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Towards a novel two-phase liquid–liquid bioreactor for microbial Cr(VI) removal from wastewaters

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ABSTRACT

The scope of the present study was to identify the most effective and less toxic combination of various organic solvents and extractants for the extraction of Cr(VI) from synthetic wastewaters with extremely high chromium concentration (up to 1,000 ppm) in order to be further used in a novel two-liquid-phase bioreactor for microbial reduction of Cr(VI). In terms of organic solvents, hexane, heptane, chloroform, ethyl-acetate, and kerosene were tested, whereas Aliquat 336, TOPO, and TPB were used as extractants. The effect of the pH of the aqueous phase and of the ratio of organic to aqueous phase were studied in terms of Cr(VI) extraction. The extraction capacities of different combinations of solvents/extractants and the addition of 1-hexanol as a stabilizer for the most effective separation of phases were also assessed. Moreover, the toxicity of each solvent was assessed using acclimated mixed consortia. The acclimated, enriched culture was further used as inoculums for the start up of a sequential batch reactor for the reduction of Cr(VI) with quite promising results. When operating the bioreactor as a two-phase system, however, the microbial consortium finally collapsed. This was attributed to the toxic effect of the extractant Aliquat 336, when being in prolonged contact with the micro-organisms.

Keywords: Hexavalent chromium; Microbial reduction; Bioremediation; Two-phase bioreactors; Anaerobic sludge

1. Introduction

Chromium has been used extensively in industrial processes such as leather tanning, electroplating, negative and film making, paints and pigments processing, and wood preservation. Through these processes, a large amount of chromium (approximately 4.500 kg/d) is discharged into the environment in the form of wastewater with high chromium concentrations. Chromium generally exists in water in two stable oxidation states: hexavalent [Cr(VI)] and trivalent [Cr(III)]. Hexavalent chromium is of particular environmental concern due to its toxicity and mobility, being a strong oxidizing agent that is carcinogenic and mutagenic [1]. Consequently, its removal from any type of wastewater that is discharged in the environment is of great importance. In contrast, Cr(III) is less

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toxic and can be readily precipitated out of solution in the form of $Cr(OH)_3$. Chromium from the anthropogenic sources is discharged into the environment mainly as hexavalent chromium. In addition to this, recent studies have shown that Cr(VI) can be formed naturally in the environment. It is for this reason that most remediation efforts target the removal of Cr(VI) primarily. According to the Environmental Protection Agency (EPA), the maximum Cr(VI) concentration in potable water is 0.05 ppm, whereas for total chromium, it is 0.1 ppm. According to the Greek legislation, the maximum daily effluent concentration for industrial wastewater should range from 1.2 to 3 ppm, whereas for potable water, the maximum allowable Cr (VI) concentration is 0.05 ppm [2].

One effective way for Cr(VI) removal from an effluent is its reduction to the less toxic and soluble Cr(III). Biological reduction of Cr(VI) through microorganisms is one of the most common and promising methods of Cr(VI) reduction to Cr(III), using either pure [3] or mixed cultures [4,5]. However, high Cr(VI) in wastewater may be inhibitory to microbial growth [6]. A possible solution to this problem could be the use of two-liquid-phase bioreactors (TLPBs), consisting of the aqueous phase (wastewater) and a proper organic phase. Such bioreactors have been used since the mid-seventies for the microbial and enzymatic bioconversion of hydrophobic/toxic substrates into products of commercial interest [7], whereas they were later applied on the biodegradation of different toxic compounds of environmental relevance, such as terpenes [8], phenols [9], and polycyclic aromatic hydrocarbons (PAHs) [10]. By principle, two-liquid-phase systems consist of an aqueous phase and a water-immiscible, i.e. organic, liquid phase present in excess of mutual saturation. The organic phase has to be water-immiscible, and also nonvolatile, non-biodegradable, and of course biocompatible with the specific microorganisms that will be used for the bioconversion. The organic phase may be added to the bioreactor, aiming: (a) to continuously remove the end-products of a biotransformation process alleviating, thus decrease in the productivity due to a product-induced feedback inhibition or toxicity; (b) to control the aqueous concentration of a toxic substrate, acting as a reservoir for controlled delivery of the inhibitory compound to micro-organisms in the aqueous phase; or (c) to enhance the biotransformation of a water-insoluble substrate by increasing its bioavailability to biocatalysts [11]. TLPBs have been tested even at industrial scale [12,13], as they allow the biotransformation of larger quantities of substrates or toxic compounds in smaller reactor volumes; reduce the toxic action of the reagents and products; and, finally, facilitate the recuperation of catalysts and products.

Based on the above-mentioned characteristics of TLPBs and with the selection of appropriate organic phases as well as biological and physicochemical conditions, it is believed that such systems could be effective for the treatment of wastewater with extremely high concentrations of Cr(VI). The creation of an emulsion, caused by mixing the two phases, will cause Cr(VI) to be transferred to the organic phase and it will then return gradually to the aquatic phase, where it will be reduced by the micro-organisms. Thus, the scope of the present study was to identify the most promising organic phase to be used for the bacterial Cr(VI) removal from high-loaded wastewaters in a novel two-phase liquid–liquid bioreactor. Research primarily focused on studying the extraction efficiency of different mixtures of solvents with proper extractants, the optimization of extraction conditions, and the biocompatibility of the solvents with an enriched bacterial culture that was developed from anaerobic sludge.

2. Materials and methods

2.1. Cr(VI) extraction process

Aqueous solutions of $K_2Cr_2O_7$ were mixed with the solvent/extractant mixture in the ratio 1:1, and were kept at 25 °C and under constant mechanical agitation of 30 rpm for 24 h. After that point, agitation was ceased and the mixture was kept at ambient temperature until formation of separate phases. Subsequently, their separation was performed using a separation funnel and the remaining Cr(VI) was quantified in the aqueous phase.

2.2. Reagents

Aliquat 336 (tricaprylylmethylammonium chloride, $CH_3N((CH_2)_7CH_3)3Cl$), 1,5-diphenylcarbazide ($C_6H_5NHNHCONHNHC_6H_5$), TBP 97% (tributyl phosphate, $CH_3(CH_2)_3O)_3PO$), TOPO 99% (trioctylphosphine oxide, $[CH_3(CH_2)_7]_3PO$), and 1-hexanol ($CH_3(CH_2)_4CHOH$) were purchased from Sigma-Aldrich Co; and potassium dichromate ($K_2Cr_2O_7$) and sodium hydroxide pellets (NaOH) from Merck Millipore. All Solvents, i.e. chloroform (CHCl3), Ethyl acetate 99.8% ($CH_3COOC_2H_5$), heptane 99% ($CH_3(CH_2)_5CH_3$), hexane 95% ($CH_3(CH_2)_4CH_3$), and kerosene (purum) were purchased from Sigma-Aldrich Co.

2.3. Analytical techniques

Volatile suspended solids (VSS) and Cr(VI) in aqueous solutions were quantified according to Standard Methods for the Examination of Water and Wastewater [14]. Especially for Cr(VI), the 3500-Cr D colorimetric method was followed.

2.4. Microbial culture

An enriched bacterial culture derived from the anaerobic sludge of the Municipal Wastewater Treatment Plant of Lycovrisi, Attica, Greece was used in all cases. The enrichment took place in Erlenmeyer flasks of 1 L total volume, under meshophilic (35˚C) and anaerobic conditions (replacement of air using CO_2: N_2, 30:70 v/v) with constant mechanical agitation of 60 rpm. The basal medium (BM) used was of the following composition (g/L): molasses, 4; NH_4Cl, 1; KH_2PO_4, 1.75; K_2HPO_4, 0.25; and 7 mL/L trace elements solution. The composition of the trace elements solution was as follows (g/L): $CaCl_2{\cdot}2H_2O$, 22.5; NH_4Cl, 35.9; $MgCl_2{\cdot}6H_2O$, 16.2; KCl, 117; $MnCl_2{\cdot}4H_2O$, 1.8; $CoCl_2{\cdot}6H_2O$, 2.7; H_3BO_3, 0.51; $CuCl_2{\cdot}2H_2O$, 0.24; $Na_2MoO_4{\cdot}2H_2O$, 0.23; $ZnCl_2$, 0.19; $NiCl_2{\cdot}6H_2O$, and 0.2; H_2WO_4, 0.01. For the acclimation of the bacteria 30 ppm of Cr(VI) in the form of $K_2Cr_2O_7$ was also added in the medium.

2.5. Toxicity tests

In order to investigate the possible toxic effect of different solvents on microbial growth, liquid batch cultures were performed with the BM described above without the addition of $K_2Cr_2O_7$. The aqueous-to-organic phase ratio was 1:1 in order to simulate extraction experiments. The cultures were incubated under anaerobic conditions at 25˚C and constant agitation of 35 ppm for 24 h, and subsequently, the aqueous/organic phases were separated. Toxicity of the different organic diluents was assessed by calculating the CFU (colony-forming units). In this direction, a 150 μl aliquot of the aqueous phase was used for the inoculation of petri dish containing solid media consisting of BM supplemented with 1.5% w/v agar. For the inoculation, the spreading method was used. After the inoculation, the plates were placed in a special jar and were incubated for 48 h under anaerobic conditions (AnaeroGen™ OXOID) at 35˚C. Toxicity was estimated in terms of colony numbers.

2.6. Bioreactor setup

The bioreactor was made of glass and was of 2 L working volume. The temperature was kept constant at 35˚C by immersing the reactor in a water bath, the operation of which was monitored online. The pH and diluted oxygen (DO ≤ 0.01 ppm) were also monitored online. A synthetic wastewater (BS with glucose 2.5 g/L as carbon source) with different Cr(VI) concentrations was used as feed, which was supplied to the reactor by a peristaltic pump. The effluent (1/2 of the working volume) was also removed using a peristaltic pump. The addition of organic phase did not affect the configuration of the bioreactor (Fig. 1), but did affect only the working volumes. Moreover, an extra peristaltic pump for the discharge of the organic phase was added. The operation of pumps, as well as the agitation system, was condoled via PC.

3. Results and discussion

3.1. Investigation of the most effective combination of organic solvent extractant for Cr(VI) extraction from aqueous solutions

In order to investigate the process of Cr(VI) ions transfer from aqueous to organic phases, five batch experiments were performed with different organic solvents, i.e. hexane (C_6H_{14}), heptanes (C_7H_{16}), chloroform ($CHCl_3$), ethyl-acetate ($C_4H_8O_2$), and kerosene. The solvents were chosen with regard to their minimum miscibility with water (creation of distinct phases) and the tolerance of bacterial growth in their presence, based on the literature [15]. Since the above solvents are hardly polar, the addition of extractants in the organic phase is necessary for the transfer of ions. As extractants, three commercially available compounds, i.e. Aliquat 336 ($C_{25}H_{54}CIN$), TOPO ($C_{24}H_{51}OP$), and TBP ($C_{12}H_{27}O_4P$), were selected.

Aqueous solutions of initial $K_2Cr_2O_7$ concentration 100 ppm (or 1,000 ppm in the case of Aliquat 336) were mixed with the solvent/extractant mixture in ratio 1:1 for 24 h, at 25˚C and under constant stirring of 30 rpm. Subsequently, separation of the aqueous from the organic phase was performed using a separation funnel and the remaining Cr(VI) was quantified in the aqueous phase. The concentrations of each extractant in the solvent were 5% v/v for Aliquat 336 and TPB, and 0.1 mol/L for TOPO. The results of extraction efficiency of different mixtures are presented in Table 1. It is apparent that the chloroform/Aliquot 5% and ethyl-acetate/Aliquat 5% are the most effective mixtures for the extraction of Cr(VI) from aqueous solutions.

However, as proven by subsequent toxicity tests of the different solvents, both chloroform and ethyl acetate cannot be used for biological reduction of Cr(VI) in a two-phase system, since they both

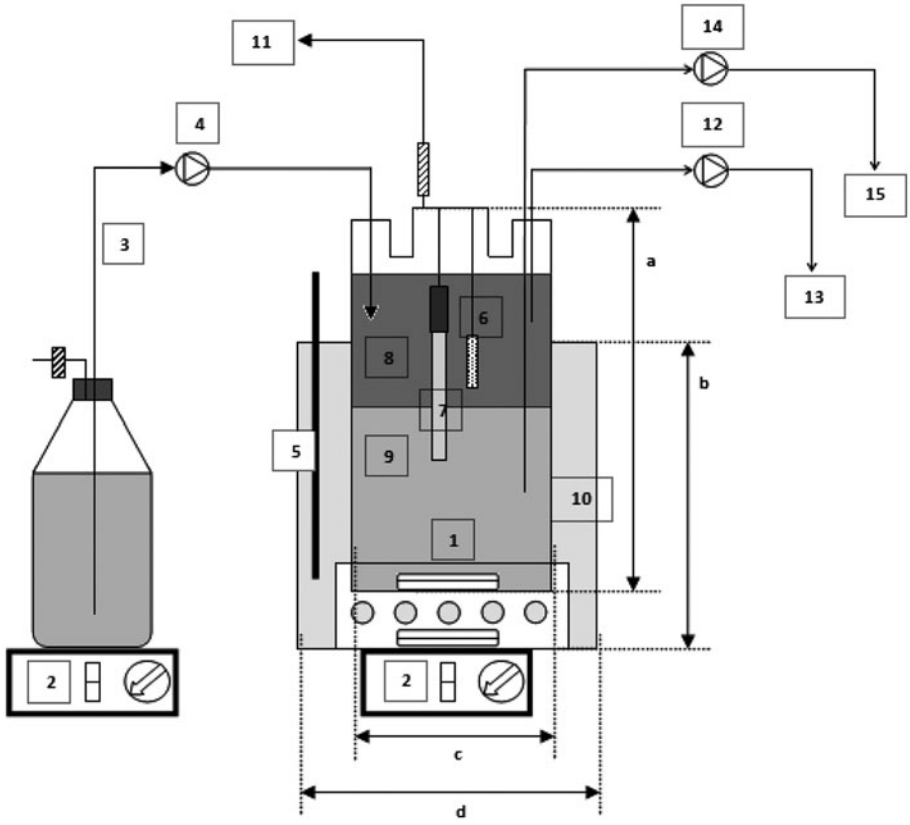

Fig. 1. Scheme of the two-phase anaerobic sequencing batch reactor. (1) Reactor, 2 L capacity ($a = 25$ cm, $c = 11$ cm), (2) magnetic stirrers, (3) influent, (4) feed pump, (5) and (6) heating resistance and temperature probe for temperature control, (7) pH probe, (8) organic phase, (9) aqueous phase, (10) Bath ($b = 20$ cm, $d = 18$ cm), (11) exhaust air, (12) discharge pump for organic phase, (13) effluent and sampling point for organic phase, (14) discharge pump for aqueous phase, and (15) effluent and sampling point for aqueous phase.

Table 1
Cr(VI) removal efficiency from aqueous solutions of $K_2Cr_2O_7$ of 1,000 (*) or 100 (**) ppm initial Cr(VI) concentration, with different solvent/extractants mixtures

	% of Cr(VI) removal			Interphase formation			$pH_{aq,initial}$		
Solvent	Aliquat*	TOPO**	TBP**	Aliquat	TOPO	TBP	Aliquat	TOPO	TBP
Hexane, C_6H_{14}	99.69	2.42	1.52	Intense	Absent	Absent	4.1	5.4	5.3
Heptane, C_7H_{16}	97.68	0.1	1.27	Intense					
Chloroform, $CHCl_3$	99.96	6.82	6.17	Absent					
Ethyl acetate, $C_4H_8O_2$	99.90	10.37	38.59	Slight					
Kerosene	98.04	0.95	0.29	Intense					

completely inhibit bacterial growth (Table 2). On the contrary, bacteria exhibited great tolerance in the presence of kerosene, thus suggesting that a kerosene/extractant mixture would be more appropriate for a two-phase liquid–liquid bioreactor. Consequently, as suggested by the experimental results presented in Table 1, the kerosene/Aliquat 336 mixture seems to be the best candidate for the operation of a two-phase liquid–liquid bioreactor. That mixture, however, although being quite efficient during extraction of Cr (VI) from aqueous solutions, has the disadvantage of forming an intense interphase when mixed with aquatic solutions, thus preventing the separation of distinct organic and aqueous phases. As a result, the formation of an interphase hinders the recovery process and the reuse of the organic solvent.

Table 2
Toxic effect of different solvent/extractant mixtures on bacterial growth

Solvent	Bacterial growth
Hexane, C_6H_{14}	−
Heptane, C_7H_{16}	+
Chloroform, $CHCl_3$	−
Ethyl acetate, $C_4H_8O_2$	−
Kerosene	++

This obstacle was overcome by the addition of stabilizer in the kerosene/Aliquat 336 mixture, and more specifically 10% v/v of 1-hexanol. The choice of 1-hexanol was based on previous studies according to which long-chain alcohols such 1-hexanol [16] and n-decanol [17] can lead to the complete separation of phases. Indeed, the addition of 1-hexanol proved to be quite successful for the separation of kerosene/Aliquat 336 mixture from water as illustrated in Fig. 2.

3.2. Optimization of conditions during Cr(VI) extraction from aqueous solutions with kerosene/Aliquat 336/1-hexanol mixture

The effect of pH is crucial for efficient bioconversions. In order to investigate the effect of the pH in the aqueous phase on the extraction process of Cr(VI), a batch experiment with kerosene/Aliquat 336/1-hexanol and aqueous solution with 1,000 ppm Cr(VI) was performed. Eight different pH values were tested, ranging from 4.3 (without adjustment) to 11. For the adjustment, 0.1M NaOH was used. As shown in Table 3, the extraction results seem to be better for acidic and neutral pH of the aqueous solution. This observation is general in agreement with previous studies regarding Cr(VI) extraction, as shown in Table 4 [18,19], as well as other heavy metals extraction from aqueous solutions using different solvent/extractant mixtures with or without the addition of stabilizers [20]. However, the opposite effect is also reported even for the same solvent/extractant mixtures, using a different stabilizer [21].

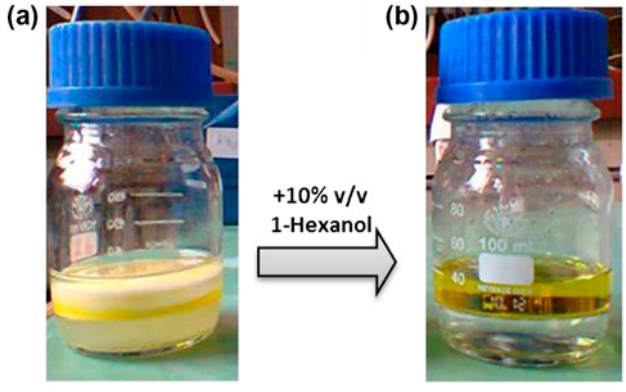

Fig. 2. Effect of 1-hexanol addition in the separation of organic to aqueous phase during the extraction of Cr(VI) with kerosene/Aliquot 336 mixture (a) formation of interface, (b) absence of interphase after addition of 1-hexanol.

For further optimization of the process, the effect of the ratio of the organic to aquatic phase on the extraction process was investigated. The internal ratio of the organic compounds was kept steady (kerosene/Aliquat, 5%)/1-hexanol, 10%), whereas the concentration of Cr(VI) in the aquatic solution was 1,000 ppm in all cases. The pH of the aqueous solution was adjusted to the values 6 and 7 in order to simulate those of industrial wastes. Batch experiments were conducted with organic:aquatic phase ratios 1:1 (control), 0.75:1, 0.5:1, and 0.25:1. The results are presented in Table 5. As shown, a 1:1 ratio seems to be more effective for both pH values tested.

3.3. Continuous Cr(VI) bacterial reduction by enriched microbial consortia

The enriched bacterial culture that was developed, as described in the Section 2, was further used for the inoculation of a bioreactor. The bioreactor was initially loaded with synthetic wastewater of 50 ppm Cr(VI) concentration and 2.5 g/L glucose as carbon source. Nitrogen and trace elements were also used as described above (BM).

The reactor was initially operated in batch mode until the complete removal of Cr(VI), as shown in Fig. 3(a). During this period, the Cr(VI) removal ratio was 13.13 ppm d^{-1}, whereas the microbial biomass growth, estimated in terms of VSS increase, was 235%. It should be emphasized that this operation was carried out only with the aqueous phase in order to assess the maximum hexavalent chromium concentration in the aqueous phase that can be effectively reduced by the specific bacterial consortium. Subsequently, the reactor was operated in sequential batch (SB) mode with increasing Cr(VI) in each operational cycle. The performance of the bioreactor for seven operational cycles is shown in Fig. 3(b). The Cr(VI) reduction rate during the operation of the reactor as SB was rather diverse, ranging from 2.8 to 15.6 ppm d^{-1}. This can be attributed to the possible variation of the suspended microbial biomass concentration during each cycle, which, however, was not quantified due to the formation of CR(III) sediments that caused extra turbidity in the aqueous phase. The

Table 3
Effect of pH on the Cr(VI) extraction efficiency of the mixture kerosene/Aliquat(5%)/1-hexanol(10%)

Initial $pH_{(aq)}$	Final $pH_{(aq)}$	Final $Cr(VI)_{(aq)}$ (ppm)	% of Cr(VI) removal
4.3	4.7	1.28	99.87
5	5.9	1.27	99.87
6	7.3	9.74	99.03
7	7.9	71.84	92.82
8	8.4	119.90	88.01
9	8.1	82.39	91.76
10	8.4	141.55	85.85
11	8.1	139.76	86.02

Table 4
Extraction efficiency of the Cr(VI) from aqueous solutions with different organic phases

Initial Cr(VI) concentration (ppm)	Solvent	Extractant	Stabilizer	% of Cr(VI) removal	$pH_{aq,initial}$/adjustment	Refs.
100	Kerosene	10% Aliquat 336	None	>98	9–10	[16]
			1% hexanol	>99		
100	Kerosene	3% TOPO	None	>70	0.1 N HNO_3	[18]
				>90	0.1 N H_2SO_4	
				>98	0.1 N HCl	
50–200	Hexane	20% TPB	5% Span 80	>20	0.2 N H_2SO_4	[19]
				>70	1 N H_2SO_4	
				>95	2 N H_2SO_4	
2.7 mM	Kerosene	3 mM Aliquat 336	10% octanol	>95	0.1 M H_2SO_4	[20]
				>65	1 M H_2SO_4	
		3 mM Alamine 336		>85	0.1 M H_2SO_4	
				>60	1 M H_2SO_4	

Table 5
Effect of organic:aqueous phase ratio on the Cr(VI) extraction efficiency of the mixture kerosene/Aliquat(5%)/1-hexanol (10%)

Initial $pH_{(aq)}$	Organic to aquatic phase ratio	Final $pH_{(aq)}$	Final $Cr(VI)_{(aq)}$ (ppm)	% of Cr(VI) removal
6	1:1	7.2	8.56	99.14
	0.75:1	7.8	15.84	98.42
	0.5:1	7.2	19	98.10
	0.25:1	7.6	50.87	94.91
7	1:1	7.8	61.26	93.87
	0.75:1	8.3	92.81	90.72
	0.5:1	8.3	129.98	87.00
	0.25:1	8.4	427.98	57.20

variation of the Cr(VI) reduction rate could further be connected to the gradual appearance of biofilm on the walls of the reactors (Fig. 4), which is actually immobilized active microbial biomass.

At the end of the seventh operational cycle, half of the reactor's working volume was replaced by the selected organic phase consisting of kerosene/Aliquat 336, 5%/1-hexanol, 10%, so that the ratio of the aqueous to the organic phase was 1:1. The aqueous phase was supplemented with fresh medium, so as to have a starting Cr(VI) concentration of 1000 ppm, the aqueous phase. In Fig. 5, the variation of hexavalent chromium concentrations in both the aqueous and the organic phases is illustrated. As shown, on the first

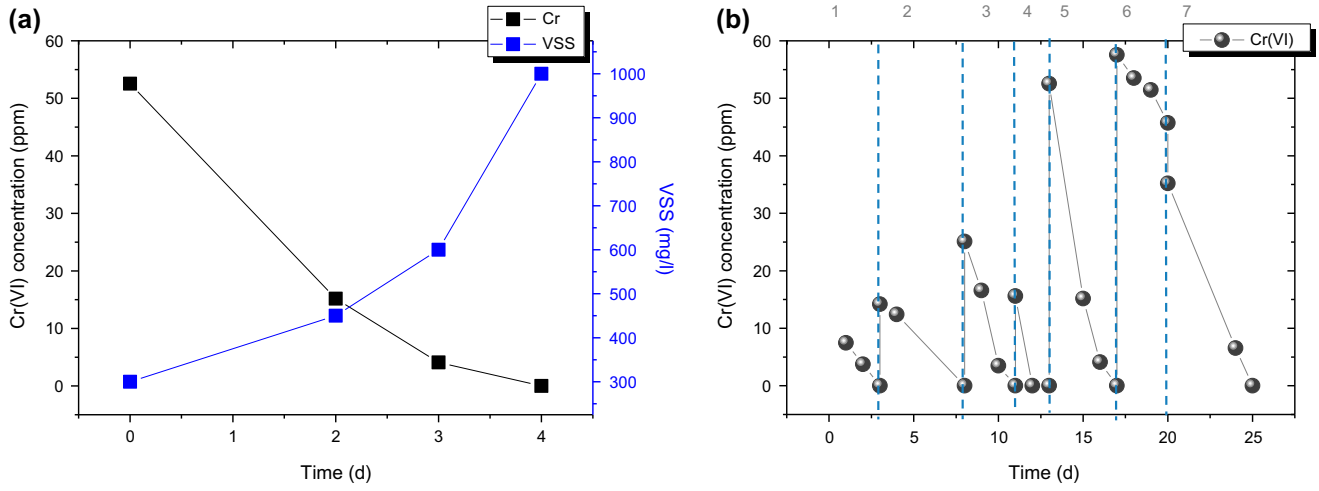

Fig. 3. Cr(VI) uptake and VVS increase (a) and Cr(VI) uptake (b) during the operation of the bioreactor at batch and SB mode, respectively.

day, ~97% of Cr(VI) was transferred from the aqueous to the organic phase as expected (Table 1). The performance of the reactor was monitored for five d, during which no change was observed in the Cr(VI) concentration; neither in the aqueous nor in the organic phase. Moreover, the pH of the aqueous phase remained constant at 6.4, whereas the biofilm that had been formed in the walls of the reactor started to detach gradually, indicating that a decay of the active biomass was taking place.

Fig. 4. Formation of biofilm on the walls of the bioreactor.

In order to further instigate this hypothesis, batch experiments were conducted with the enriched cultures using the BM without the addition of Cr(VI), and with high initial concentration of VSS (bacterial biomass). The medium was supplemented with Aliquat 336 in concentrations ranging from 5 to 0.1%. The cultures were incubated under anaerobic conditions at 25°C and constant agitation of 35 ppm for 24 h, and were subsequently used for the inoculation of solid cultures according to the protocol described in Section 2.5. Unfortunately, it was observed that the presence of Aliquat 336 was completely inhibitory for bacterial growth even at the concentration of 0.1% (data not shown).

The bibliographic data on the toxicity of Aliquat 336 on bacterial species are rather controversial. In a

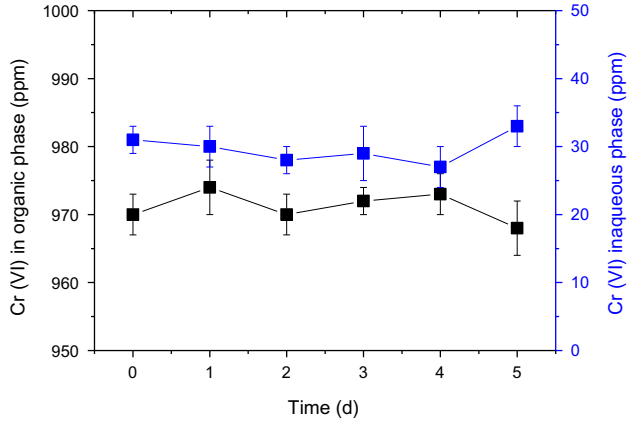

Fig. 5. Cr(VI) concentration in the aqueous and the organic phase of the TPLLB.

study with mixed culture consisting of *Bacillus cereus*, *B. pantothenticus*, *B. coagulans*, *Pseudomonas aeroginosa*, *and Lactobacillus plantarum*, no toxic effect of Aliquat 336 was reported even when the growth medium was supplemented up to its saturation limits [22]. Coelhoso et al. [23], on the other hand, reported that Aliquat 336 completely inhibited the growth of *Lactobacillus rhamnosus* when dissolved in the cultivation medium up to its saturation level and even at 10% saturation; when used up to 1% of saturation, microbial growth was not significantly affected [23]. It has to be mentioned that, in both cases, no organic phase was present during the incubation. The inhibitory effect of Aliquat could indeed be attributed to its transfer to the aqueous phase. Indeed, the relative short aliphatic chains of Aliquat 336 (mixture of C_8 and C_{10} chains with C_8 predominating), although ensuring high solubility in the organic phases, do not prevent its diffusion into the aqueous (polar) phase. The use of an Aliquat 336-based extractant with longer aliphatic chains could reduce the toxic effect of the bacterial consortium that was used in this study. Further experiments are carried out at the moment in this direction.

4. Conclusions

Two-phase liquid–liquid bioreactor could be used for the efficient extraction of Cr(VI) from wastewaters with high load. In the present study, an enriched mixed culture was developed, whereas the suitability of different solvent/extractant mixtures was evaluated with regard to the highest Cr(VI) removal capacity as well as the less toxic effect on the enriched culture. The mixture of kerosene/Aliquat 336 (5%) seems to be the most promising when used in ratio 1:1 with the aquatic phase. The addition of 1-hexanol in the organic phase was also proven to be necessary for the complete separation of the phases after the extraction process. Subsequently, the bioreactor was operated under meshophilic and microaerobic conditions for one month, initially at batch, and afterwards at continuous mode. The enriched culture was shown to be sufficient for the complete Cr(VI) removal up to 55 ppm. However, the system demonstrated rather variable removal rates, which were attributed mainly to the formation of biofilm on the walls of the reactor. When operating the bioreactor as a two-phase system with high initial chromium (VI) concentrations (of the order of 1,000 ppm), however, the microbial consortium collapsed. This was attributed to the toxic effect of Aliquat 336. A possible remedy is to replace Aliquat 336 with a less water-soluble extractant.

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