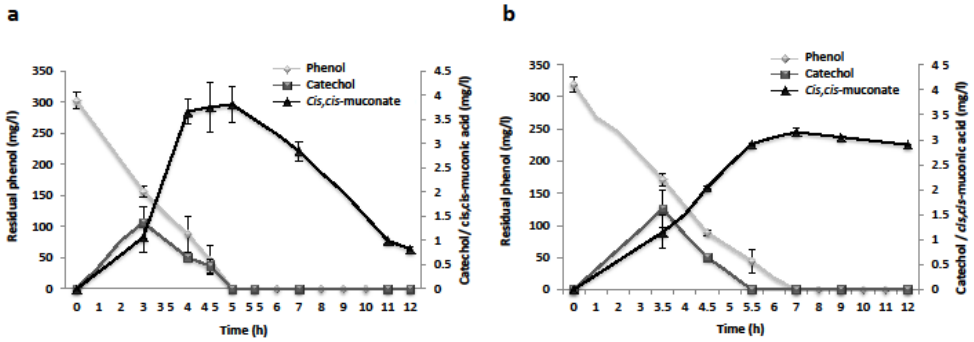
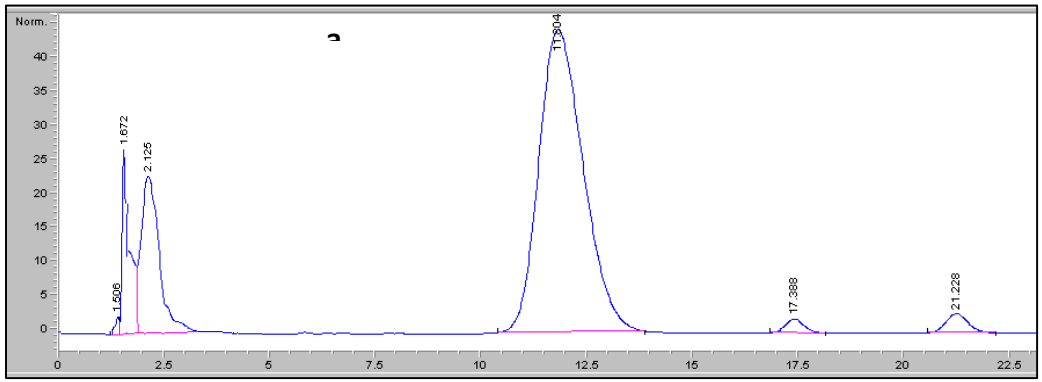


Figure 7: Typical chromatograms obtained from samples of reaction medium containing phenol and Cr(VI), when a phenol removal of (a) 50% and (b) 100% was achieved. Peaks: 1: Catechol; 2: *cis,cis*-muconate; 3: phenol; 4 and 5: unidentified.



a

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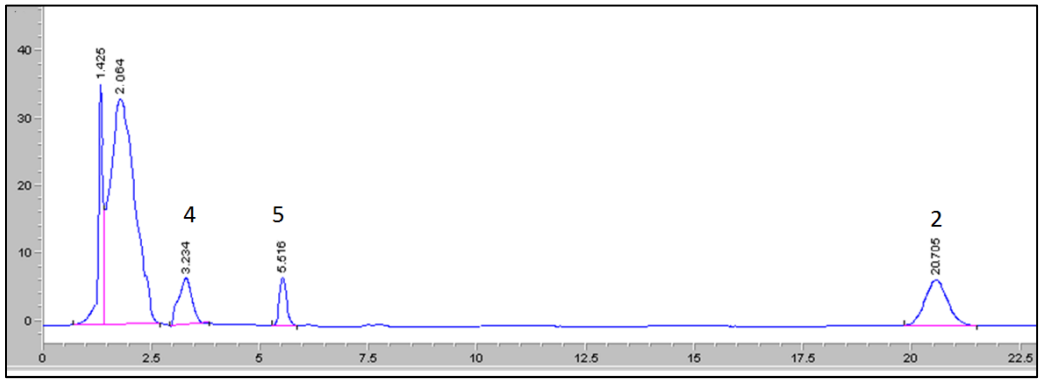
3

1



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