Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Optimization of simultaneous removal of Cr (VI) and phenol by a native bacterial consortium: its use for bioaugmentation of co-polluted effluents

O.M. Ontañon, P.S. González and E. Agostini

Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina

Keywords
bacterial consortium, chromium, phenol, simultaneous bioremediation, tannery.

Correspondence
Paola S. González, Departamento de Biología Molecular, FCEFQyN, UNRC. Ruta 36 Km 601, CP 5800 Río Cuarto (Cba), Argentina.
E-mail: pgonzalez@exa.unrc.edu.ar

2015/0986: received 15 May 2015, revised 8 July 2015 and accepted 21 July 2015

doi:10.1111/jam.12913

Abstract

Aims: This study was designed to isolate, identify and characterize microorganisms or mixed cultures capable of simultaneously removing Cr (VI) and phenol in the surrounding area of a tannery localized in Elena, Córdoba, Argentina. In addition, nutritional and physical factors were optimized in order to improve the removal efficiency in a real effluent.

Methods and Results: The consortium SFC 500-1, composed of two bacterial strains belonging to *Acinetobacter* and *Bacillus* genus, was isolated from the heavily polluted wastewater discharge channel of a local tannery. SFC 500-1 was able to remove phenol at environmentally relevant concentrations (1000 mg l^{-1}) and reduce Cr (VI) to Cr (III), which was immobilized in the bacterial biomass. The consortium simultaneously removed these contaminants under a wide range of physicochemical conditions and different growth media, even in a tannery effluent.

Conclusion: The ability of SFC 500-1 to simultaneously reduce Cr (VI) and degrade phenol in different synthetic growth media and even in the effluent from which it was isolated with high efficiency makes this consortium a potential candidate for the biotreatment of effluents.

Significance and Impact of the Study: This finding is important, taking into account that industrial effluents present complex mixtures of toxic substances as well as native flora which often affect the bioremediation process. Considering the ecological advantages of using native bacteria for bioremediation, as well as the high efficiency of the consortium SFC 500-1 to simultaneously remove Cr (VI) and phenol, this could be a suitable biological system to improve the biotreatment of polluted effluents through a bioaugmentation strategy.

Introduction

In the last century, industrial development, urbanization and excessive agricultural practices have caused a marked increase in the chemical content in nature, generating serious environmental pollution problems. The main environmental pollutants listed by the US EPA (Environmental Protection Agency of the United States) are organic compounds such as hydrocarbons, phenols, benzene, toluene, solvents and pesticides, as well as inorganic substances such as heavy metals, including chromium. In general, polluted sites are co-contaminated with a mixture of pollutants which represent dangerous risks to human, animal and vegetal life (Sandrin and Maier 2003). Chromium (Cr), especially in its trivalent (Cr III) and hexavalent (Cr VI) forms and in aromatic compounds such as phenol, is discharged in industrial processes like wood preservation, petroleum refining and leather tanning and finishing, among others. As a consequence, these pollutants can be simultaneously found in wastewater treatment plants, groundwater and surface water (Goldstone *et al.* 1990; USEPA 1990). In

Argentina, Greenpeace reports revealed strong chromium and phenol pollution due to the indiscriminate discharge of tannery waste in waters used for human consumption (http://www.greempeace.org.ar: Campaña Riachuelo 2012, http://www.cicplata.org). Therefore, with the aim of reducing and/or removing the level of harmful substances from the environment, numerous biological systems have been developed for the treatment of contaminated sites. Among them, bioremediation is a method that involves the metabolic capabilities of microorganisms for the detoxification of several compounds, either through total degradation, chemical transformation or retention, preventing their mobilization in the environment (Megharaj *et al.* 2011). There is extensive literature showing the superiority in this aspect of bacteria isolated from contaminated areas compared to those isolated from noncontaminated areas, possibly due to selective pressure and vertical or horizontal gene transfer (Wang *et al.* 2007). In this sense, the introduction of consortia in the environment is considered the most appropriate alternative for remediating different contaminants (Supaphol *et al.* 2006). The main benefit of using these consortia lies in their ability to degrade a variety of substrates without the harmful accumulation of toxic intermediates (Acuna-Arguelles *et al.* 2003). In this context, bacterial Cr (VI) reduction and simultaneous phenol degradation may represent a beneficial biological process. The electron donors for Cr (VI) reduction are generally limited to amino acids and aliphatic compounds. Thus, the use of phenol as an electron donor for microbial Cr (VI) reduction could represent an alternative for the simultaneous removal of both pollutants (Liu *et al.* 2008). Nevertheless, the use of microbial consortia for simultaneous Cr (VI) and phenol removal in different media and effluents has not been widely studied, particularly when it comes to indigenous bacterial isolates. For this reason, the aim of the present work was to isolate, identify and characterize micro-organisms or mixed cultures capable of simultaneously removing Cr (VI) and phenol in the surrounding area of a tannery localized in Elena, Córdoba, Argentina. In addition, nutritional and physical factors were optimized in order to improve the removal efficiency in a real effluent. This information would be useful to develop a bioremediation process for wastewater treatment plants.

Materials and methods

Characterization of the polluted area and collection of samples

Sediment and effluent samples were collected from one of the discharge points of a tannery located in Elena, Córdoba province, Argentina (32°34′ South latitude and 64°23′ West longitude), in order to isolate individual or mixed bacterial strains able to remove Cr (VI) and phenol. Total Cr and/or Cr (VI) as well as phenol concentrations were determined in all collected samples. Some physicochemical parameters such as pH values, temperature, Chemical Oxygen Demand (COD), total dissolved solids, electrical conductivity and sulphates were also analysed in the effluent.

Different samplings were carried out over the course of 2 years to detect variations in the content of pollutants over time. Cr (VI) and total Cr were determined by diphenylcarbazide (DPC) technique (APHA 1998) and inductively coupled plasma atomic emission spectroscopy (ICP-AES) respectively. Total phenols were measured using the method of 4-aminoantipyrine, as it is described in section Phenol determination (Wagner and Nicell 2002).

Culture media for bacterial isolation, maintenance and removal assays

The culture media employed in this research and their composition (g l^{-1}) are detailed below: nutritive medium (NM) (tryptone 5·0; yeast extract 3·0; $CaCl_2$ 0·65) (Beringer 1974); MM9 medium (Na_2HPO_4 2·0; KH_2PO_4 9·0; NaCl 2·5; NH_4Cl 1·0); MM1 medium (NH_4Cl 1·0; $MgSO_4$ 0·2; $FeSO_4$ 0·001; $CaCl_2$ 0·001; K_2HPO_4 0·5); MM2 medium (KH_2PO_4 1·0; K_2HPO_4 1·0; $MgSO_4$ 0·2; $CaCl_2$ 0·1; NaCl 0·2; $MnSO_4$ 0·01; NH_4NO_3 1; $FeCl_3$ 0·05). MM9 medium supplemented with two different combinations of yeast extract and tryptone as additional carbon sources was also used. These media were called MYT ½ (tryptone 0·25% and yeast extract 0·15%) and MYT ¼ (tryptone 0·12% and yeast extract 0·07%). Solid medium NMA was prepared using NM supplemented with agar (1·2%).

Isolation of bacterial strains able to tolerate and remove Cr (VI) and/or phenol

For bacteria isolation, 10 g of sediment or 10 ml of effluent were mixed with 90 ml of sterile solution of NaCl 0·9%, kept some minutes and used for inoculation of NM with phenol (100 mg l^{-1}) and Cr (VI) (10 mg l^{-1}). These media containing the contaminants were incubated 48 h at 28°C and 150 rev min^{-1}. An aliquot of each culture was then used for inoculating other media containing different concentrations of pollutants: Medium MM9 amended with phenol (300–500 mg l^{-1}) plus Cr (VI) (5–20 mg l^{-1}) and NM containing phenol (500–750 mg l^{-1}) plus Cr (VI) (20–50 mg l^{-1}). Serial dilutions in NaCl 0·9% of each one of these cultures were spread on plates containing NMA.

All morphologically different colonies were microscopically examined by Gram staining. Those mixed cultures that coexisted at the highest pollutant concentrations and dilutions above 10^{-5} were used as bacterial consortia and grown together (in their natural proportion) in agar plates containing Cr (VI) (20 mg l^{-1}) and phenol (100 mg l^{-1}).

The most tolerant pure or mixed cultures were analysed on the basis of their removal capabilities. For Cr (VI) removal assays, NM amended with 50 mg l^{-1} of the metal were used, whereas for phenol removal assays MM9 with phenol (100 mg l^{-1}) as sole carbon source was employed. Studies of simultaneous removal of both pollutants were performed in NM and MM9 supplemented with Cr (VI) (25 mg l^{-1}) and phenol (100 mg l^{-1}).

Characterization of the selected consortium

Maintenance and identification

The consortium was weekly subcultured in plates containing NMA plus Cr (VI) (20 mg l^{-1}) and phenol (100 mg l^{-1}). To ensure a constant proportion of each strain, a bacterial count was performed regularly.

Individual strains which compose the selected consortium were isolated and characterized employing morphological, biochemical and molecular methods. For isolation, single colonies were taken from serial dilutions of the consortium, visualized by Gram staining and spread in separate agar plates containing both pollutants.

Identification of both strains through amplification of 16S rRNA gene was carried out by PCR using an universal primers set: forward (5′-CCAGCAGCCGCGGTAATACG-3′) and reverse (5′-TACCAGGGTATCTAATCC-3′). Sequencing was performed by the 'MacroGen' company (Seoul, Korea). The sequences were compared and identified using BLAST (Altschul *et al.* 1997) and deposited in GenBank.

Cr (VI) and phenol removal assays

Cr (VI) and phenol removal assays were performed in Erlenmeyer flasks containing 20 ml of the corresponding medium supplemented with different Cr (VI) (10; 20; 50 and 100 mg l^{-1}) or phenol (100; 300; 500; 750; 1000 mg l^{-1}) concentrations. For phenol removal assays, MM9 and NM media were used, whereas Cr (VI) removal was evaluated only in NM.

All flasks were inoculated with a bacterial culture grown overnight in NM to achieve an initial absorbance of 0·05 at 600 nm. Then, they were incubated at 150 rev min^{-1} and 28 ± 2°C. Abiotic controls were performed using noninoculated media supplemented with Cr (VI) and phenol to evaluate the loss of the contaminant by evaporation. Growth controls without contaminants were performed in NM and MM9 medium to determine the capability of the consortium to grow in control conditions.

Based on previous growing studies, the growth controls without phenol were performed in MM9 medium supplemented with yeast extract (0·5%) as nutrient source.

At predetermined time intervals, aliquots were withdrawn for bacterial growth evaluation and determination of residual Cr (VI) and phenol (See Determination of bacterial growth and pollutant concentration). Removal rates were calculated by plotting contaminant concentrations *vs* time and fitting the data to a linear regression model.

Simultaneous removal assays and determination of optimal conditions

The ability of the selected bacterial consortium to simultaneously remove Cr (VI) 25 mg l^{-1} and phenol 300 mg l^{-1} was analysed at diverse growth conditions. For that, three mineral media called MM1, MM2 and MM9 with phenol as only carbon source were employed. Medium MM9 supplemented with different tryptone and yeast extract concentrations as additional carbon sources (MYT ¼ and MYT ½) and NM were also used.

The selected culture medium was used for the optimization of other experimental conditions, such as pH (4–12), temperature (20–40°C) and agitation rate (120–250 rev min^{-1}). In these assays, all parameters were kept almost constant, varying only the analysed condition.

After that, the removal of 12 different combinations of Cr (VI) (10, 25 and 50 mg l^{-1}) with phenol (100, 300, 500 or 750 mg l^{-1}) was studied employing selected optimal conditions.

All of these experiments were monitored over the course of 72 h. For each condition, noninoculated controls were employed. Growth and residual Cr (VI) and phenol concentrations were periodically determined. Removal percentages and rates were calculated.

Determination of bacterial growth and pollutant concentration

Microbial growth

Microbial growth was evaluated in all individual experiments by measuring the absorbance at 600 nm using a Beckmann DU630 spectrophotometer.

Cell viability along the process of simultaneous removal was monitored employing the selected optimal conditions. At 6-h intervals, samples were withdrawn and a bacterial count was carried out through the plate count method (Spencer and Ragout 2004). Samples were serially diluted in sterile 0·9% NaCl solution and placed by triplicate in NMA plates. Total colony forming units (CFU)/ml were calculated and plotted. Colonies corresponding

to each strain were identified in accordance with their morphological characteristics. As additional controls, appropriate dilutions of each culture were spread on plates to confirm the presence of the two strains forming the consortium at the end of all experiments.

Phenol determination

Residual phenol concentration was spectrophotometrically evaluated in supernatants according to Wagner and Nicell (2002) using a Beckman DU640 spectrophotometer. Samples of 100 μl were mixed with 700 μl of sodium bicarbonate (pH 8), 100 μl of 4-aminoantipyrine (20·8 mmol l^{-1}) and 100 μl of potassium ferricyanide (83·4 mmol l^{-1}). After 5 min, absorbance at 510 nm was measured. The absorbance data were converted to phenol concentrations using a calibration curve from 0 to 100 mg l^{-1} with an r^2 of 0·995.

Chromium determination

Cr (VI) in supernatants was determined at 540 nm after reaction with DPC in acid solution. The reaction mixture contained 500 μl of H_2SO_4 0·2 mol l^{-1}, 200 μl of DPC (5 mg l^{-1}) and 500 μl of sample in a final volume of 5 ml. The absorbance data were converted to Cr (VI) concentrations using a calibration curve from 0 to 10 mg l^{-1}, with an r^2 of 0·988. To analyse the effluent, it was centrifuged and its pH was neutralized before Cr (VI) determination.

Total Cr concentration in supernatants was determined by AAS at the end of the assay (APHA 1989). Cr (III) concentration was calculated by difference between total Cr and Cr (VI).

For Cr quantification in biomass, cells were obtained by centrifugation (15 000 ***g***- 15 min), washed three times with saline solution (0·9% NaCl), dried and weighted. Dry biomass was digested with HNO_3, and Cr (III) and Cr (VI) were analysed by AAS using a Perkin Elmer Analyst in a specialized laboratory.

Reagents

All reagents used in the experiments were of analytical grade and purchased from Merck (Germany) and Sigma-Aldrich (MO, USA). Phenol and Cr (VI) have purity in excess of 99·5%. All solutions and culture media were prepared using deionized water.

Statistical analysis

All the experiments were carried out at least three times in triplicate. Data were analysed using ANOVA, followed by Tukey test ($P < 0·05$), through INFOSTAT (ver. 2012 E Universidad Nacional de Córdoba, Córdoba, Argentina) software.

Results

Characterization of tannery sediment and effluent

Samples of effluents and sediments in contact with them were collected from the wastewater discharge channel of a local tannery over 2 years, encompassing the four seasons. The physicochemical characteristics of the effluents as well as the content of Cr and phenol were evaluated.

According to the season, the temperature of the effluents varied between 14 and 26°C, but the pH was alkaline in all samples, reaching an average value of 10. Other remarkable physicochemical parameters of these effluents were total dissolved solids (up to 18 000 mg l^{-1}), electrical conductivity (8 600 $\pm$ 1300 mS cm^{-1}), sulphates (up to 980 mg l^{-1}) and COD (up to 2000 mg l^{-1}), all of them exceeding the limits established by national legislation.

As can be seen in the Table 1, total Cr concentrations registered in effluents were under 0·45 mg l^{-1}, whereas concentrations of phenols were up to 17 mg l^{-1}, which widely exceeded the guideline values that establish 0·1 mg l^{-1} as the maximum value for discharge water going into natural water sources. In the sediments, the total Cr concentration was higher than 2500 mg kg^{-1} in all samplings, while concentrations of phenols were below detection limits.

Although significant fluctuations were evidenced in some of the analysed parameters, they were not seasonally affected. Variations in the composition of samples might have been related to changes in the tanning process in the different samplings.

Table 1 Content of chromium and total phenols in effluent and sediment from the sampling area

		Guideline values	
Analysed pollutant	Detected concentration	Industrial use	Residential use
Sediment (mg kg^{-1})			
Total Cr	2500–6770	800*	250*
Total phenols	<0·1	10*	1*
Effluent (mg l^{-1})			
Total Cr	0·01–0·45	2†	0·05‡
Cr (VI)	0·01–0·15	0·2†	0·005‡
Total phenols	10·8–17·0	0·1†	0·001‡

*Maximum concentration accepted for industrial or residential soils respectively (Decree 831/93, Argentinean Law of dangerous wastes)
†Maximum concentration accepted for discharge water into natural water sources (Decree 831/93, Argentinean Law of dangerous wastes).
‡Maximum concentration allowed by the Argentinean Food Code.

Isolation of micro-organisms able to tolerate and remove Cr (VI) and phenol

Thirty eight tolerant microbial cultures were isolated from effluent and sediment samples from the tannery. The isolates were classified based on their macro and microscopic characteristics by Gram staining and their removal capabilities were studied. Most of them removed neither of the two compounds, either individually or simultaneously. Only 30% of the isolates were able to remove between 10 and 35 mg l^{-1} of Cr (VI) in NM, and around 15% of them could remove at least 50 mg l^{-1} of phenol as only carbon source in MM9 medium.

Three consortia were efficient in simultaneously removing Cr (VI) and phenol. A consortium called SFC 500-1 with the best removal capabilities, both in NM and MM9 medium, was selected to conduct this study.

Characterization of the consortium SFC 500-1

Identification

Two bacterial strains, identified as *Bacillus* sp. SFC 500-1E and *Acinetobacter* sp. SFC 500-1A by sequencing of DNAr 16S were present in the mixed culture. The sequences were deposited in GenBank under accession numbers JQ701739 and JX198426 respectively.

Growth and Cr (VI) removal

Growth and Cr (VI) removal were monitored at different initial Cr (VI) concentrations (10–100 mg l^{-1}) in NM at pH 7 and 28°C. Figure 1 shows a negative effect of Cr (VI) on microbial growth, as its development was greater in control conditions without the contaminant than in the presence of Cr (VI). Moreover, the increase in the concentration of Cr (VI) was associated with a significant decrease in growth.

Regarding Cr (VI) removal, SFC 500-1 was able to totally remove 10 mg l^{-1} of Cr (VI) before 24 h had elapsed. For higher Cr (VI) concentrations a partial removal was observed after 72 h. Maximal removal of 35 mg l^{-1} was achieved when 50 mg l^{-1} were supplemented. During the first 24 h, the removal rate increased after increasing the initial Cr (VI) concentration, reaching a value of 1 mg l^{-1} h^{-1} for 50 mg l^{-1}. When the concentration was 100 mg l^{-1}, removal rate decreased (results not plotted).

Growth and phenol removal in MM9 medium and NM

Growth and phenol removal by SFC 500-1 was evaluated in MM9 medium and NM supplemented with phenol (100–1000 mg l^{-1}) (Fig. 2). Regarding microbial growth, phenol addition did not significantly affect the microbial development with respect to the control without the contaminant in NM. Although bacterial growth in MM9 medium was lower than in NM, it was favoured by the addition of the highest phenol concentrations.

On the other hand, the consortium was capable of fully removing phenol up to 1000 mg l^{-1} in both culture media in less than 72 h. Figure 2 shows that phenol removal rates increased at higher phenol concentrations in MM9 medium. A similar effect was observed in NM for the lowest concentrations; however, the removal rate strongly diminished when 1000 mg l^{-1} were incorporated. In all cases, removal rates were higher in MM9 medium than in NM.

Simultaneous Cr (VI) and phenol removal

Effect of culture media. Simultaneous removal of Cr (VI) (25 mg l^{-1}) and phenol (300 mg l^{-1}) was evaluated in different synthetic culture media to analyse the effect of their nutritional composition on the remediation capabilities of SFC 500-1. Although phenol 300 mg l^{-1} was fully

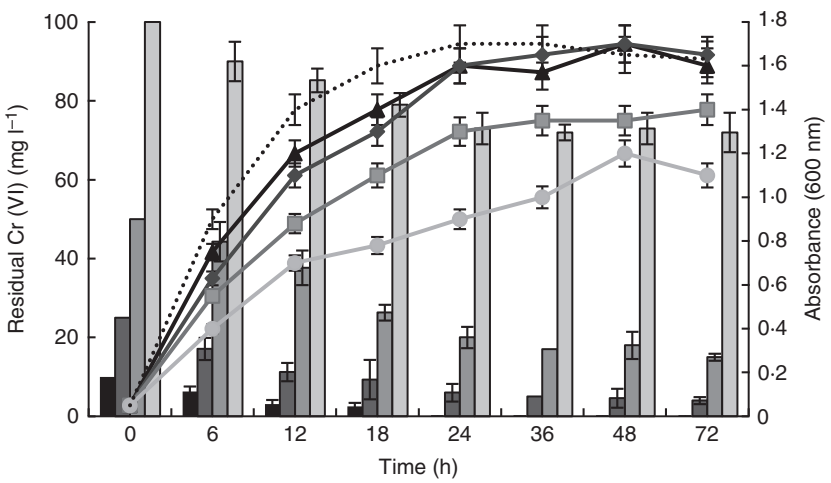


Figure 1 Time-course of Cr (VI) removal and growth by the consortium SFC 500-1. Lines represent growing in NM with different Cr (VI) concentrations: 10 mg l^{-1} (▲), 25 mg l^{-1} (⬟), 50 mg l^{-1} (▣), 100 mg l^{-1} (○) and without Cr (VI) (--). Bars indicate the residual concentrations of Cr (VI) along the time: 10 mg l^{-1} (■), 25 mg l^{-1} (▩), 50 mg l^{-1} (▩), 100 mg l^{-1} (▩). The error bars represent standard errors.

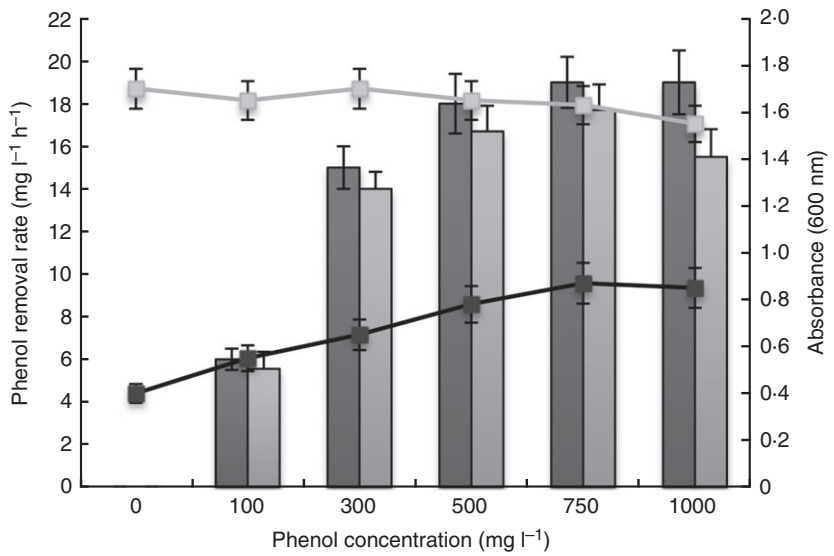


Figure 2 Maximal phenol removal rate (bars) and maximal microbial growth (lines) achieved for the consortium SFC 500-1 in MM9 medium (■) and NM (□) supplemented with different phenol concentrations (100–1000 mg l^{-1}). The growth in control conditions (without phenol) in the both media is also plotted (represented as phenol 0 mg l^{-1}). The error bars represent standard errors.

degraded in almost all evaluated conditions, Cr (VI) removal varied according to the culture medium used. Figure 3 shows that, irrespective of their composition, in those mineral media with phenol as only carbon source, Cr (VI) removal did not exceed 27%. However, removal percentages significantly increased ($P < 0{\cdot}05$) to 40 and 62% when mineral medium MM9 was supplemented with yeast extract and tryptone as additional electron sources (MYT ¼ and MYT ½ respectively). When experiments were performed using NM, a maximal Cr (VI) removal value of 80% and complete phenol degradation were detected. In a similar way, bacterial growth increased in those media containing a higher concentration of organic compounds (data not shown).

In noninoculated controls, pollutant removal was not detected towards the end of the experiment.

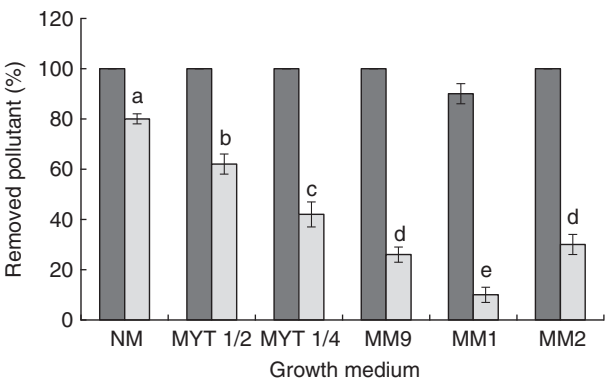


Figure 3 Simultaneous Cr (VI) (□) and phenol (■) removal by the consortium SFC 500-1 in different culture media. Different letters indicate statistically significant differences ($P < 0{\cdot}05$) in Cr (VI) removal. The error bars represent standard errors.

According to the results obtained, NM would be the most suitable medium to achieve efficient simultaneous removal by SFC 500-1.

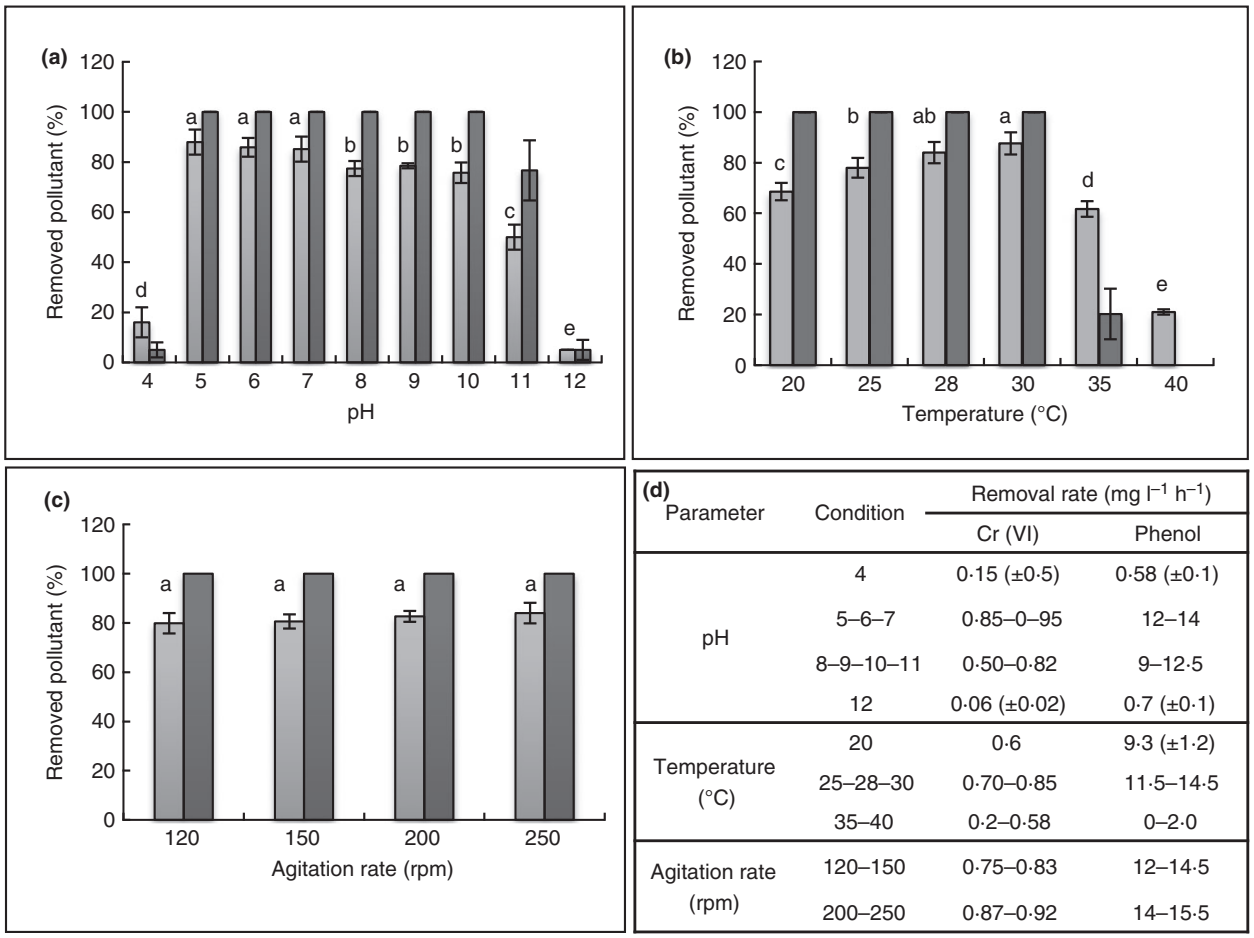
Selection of optimal pH, temperature and agitation rate

Some factors affecting the capability of SFC 500-1 for the removal of Cr (VI) and phenol, such as pH, temperature and agitation rate, were investigated in NM using Cr (VI) 25 mg l^{-1} and phenol 300 mg l^{-1} (Fig. 4).

SFC 500-1 was able to biotransform Cr (VI) and phenol even at acid and alkaline pH values. At a pH of 5–10, Cr (VI) removal higher than 75% and complete phenol removal were achieved. At pH 11 the removal of both pollutants was negatively affected, and the extreme conditions (pH 4 and 12) strongly inhibited the process (Fig. 4a). The optimal pH range to achieve the highest Cr (VI) reduction was between 5 and 7, and the maximum phenol degradation rates were obtained at pH 6–7 (Fig. 4d). Thus, neutral or slightly acidic pHs are likely the most favourable for the simultaneous removal of both compounds.

Incubation temperature also affected removal capability (Fig. 4b). The optimal temperatures registered were between 28 and 30°C and an increase in Cr (VI) removal and phenol degradation rate was observed when temperatures rose from 20°C to 30°C. However, when incubation was carried out at 35 or 40°C, a marked removal decrease was observed, with total inhibition of phenol degradation at 40°C.

On the other hand, changes in agitation rates from 120 to 250 rev min^{-1} did not significantly affect the removal of pollutants (Fig. 4c). Nevertheless, at higher agitation rates (200–250 rev min^{-1}) the maximal removal rates were obtained.



(d)

Parameter	Condition	Removal rate (mg l^{-1} h^{-1})	
		Cr (VI)	Phenol
pH	4	0·15 (±0·5)	0·58 (±0·1)
	5–6–7	0·85–0–95	12–14
	8–9–10–11	0·50–0·82	9–12·5
	12	0·06 (±0·02)	0·7 (±0·1)
Temperature (°C)	20	0·6	9·3 (±1·2)
	25–28–30	0·70–0·85	11·5–14·5
	35–40	0·2–0·58	0–2·0
Agitation rate (rpm)	120–150	0·75–0·83	12–14·5
	200–250	0·87–0·92	14–15·5

Figure 4 Simultaneous Cr (VI) (□) and phenol (■) removal by SFC 500-1 at different culture conditions: (a) pH, (b) Temperature, (c) Agitation rate. Removal rates for both contaminants in each tested condition are tabulated in d. Different letters indicate statistically significant differences (SSD) ($P < 0·05$) in Cr (VI) removal. The error bars represent standard errors.

Finally, an evaluation of the concentration of pollutants and their distribution, and bacterial growth in NM were carried out employing the selected optimal conditions (pH 7, 28–30°C, 200 rev min^{-1}). In these experiments, microbial development increased by more than a logarithm throughout 24 h and a prevalence of *Acinetobacter* sp. over *Bacillus* sp. was detected. The proportion of *Bacillus* sp. in the mixed culture compared to *Acinetobacter* sp. always remained below 13% (Data not plotted).

The removal process was associated with microbial growth, as maximal Cr (VI) removal and complete phenol degradation were observed in less than 24 h. Also, a slight additional decrease in Cr (VI) concentration was achieved between 24 and 72 h (Fig. 5a).

To evaluate the mechanisms involved in Cr (VI) removal by SFC 500-1, such as reduction and/or biosorption, extra and intracellular Cr (VI) and Cr (III) concentrations were determined at the end of the experiments (Fig. 5b).

Cr (VI) was mainly reduced to Cr (III) (around 75%), which was detected both in supernatant and biomass. Sixteen per cent of it remained as Cr (VI) in supernatant. After 72 h of incubation, the total accumulated Cr was 6·1 mg per gram of dry biomass.

Effect of Cr (VI) and phenol concentrations on removal capability. The efficiency of the mixed culture SFC 500-1 to simultaneously remove Cr (VI) (10, 25 or 50 mg l^{-1}) and phenol (100, 300, 500, 750 mg l^{-1}) was assessed in NM.

Cr (VI) was partially removed up to 30 mg l^{-1} when 50 mg l^{-1} were added in combination with phenol 300 and 500 mg l^{-1}. Removal rates increased from 0·35 mg l^{-1} h^{-1} to 0·95 mg l^{-1} h^{-1} approx. when Cr (VI) concentration was increased from 10 to 50 mg l^{-1} (Fig. 6). Such process was not significantly affected by the addition of phenol concentrations up to

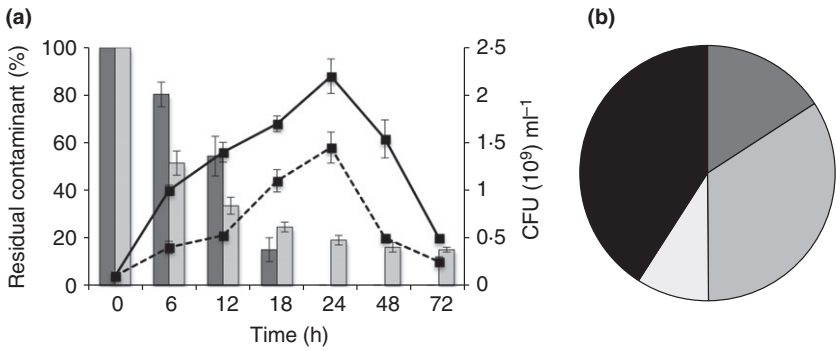


Figure 5 (a) Growth and simultaneous removal of Cr (VI) and phenol by the consortium SFC 500-1. Growth is plotted as viable cells count in the presence (dotted line) and in the absence (complete lines) of these pollutants. Bars represent residual content of Cr (VI) (□) and phenol (■). The error bars represent standard errors. (b) Distribution of Cr species in supernatants and cell biomass after the removal process (72 h). (■) Cr (VI) Supernatant; (▨) Cr (III) Supernatant; (□) Cr (VI) Biomass and (■) Cr (III) Biomass.

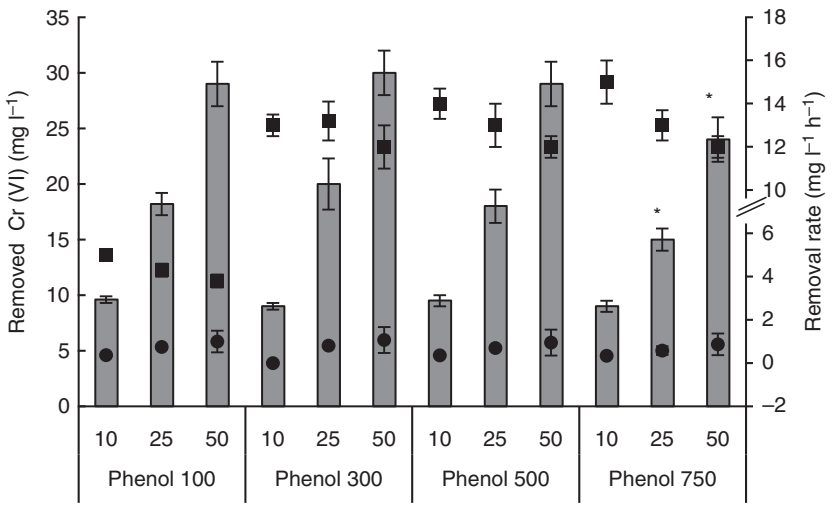


Figure 6 Removal of Cr (VI) (10; 25; 50 mg l^{-1}) by the consortium SFC 500-1 in the presence of different phenol concentrations (100; 300; 500; 750 mg l^{-1}). Residual Cr (VI) (■) concentrations are plotted with bars. Symbols indicate Cr (VI) (•) and phenol (■) removal rates. The error bars represent standard errors. (*) indicate those conditions in which Cr (VI) removal was significantly affected by increasing in phenol concentrations ($P < 0{\cdot}05$).

500 mg l^{-1}. However, when 750 mg l^{-1} of phenol were added to the culture medium, a significant decrease ($P < 0{\cdot}05$) in the removal of 25 and 50 mg l^{-1} of Cr (VI) was observed.

Furthermore, complete phenol removal was achieved in the presence of different Cr (VI) concentrations with a maximal removal rate of 15 mg l^{-1} h^{-1} for phenol 750 mg l^{-1} (Fig. 6). The increase in Cr (VI) concentrations caused a decrease in phenol removal rates.

Simultaneous removal in tannery wastewater. The isolated consortium SFC 500-1 was tested for its ability to remove both contaminants in the tannery effluent previously characterized (3·1) (Fig. 7). Experiments were carried out *ex situ* in Erlenmeyer flasks employing optimal conditions of temperature and agitation (28°C, 200 rev min^{-1}), although the pH of the effluent was close to 10. Contaminants were added to the effluent at a final concentration of 25 and 300 mg l^{-1} for Cr (VI) and phenol respectively.

Under these conditions, the consortium was able to remove all phenol incorporated and around 40% of Cr (VI) in 72 h. The removal capability of effluent-native micro-organisms was also detected. However, they reduced only 20% of phenol and 10% of Cr (VI) in the same period of time.

Discussion

Bioremediation has emerged as an eco-friendly strategy to treat environmental pollution. As most polluted sites contain complex mixtures of chemicals, including inorganic and organic pollutants such as Cr and phenol, it is important to find bacterial cultures that can tolerate and remove multiple contaminants. However, few biological systems have shown suitable detoxification potential when simultaneously exposed to different kinds of

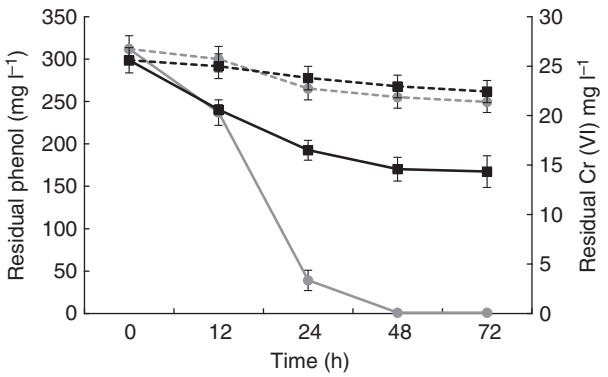


Figure 7 Cr (VI) and phenol simultaneous removal in a tannery effluent. Dotted lines indicate the removal achieved by native micro-organisms (Noninoculated). Complete lines represent the removal achieved by native micro-organisms plus the consortium SFC 500-1. (–●–) SFC 500-1 Phenol; (–■–) SFC 500-1 Cr (VI); (-·●·-) Noninoculated Phenol and (-·■·-) Noninoculated Cr (VI).

pollutants (Chen *et al.* 2011; Gunasundari and Muthukumar 2013).

To obtain bacterial cultures able to simultaneously remove Cr (VI) and phenol, we isolated micro-organisms from a waste discharge channel belonging to a local tannery polluted with phenols and heavy metals. The study of this area over time revealed that Cr concentration in sediments was approx. between 3 and 8·5 times above the accepted limits for industrial soils in our country. Moreover, the phenol content in effluents exceeded by more than 100 times the guideline values given by the Argentinian law of hazardous wastes (National Law from Argentina No. 24.051, Decree 831/93) and international standards (USEPA 1979; ATSDR 1998).

From such sediments and effluents 38 bacterial strains tolerant to Cr (VI) and/or phenol were isolated, although most of them were not able to remove these compounds. Among the isolates, a consortium composed of two bacterial strains belonging to genus *Acinetobacter* and *Bacillus* was selected on the basis of its capability for simultaneous removal.

An exhaustive analysis of the selected consortium, called SFC 500-1, revealed its tolerance and removal capability even at high Cr (VI) and phenol concentrations. However, microbial growth was more strongly affected by Cr (VI) than by phenol. This result might be due to the fact that Cr (VI) is not an essential metal for microbial growth while phenol, because of its organic nature, can be susceptible to bacterial mineralization and used as carbon source (Cervantes *et al.* 2001; Basha *et al.* 2010). Regarding the remediation assays, Cr (VI) concentrations up to 35 mg l^{-1} were removed before 72 h had passed, although the maximum removal rate was achieved within the first 24 h and then the process declined. This time-limited removal could be associated with the electron source consumption, as well as to the loss of active cells or cellular death induced by Cr oxidative potential (Ramírez-Díaz *et al.* 2008). By contrast, the consortium was able to completely remove phenol up to 1000 mg l^{-1} at a high rate in both MM9 and NM media. Removal and microbial growth were improved in MM9 medium by increasing phenol concentrations. This could be related to the consumption of phenol as carbon source to promote bacterial growth, suggesting the presence of a metabolic pathway for phenol oxidation in the consortium. Besides, SFC 500-1 exhibited high phenol removal efficiency in NM, which contains easily assimilable carbon sources like organic acids and amino acids. Such behaviour could be a significant advantage for its application in a complex environment. Other bacterial strains have instead shown catabolic repression in phenol degradation caused by the presence of rich carbon sources (Ribeiro Bastos *et al.* 2000).

As the presence of heavy metals affects the microbial growth and the remediation of organic contaminants, the simultaneous removing of Cr (VI) and phenol could be a complex problem (Sandrin and Maier 2003). In addition, industrial effluents often have fluctuations in their composition, physicochemical parameters and concentration of contaminants, which can generate unfavourable conditions for the establishment and maintenance of microbial communities with bioremediation ability (Kibret *et al.* 2000; Tadesse *et al.* 2004). Hence, the selection of micro-organisms for the biotreatment of effluents depends not only on their removal efficiency but also on their adaptability to different environmental conditions. Effluents co-contaminated with Cr and phenol can be poor nutritionally, such as those from metallurgic and chemical industries, or they can present high organic matter content, such as the effluents from tannery, paper, textile and pharmaceutical industries (Tiku *et al.* 2010; Tripathi *et al.* 2011; Gunasundari and Muthukumar 2013). In this context, the consortium SFC 500-1 showed high versatility to simultaneously remove Cr (VI) and phenol both in rich and mineral media; but those media with high nutrient content were more suitable due to clearly favoured Cr (VI) removal. The easily biodegradable organic matter could act as electron donor for the reduction of metals and also as cell protection against the toxicity of contaminants (Polti *et al.* 2007).

Additional assays demonstrated that SFC 500-1 achieved successful Cr (VI) and phenol removal over a wide range of pH values (5–11), temperatures (20–35°C) and agitation rates (120–250 rev min^{-1}). These results confer to this consortium certain advantages for its use in the treatment of effluents, considering that many bacteria employed in bioremediation are efficient in a

restricted range of environmental conditions (Wang and Xiao 1995; Shourian *et al.* 2009).

Under optimal operational conditions (pH 7, 28°C, 200 rev min^{-1}) SFC 500-1 achieved a high simultaneous removal rate after 24 h. At this time the maximum biomass growth was also reached, being *Acinetobacter* sp. the major bacterial strain in the whole experiment. This last finding could be related to the involvement of each strain in the removal of pollutants. As far as this is concerned, some authors have reported that the relative abundance of bacterial species within certain natural consortia did not change significantly during the detoxification of pollutants, noting the prevalence of those strains with the best bioremediation ability (Desai *et al.* 2008). Furthermore, Cr (III) was the predominant product of Cr (VI) reduction detected in supernatant after incubation for 72 h, which is strong evidence of reductase activity in SFC 500-1. On the other hand, considering that most of the Cr (III) formed was associated with the cellular biomass, mechanisms of absorption, adsorption and/or accumulation could be involved. As it is well known, Cr (III) accumulation induces cell damage, which could explain the viable cell loss after the removal process (Cervantes and Campos-Garcia 2007).

The consortium SFC 500-1 achieved high removal percentages for 12 different combinations of Cr (VI) and phenol concentrations. In these experiments, all phenol concentrations were completely removed in the presence of Cr (VI), but high Cr (VI) concentrations slowed down the process rate. This could be related to the capability of Cr (VI) to bind itself to functional groups of proteins, such as those involved in phenol detoxification (Sandrin and Maier 2003; Ramírez-Díaz *et al.* 2008). Moreover, the addition of phenol 750 mg l^{-1} caused a significant diminution in the removal of Cr (VI) (25–50 mg l^{-1}), indicating a negative effect of combining high concentrations of heavy metals and aromatic compounds in bioremediation (Nkhalambayausi-Chirwa and Wang 2000; Song *et al.* 2009).

Based on this background and considering that the main goal of bioremediation is the application of microorganisms for the detoxification of real polluted areas, we evaluated the simultaneous removal of Cr (VI) and phenol by the consortium SFC 500-1 in the effluent from which it was isolated. The results obtained demonstrated that inoculation of SFC 500-1 in tannery wastewaters significantly enhanced the removal achieved by native micro-organisms. These assays constitute our preliminary attempt to apply native strains in the bioaugmentation of polluted effluents. *In situ* and pilot scale experiments are currently being conducted in our laboratory.

The removal of heavy metals and organic pollutants is a crucial issue. In the present work, the evaluation of Cr (VI) and phenol removal by isolated micro-organisms from a local tannery revealed that mixed cultures were more effective for simultaneous bioremediation than single strains. The consortium SFC 500-1, exhibited great potential to reduce Cr (VI) and biodegrade phenol simultaneously as well as independently and was able to jointly remove 12 different combinations of the pollutants. Phenol as only carbon source allowed microbial growth, suggesting that it could be degraded. Cr (VI) was reduced to Cr (III), which was associated with cellular biomass.

In conclusion, the removal of heavy metals and organic pollutants is a crucial issue. In the present work, the evaluation of Cr (VI) and phenol removal by isolated microorganisms from a local tannery revealed that mixed cultures were more effective for simultaneous bioremediation than single strains. One of them, the consortium SFC 500-1, exhibited great potential to reduce Cr (VI) and biodegrade phenol simultaneously as well as independently. The consortium was able to jointly remove 12 different combinations of the pollutants: up to 23 mg l^{-1} of Cr (VI) and 750 mg l^{-1} of phenol were removed in less than 72 h at a wide range of pH values and temperatures. Phenol as the only carbon source allowed microbial growth, suggesting that it could be degraded. Cr (VI) was reduced to Cr (III), which was associated with cellular biomass.

The ability of SFC 500-1 to simultaneously reduce Cr (VI) and degrade phenol in different synthetic growth media and even in the effluent from which it was isolated with high efficiency makes this consortium a potential candidate for the biotreatment of effluents.

Acknowledgements

P.S.G and E.A. are members of the research career from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Argentina). O.M.O has a fellowship from CONICET. This work was supported by grants from PPI (SECyT- UNRC), CONICET, MinCyT Córdoba and PICT (FONCyT). We thank Prof. Florencia Sgarlatta for language correction of the manuscript.

Conflict of Interest

No conflict of interest declared.

Compliance with ethical standards

Disclosure of potential conflicts of interest

We have no conflict of interest. The manuscript has not been submitted to another journal and all authors agree to submit it to Journal of Applied Microbiology. If the

manuscript is accepted, the authors will transfer the copyright of the article to the publishers.

There are not conflicts of interest between the authors of the manuscript and the organisms that finance this investigation. The authors belong to Universidad Nacional de Río Cuarto, which is a public organism. This research work was not supported by any private financial entity.

The funding support is being provided by these public organisms: Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Universidad Nacional de Río Cuarto (SECyT- UNRC) and Ministerio de Ciencia y Tecnología, Córdoba province (FONCyT, MinCyT). These organisms have been cited in the Acknowledgements section.

Research involving Human Participants and/or Animals

In this research work, animals or human have not been involved.

Informed consent

All authors have read the Ethical Rules and agree with them.

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