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Short communication

Chromate reduction by *Arthrobacter* CR47 in biofilm packed bed reactors

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Abstract

Bacterial strain Cr47 was isolated from a landfarming process soil sample. It was identified, by 16s rDNA sequencing, as *Arthrobacter* sp. The time course of the Cr(VI) reduction was monitored in batch operated packed bed biofilm reactors (12mL void volume) and in recirculating packed bed biofilm reactors (100 mL void volume) inoculated with bacterial strain Cr47. The reduction was evaluated with, 30 mg L^{-1} Cr(VI) laboratory solutions prepared with $K_2Cr_2O_7$ and enriched with glucose-minimal medium, and with 30 mg L^{-1} Cr(VI) industrial model solutions prepared with chrome plating waste waters enriched with sucrose-minimal medium. Under batch mode the reduction reaction by the biofilm seemed to fit well an exponential-decay model with a first order kinetic parameter of 0.071 mg(L h)$^{-1}$ Cr(VI). In the recirculating reactor, monitored after 4 weeks from inoculation and fed with laboratory solutions the removal rate was 0.79 mg(L h)$^{-1}$. In the reactor fed with the industrial model solutions the maximum Cr(VI) removal rate attained was 0.49 mg(L h)$^{-1}$. *Artrobacter* sp. packed bed biofilm reactors achieved Cr(VI) reduction rates comparable to other aerobic and anaerobic fixed film bioreactors previously reported.

Keywords: Chromate; Biofilm reactor; Biological treatment; Recirculating reactor; Reduction; Kinetics

1. Introduction

Cr(VI) (chromates) are a common residue of industrial processes such as, leather tanning, corrosion control, plating and pigment manufacture. In a study published by DAMA-IDEAM (Bogota's municipal environmental protection agency) on industrial effluents in Bogotá in the year 2002 was reported that the two kinds of economical activity that produced waste water discharges contaminated with Cr(VI) were the leather tanning and the metal plating industry. The percentage of those discharges that did not complied with the environmental regulation (0.5 mg L^{-1}) was 54.9% and 78.8% respectively. The average concentration of Cr(VI) in the discharges from the leather tanning industry was 13.23 mg L^{-1}, and 61.71 mg L^{-1} in the metal plating industry. This is causing a discharge of 450 kg day^{-1} of chromium to the city's river. There are reports of higher chrome urine levels in the general population (not directly involved in the tannery or chrome plaiting labor) that lives and works in those tannery and chrome plating districts. The estimated percentage of this population with urine chromium levels above the accepted 10 μg L^{-1} limit is 6.3%. Health problems that have been associated with labor in industries that use or produce chromates, has become in Bogota's case a public health issue. The great majority of those small tanneries and chrome plating shops will loose their financial viability if they had to implement traditional treatment methods, which require the use of large quantities of expensive chemical reagents.

Chromates are highly soluble in water which gives them a high mobility in aquatic environments. Chromates are strong oxidizing agents that can react with nucleic acids producing mutagenic and carcinogenic effects [1,2]. The most widely accepted hypothesis of the mechanisms of Cr(VI) toxicity involved entry into the cells by the sulfate transport channels and subsequent intracellular reduction to Cr(V) and reactive oxygen species. However According to recent data [3,4], extracellular reduction of Cr(VI) in animals may occur in the initial steps of Cr(VI) metabolism. Evidence is mounting that observed long lived Cr(V) complexes are stabilized by a combination of extra-

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cellular and intracellular species. In either case, typical aerobic, one electron reduction of Cr(VI), results in the formation of reactive oxygen species, however if they are formed extracelluarly damage to DNA can be reduced.

A great variety of bacteria, including strains from *Pseudomonas* sp., *Bacillus* sp. sulphate-reducing bacteria (SRB), and *Microbacterium* sp., have the ability of reducing Cr(VI) [5–9]. These bacteria reduce the highly toxic and soluble Cr(VI) to Cr(III) which spontaneously forms insoluble oxides (solubility <0.01 mg L^{-1}) and hydroxides at pH above 5, making the metal ion less bioavailable and less toxic [5,10]. The Cr(VI) reduction mechanism in the *Arthrobacter* sp. has been elucidated in recent studies with *Arthrobacter oxydans*. Its been reported that macromolecules at the cell wall of *A. oxydans* could act as ligands and electron donors to Cr(VI) and as stabilizers of Cr(V) intermediates. The reduction of Cr(VI) in *A. oxydans* begins with the formation of Cr(V)-diol complexes at the surface of bacteria [4].

Besides this ability to chemically transform Cr(VI), bacterial cells are excellent nucleation sites for the formation of Cr(III) complexes [6,12]. This is a desirable property for biological waste water treatments where biomass is usually separated, from the water, by sedimentation. Biofilms have been reported to be more resistant to Cr(VI) toxicity than suspended growth cells [11,13], concentration gradients, complex formation, ion entrapment and the stationary phase of the biofilm biomass are possible reasons for the superior resistance [11].

Conventional methods for removing chromates from industrial waste waters usually include chemical reduction, with sulfurous acid, followed by NaOH-induced precipitation [14]. This process has high energy costs and requires big quantities of chemical reagents. Other methods immobilize the chromate ions in a solid phase, without reducing it; these include ionic exchange, adsorption in activated carbon, among others. These methods may have low costs but desorption usually occurs, when the adsorbent solid is deposited in landfills, contaminating ground waters.

The use of biofilm packed bed reactors for the biological reduction of Cr(VI) has been previously reported by Chirwa and Wang [13]. *Bacillus* sp. was used in that work for the transformation of Cr(VI) into Cr(III). Dermou et al. [15] also reported the biological reduction of Cr(VI) in a pilot-scale tricking filter inoculated with an industrial sludge containing strains from the *Acinetobacter* sp. Other studies have reported the use of anaerobic bacteria and conditions in film fixed bioreactors to reduce Cr(VI) [16,17]. Among these micro-organisms, sulphate-reducing bacteria (SRB) are known to readily use hydrogen and indirectly reduce Cr(VI) by hydrogen sulphide or by using Cr(VI) as a terminal electron acceptor [16].

The aim of this study is to use the Colombian native Cr(VI)-reducing *Arthrobacter* Cr47 to form Cr(VI)-reducing biofilms in gravel packed bed reactors. The Cr(VI) reduction, in water contaminated with the average reported concentration of Cr(VI) for the discharges in Bogotá, was evaluated. The time course of the Cr(VI) reduction was monitored in batch operated packed bed biofilm reactors and in flow-through packed bed biofilm reactors with recirculation flask. To further evaluate the capacity, of the proposed system, to treat an industrial waste water contaminated with Cr(VI), tests were done with Cr(VI) industrial model solutions prepared with a chromic acid bath from a chrome plating industry. The Carbon source in the growth medium was changed from anhydrous glucose to commercial sucrose, a more available and cheaper sugar (in the Colombian market). For industrial applications the sugar cane molasses, a by-product of the sugar production, that contains mostly sucrose can be used as growth medium.

2. Materials and methods

2.1. Bacterial strain

The strain used in this study, for its potential as a chromate reducer, *Arthrobacter* Cr47, is an aerobic, non-spore-forming bacterium. The strain was isolated from a soil sample, contaminated with hydrocarbons and heavy metals, taken from a landfarming process in Cundinamarca, Colombia. It was selected from a group of 14 strains isolated, from the same soil sample, for its ability to grow in the presence of chromate and for its higher Cr(VI) reduction rate. It was identified using 16S rRNA analysis. The gene was amplified by PCR, first with external primers GM3F (5′ AGAGTTTGATCMTGGC 3′) and GM4R (5′AAGTCGTAACAAGGTA 3′) and then with internal primers 357F (5′ACTCCTACGGGAGGCAGCAG 3′) y 1087R (5′GGGTTAAGTCCCGCAACGAG 3′). The sequence obtained was compared with the available sequences in the internet web site Ribosomal Database (http://rdp.cme.msu.edu/index.jsp).

2.2. Media

Arthrobacter Cr47 was grown and maintained in Luria broth: 5 g L^{-1} of yeast extract, 10 g L^{-1} NaCl and 10 g L^{-1} triptone. For chromate reduction, a glucose-minimal medium was used. This was made in two parts, autoclaved separately, and mixed at room temperature prior to use. Part A consisted of 10 g L^{-1} of glucose, 2.67 g L^{-1} of NH_4Cl, 5.35 g L^{-1} of Na_2HPO_4. Part B (6 mL per L of A) consisted of, 0.1 g L^{-1} of $CaCl_2{\cdot}2H_2O$, 10 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 0.07 g L^{-1} of $MnSO_4{\cdot}7H_2O$, 0.4 g L^{-1} of $FeSO_4{\cdot}7H_2O$. The sucrose-minimal medium contained the same salts but 30 g L^{-1} of sucrose, and it was not autoclaved, the sucrose and salts were dissolved together in tap water over a heater.

2.3. Cr(VI) analysis

Chromate reducing activity was measured as the decrease of Cr(VI) with time using the colorimetric reagent diphenylcarbazide [19]. The diphenilcarbazide solution was prepared by dissolving 250 mg of 1,5-diphenylcarbazide in 50 mL of acetone, the solution was stored in a brown bottle at 4 °C [20]. The reaction mixture was done in essay plastic tubes as follows, for the upper part of the concentration range (30–5 mg Cr(VI) L^{-1}): 4.72 mL of distilled water, 160 μL of sample, 20 μL of H_2SO_4 10% v/v, and 100 μL of the diphenylcarbazide solution, giving a total volume of 5 mL. For the concentration

range between 5 mg L^{-1} and 0.5 mg L^{-1}: 3.88 mL of distilled water, 1000 μL of sample, 20 μL of H_2SO_4 10% v/v, and 100 μL of the diphenylcarbazide solution. The mixture was left to react for 5–10 min and then spectrophotometric measurements were made at 540 nm, using distilled water as blank. The samples were centrifuged for 4 min at 12 rpm, before preparing the colorimetric reaction mixture, to avoid turbidity interference in the measurements. When it was not possible to centrifuge the sample, the reaction mixture before adding the diphenylcarbazide was used as blank in the spectrophotometric measurements.

2.4. Growth profile

For the growth profile 30 mL of liquid minimal medium with Tris (MMS-T) [21] and 1% glucose was used. The required volume of inoculum for an initial O.D. of 0.003 at 660 nm was centrifuged and washed twice with MMS-T before the inoculation. The growth was monitored at two different $K_2Cr_2O_7$ concentrations (0.15 and 0.3 mM) and a control with no metal ion. After inoculation initial O.D. at 660 nm was verified and the inoculated media was incubated at 30 °C with shacking at 180 rpm. The O.D. was monitored every 24 h for 168 h.

2.5. Experimental set-up: batch operated packed columns

PVC tubes of 1/2 an inch nominal diameter and a length of 10 cm, with fabric filters at both ends, for gravel retention. The columns were filled with approximately 20 g river gravel with a mean diameter of 3 mm; the void volume inside the packed column was 12 mL.

2.6. Experimental set-up: SBR packed columns with recirculation

PVC tubes of 1 inch nominal diameter and a length of 40 cm, with fabric filter, for gravel retention. The columns were filled with river gravel with a mean diameter of 3 mm; the void volume inside the packed column was 100 mL. The total volume of the batch treated in each cycle was 500 mL. The columns where installed vertically; the batches were pumped through the column from bottom to top at a flow rate of 100 mL min^{-1} and reticulated back to the flask. For temperature control a flexible resistance, coiled around the packed column, and a thermostat set at 30 °C were used. In Fig. 1 a diagram of the experimental set-up is shown.

2.7. Inoculation and biofilm formation

Arthrobacter Cr47 was cultured aerobically with shaking at 30 °C for 24 h in 50 mL of Luria broth. Cells were harvested by centrifugation (4 min at 12 rpm) and resuspended in 500 mL of glucose or sucrose-minimal medium, with out Cr(VI). The inoculated medium was recirculated trough the column for a period of 1 week. The temperature was kept at 30 °C, and the media, in the recirculation flask, was continuously aerated with an air compressor. At the eighth day the cells were harvested by centrifugation and resuspended in a new 500 mL batch of aerated glucose or sucrose-minimal medium, the refreshed inoculum was recirculated for another 8 days. The same procedure was repeated during 4 weeks. At the end of the fourth week, before starting the Cr(VI) reduction experiments, the columns were thoroughly flushed with tap water, to remove unattached biomass. For the batch operated columns a similar procedure was used but with 10 mL batches and no recirculation (static liquid phase). The growth medium solutions were saturated with air at the beginning of each new cycle. With the specified volume the batch-operated packed beds were completely flooded.

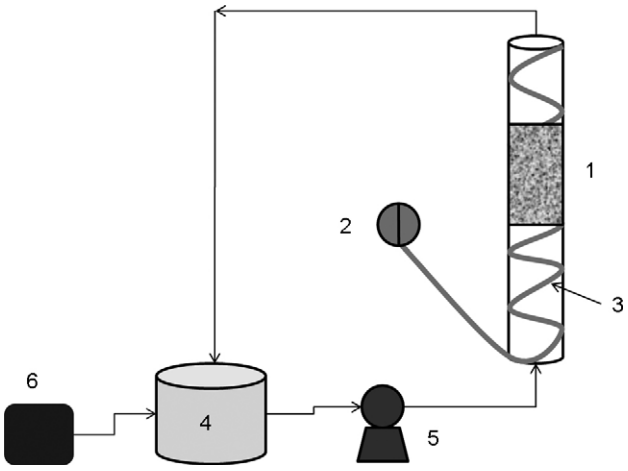

Fig. 1. Recirculating packed bed biofilm reactor. (1) Biofilm packed bed column, (2) thermostat, (3) electric heater, (4) recirculation flask (sampling), (5) pump and (6) air compressor.

2.8. Column operation

Batches containing 500 mL of glucose-minimal medium (10 mg L^{-1} electron donor) with 500 μL of a $K_2Cr_2O_7$ 300 mM solution were placed in the recirculation flask and pumped through the column. The $K_2Cr_2O_7$ solution was continuously aerated, in the recirculation flask, with an air compressor. Samples were taken from the recirculation flask to determine the Cr(VI) concentration. Before the beginning of each operating cycle the columns were thoroughly flushed with tap water, to remove unattached biomass. For the abiotic control, batches containing 500 mL of distilled water with 500 μL of a $K_2Cr_2O_7$ 300 mM solution were poured into the column, the electron donor was intentionally excluded from the mix to minimize contamination. To prepare the 30 mg Cr(VI) L^{-1} solutions with the chromium acid plating bath, which had a concentration of 76.9 mg Cr(VI) L^{-1}, 200 μL were mixed with 500 mL of sucrose-minimum medium (30 mg L^{-1} electron donor). For the batch operated columns a similar procedure was used but with 10 mL batches and no recirculation. The Cr(VI) and media solutions were saturated with air before the beginning of the operating cycle. Samples were taken from the top of the columns to determine Cr(VI) concentration. Before each sampling the columns were shaken, as an attempt to obtain an average of the Cr(VI) concentration inside the packed bed.

3. Experimental results

3.1. Bacterial strain identification

The bacterial strain Cr47 isolated from soil a sample from a landfarming process was identified as *Arthrobacter* sp (97–99% similitude with reported sequences); which belongs to the usual soil microbial population. Soil bacteria might be the best choice for packed bed bioreactors, because this kind of bacteria is well adapted for growth and biofilm formation in porous granular media. The Cr(VI) reduction mechanism in the *Arthrobacter* sp. has been elucidated in recent studies with *A. oxydans*. It has been reported [3,4,18] that the mechanism responsible for chromate resistance in *A. oxydans* is a positively charged surface protein and a capsule that plays a role in the entrapment and chemical transformation of Cr(VI).

The effect of two different Cr(VI) concentrations in the growth kinetics of suspended biomass for bacterial strain Cr47 were evaluated. The growth profile, monitored for seven days, allowed the observation of the different growth stages. Growth was significantly inhibited by chromate, but the twofold increase in chromate concentration (from 15 mg L^{-1} to 30 mg L^{-1}) did not reflect significantly in the growth kinetics. With an initial Cr(VI) concentration of 30 mg L^{-1} after a longer lag phase, bacterial strain Cr47 reached the exponential phase and significant growth was observed.

3.2. Batch operation

The results of four batch operating cycles, in the biofilm packed bed reactors, after 4 weeks from inoculation and being fed with potassium dichromate solutions enriched with glucose-minimal salt medium, are shown in Fig. 2. Although completely mixed conditions, are not expected under this operating mode, the sampling procedure used and the small size of the packed bed (10 mL void volume), minimizes the effect of the phenomena that could lead to the creation of highly differentiated zones inside the bioreactor. Allowing an estimate of the overall Cr(VI) reduction capacity of the reactor, to be elucidated. Complete reduction of Cr(VI) (100% removal) was attained in about 26 h. The operating cycles were stable and replicable. The concentration–time data points, of each cycle, had a behavior that fitted well to an exponential-decay model ($S_a = S_{a0}e^{-k_b t}$). The value for the kinetic parameter k_b that resulted from fitting the model to the experimental data is 0.071 ± 0.012 mg(L h)$^{-1}$. No substantial change in the Cr(VI) concentration was observed in the abiotic control compared to the one observed in the inoculated column. This indicated that adsorption of Cr(VI) ions in the solid biofilm support (gravel) or physicochemical reduction were unimportant in the Cr(VI) removal process in this reaction system. In the batch reactors all the Cr(VI) solution volume is in contact with the packed bed at all times and therefore the initial biomass distribution in the system is more uniform. This is different from the recirculating SBR, where at the beginning of each cycle two distinct zones exist a biomass-rich fixed film bed and an almost biomass-free aeration basin.

3.3. SBR operation with recirculation—laboratory dichromate solutions

The result of three consecutive cycles in the recirculating reactor, after 4 weeks from inoculation and fed with potassium dichromate solutions enriched with glucose-minimal salt medium, are shown in Fig. 3. Complete Cr(VI) removal from the 500 mL recirculation batch was attained in about 45 h. After the first 20 h of operation a significant amount of unattached biofilm sludge could be observed in the recirculation flask. This reflected in the reduction data, where at 20 h the Cr(VI) removal rate increased significantly. During the first 20 h of operation, the Cr(VI) reduction was done almost entirely by the biomass immobilized in the gravel bed. The reduction rate in the volume that stays in the recirculation flask (400 mL) was very small during this first period of time, due to the very low concentration of biomass in this part of the reaction system. During this period the Cr(VI) removal rate had a value of 0.5 mg(L h)$^{-1}$. At 20 h the unattached sludge began to be important in the overall Cr(VI) removal efficiency of the recirculating reactor, and therefore a

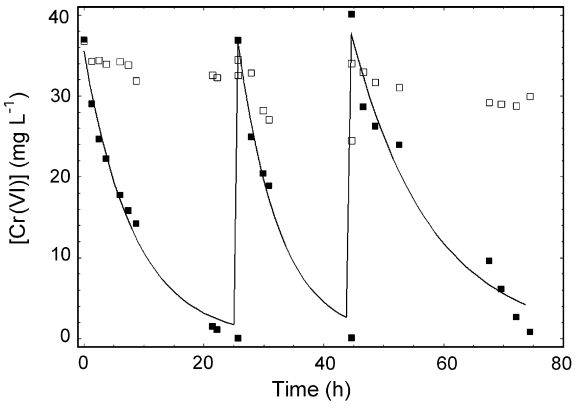

Fig. 2. (■) Operating cycles of the biofilm packed bed reactor under batch operation; (□) time course of Cr(VI) concentration in the abiotic biofilm packed bed reactor; (–) exponential decay model fit. Shortest operating cycle 25.72 h.

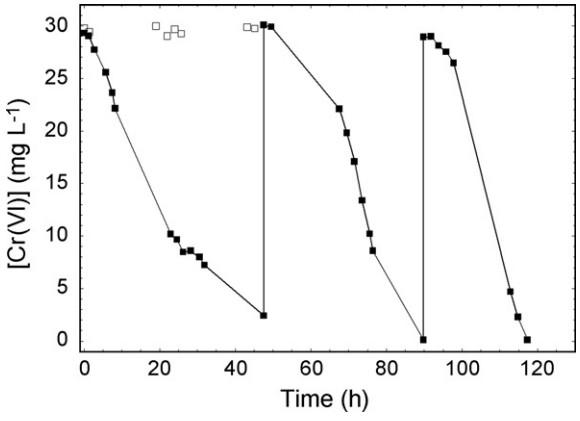

Fig. 3. (■) Operating cycles in the biofilm packed bed reactor under SBR recirculating mode. Laboratory potassium dichromate solutions enriched with glucose-minimum salt medium. (□) Time course of Cr(VI) concentration in the abiotic biofilm packed bed reactor. Shortest operating cycle 27.5 h.

sharp increase in the Cr(VI) reduction rate to 0.9 mg(L h)$^{-1}$ was observed. This trend in the Cr(VI) reduction rate contrasts with the smoother exponential behavior observed in the batch operated reactors. Where the shorter operating cycles and the initial uniform biomass distribution make the Cr(VI) reduction rates less dependent on unattached biomass growth.

3.4. SBR operation with recirculation—industrial model waste water

To further determine the industrial feasibility of the SBR packed bed biofilm reactor, the experimental apparatus was operated using industrial waste water enriched with an industrial grade carbon source. Although Cr(VI) was fed after 4 weeks from inoculation, in this case, differently from the column operated with laboratory solutions, the system did not reach an steady state until the eight week. Three operating cycles of chromate reduction observed in the SBR recirculating reactor, after 8 weeks from inoculation and fed with chrome plating bath solutions enriched with sucrose-minimal medium, are shown in Fig. 4. Complete Cr(VI) removal from the 500 mL batch was attained in about 70 h. In this case, the increase of turbidity, as an indicator of unattached biomass, in the recirculation flask, was slower. This is reflected in the smoother behavior of the concentration–time data. Although the increase in the Cr(VI) removal rate at 20 h is present, it is much less pronounced than in the system with dichromate solution and glucose as electron donor. Also, as stated above, to reach the stable cycles shown in Fig. 4 it was required twice the time taken in the system with laboratory dichromate solutions enriched with glucose. We sate two possible hypotheses for the slower growth of attached and unattached biomass in the industrial model system. A first cause might be a slower metabolic pathway for sucrose in the bacterial strain Cr47. A secondary cause could be related to the presence of other toxic substances in the industrial waste water that could further inhibit the growth of Cr47.

In all the cases and operating modes that were studied in this work, after the start up period was completed, the operating cycles reached stability and reproducibility indicating that chromate reduction capacity of the biofilm is not lost over time. At the end of each reduction cycle a sedimentable sludge was formed in the recirculation flask.

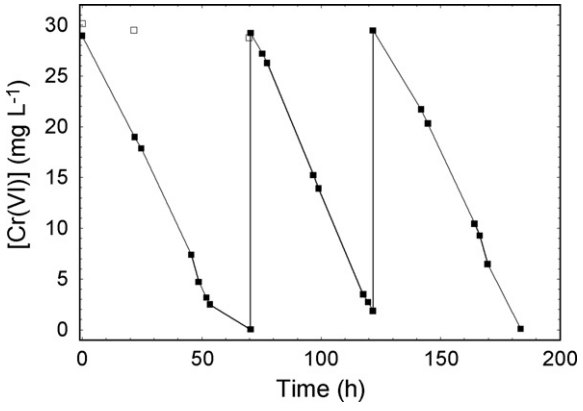

Fig. 4. (■) Operating cycles in the biofilm packed bed reactor under SBR recirculating mode. Chrome plaiting waste water solutions enriched with sucrose-minimum salt medium. (□) Time course of Cr(VI) concentration in the abiotic biofilm packed bed reactor. Shortest operating cycle 51.25 h.

4. Discussion

The packed bed recirculating SBR bioreactor described in this study is a promising alternative for a more economical way of treating the chromate contaminated effluents of the small tanneries and chrome plating shops. The SBR with recirculation bioreactor is a very convenient set-up for the required small-scale industrial application. Although there is an initial uneven biomass distribution in the system, due to the relatively large volume chosen for the recirculation flask, the system reaches very high reduction rates after 20 h of operation. The recirculation flask also serves as an aeration basin, that contributes well to the formation of sedimentable sludge. The flushing of the packed bed before each cycle ensured that the only biomass present in the reaction system at the beginning was the attached biomass. Therefore biofilm formation by *Arthrobacter* Cr47 in the support media allows excellent biomass retention; eliminating the need for sludge recirculation and the pumping energy costs that this implies. The bacterial strain used, was also capable of standing the non-sterile conditions (possible contamination) present through all the start up and operating cycles.

Chirwa and Wang [13] reported Cr(VI) reduction in a fixed film bioreactor. They inoculated their bioreactor with a *Bacillus* sp. strain and operated it in a continuous mode with a high recycle ratio. After 24 days from inoculation their bioreactor removed about a 50 mg L^{-1} in a 24 h detention time. That means an average reduction rate of approximately 2 mg(L h)$^{-1}$, which is about twice the maximum rate observed in our biofilm bioreactors. However with a reactor configuration and operation mode more similar to the one presented in this work, Dermou et al. [15] reported the reduction of 30 mg L^{-1} Cr(VI) in an aerobic trickling filter inoculated with a consortium containing *Acinetobacter* sp. and with sodium acetate as carbon source. After a start up (biofilm formation) period of 40 days, they achieved a minimum operation cycle of 19 h, under batch mode operation. Under the same mode our batch reactors achieved a minimum operating cycle of 25.7 h, after 28 days from initial inoculation. Under SBR mode Dermou et al. [15] trickling filter reached operating cycles as short as 40 min. However this was only attained after 50 days from inoculation. For the operations periods monitored in our study (up to 35 days) their operating cycles were of around 30 h. This is actually very close the minimum operating cycle that we observed in the reactor fed with the dichromate solutions. The reductions rates that we report are also comparable to the ones reported by Battaglia-Brunet et al. [16], in anaerobic fixed film bioreactors inoculated with *Desulfomicrobium* sp. With a feed containing 40 mg L^{-1} Cr(VI), 600 mg L^{-1} sulfate and H_2, they reported a reduction rate of 1 mg(L h)$^{-1}$. Our SBR operated reactors achieved quite similar values, 0.79 mg(L h)$^{-1}$ for the one being fed with dichromate laboratory solutions, and 0.49 mg(L h)$^{-1}$ for the column fed with industrial model solutions. Although the cited studies all used different reactor set-ups, different beds, and different electron donors, and

for that reason the comparison is not completely accurate, it does shows that the *Artrobacter* sp. packed bed biofilm reactors achieved Cr(VI) reduction rates comparable to other aerobic and anaerobic fixed film bioreactors.

5. Conclusions

Colombian native bacterial strain Cr47 was isolated from a contaminated soil sample and selected for its ability to grow in the presence of chromates and for its capacity to yield a higher Cr(VI) reduction rate than other strains present in the same soil sample. The results obtained in this study show that the technical feasibility of a *Arthrobacter* Cr47 biofilm packed bed bioreactors, in batch and recirculating mode, to remove Cr(VI) from water contaminated with concentrations up to 30 mg L^{-1} is promising. The recirculating mode will probably be more appropriate for industrial-scale applications, because in this type of system better mixing and aeration can be attained, improving biomass growth and performance.

Tests were made with solutions prepared with chrome plating baths enriched with sucrose as model industrial waste water. The Cr(VI) reduction results were satisfactory. We gathered enough evidence to state that, at least for waste waters from the chrome plating industry the biological treatment is a feasible alternative to the traditional methods. Bacterial strain *Arthrobacter* Cr47 proved to be very well suited for biofilm formation, in the support media used, and for the formation of sedimentable sludge.

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