TOWARDS A NOVEL TWO PHASE LIQUID:LIQUID BIOREACTOR FOR MICROBIAL Cr(VI) REMOVAL FROM WASTEWATERS

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Abstract

The present study aims to the development of a novel two phase liquid-liquid bioreactor (TPLLB) for the microbial reduction of Cr(VI) in synthetic wastewaters with extremely high chromium concentration (up to 1000 ppm). Research has primarily focused on the identification of the most effective and less toxic combination of organic solvent- extractant and the optimization of extraction conditions. In terms of organic solvents, hexane, heptane, chloroform, ethyl-acetate and kerosene have been tested, whereas as extractants Aliquat 336, TOPO and TPB were used.

Although, the chloroform/Aliquat 336 and ethyl-acetate/Aliquat 336 mixtures proved to be most effective for the extraction of Cr(VI) from aqueous solutions, they were both quite inhibitory on microbial growth. Microorganisms on the other hand have exhibited great tolerance to kerosene, which however failed to separate effectively from the aqueous phase when used together with Aliquat 336, forming an interphase. This problem was overcome by the addition of 1-hexanol as stabilizer, which had no toxic effect on microbial growth. Subsequently, an enriched mixed microbial culture derived from anaerobic sludge, with high Cr(VI) reduction rate (15ppm/day) and tolerance to the organic diluents was developed. The acclimated, enriched culture was further used for the reduction of Cr(VI) in the TPLLB under mesophilic and microaerobic conditions, with quite promising results .

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 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, while 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, while 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). However, high Cr(VI) in wastewater may be inhibitory to microbial growth [3]. A possible solution to this problem could be the use of two-phase bioreactors, consisting of an organic and an aqueous phase. Such bioreactors have been tested extensively for various bio-transformations even at industrial scale [4-6], as they allow the transformation of larger quantities with smaller volumes (smaller reactor volume), reduce the toxic action of the reagents and products, and, finally, facilitate the recuperation of catalysts and products. Based on these characteristics and with the selection of appropriate biological and physicochemical conditions, such systems could treat 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 microorganisms.

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. The enriched bacterial culture that was used was derived from anaerobic sludge and its biocompatibility with several organic solvents was tested via toxicity tests. Concurrently, the extraction efficiency and the optimization of extraction condition were investigated for mixtures of the solvents with proper extractants. Subsequently, the bioreactor was loaded with synthetic wastewater of various Cr(VI) concentrations, up to 55ppm, and was inoculated with the enriched culture. Its performance was evaluated in batch and sequential batch mode.

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 ratio 1:1 and were kept at 25^{o}C and under constant mechanical agitation of 30rpm for 24h. 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; 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) from Sigma-Aldrich Co.

2.2. 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 [7]. Especially for Cr(VI) the 3500-Cr D colorimetric method was followed.

2.3. 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 1L total volume, under meshophilic (35^{o}C) and anaerobic conditions (replacement of air using CO_2:N_2, 30:70v/v) with constant mechanical agitation of 60rpm. The medium 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; $NiCl_2 \cdot 6H_2O$, 0.2; H_2WO_4, 0.01. For the acclimation of the bacteria 30ppm of Cr(VI) in the form of $K_2Cr_2O_7$ was also added in the medium.

2.4. Toxicity tests

In order to investigate the possible toxic effect of the 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 35ppm for 24 hours, and subsequently the aqueous / organic phases were separated. An 150 μl aliquot of the aqueous phase was then used for the inoculation of solid media (BM supplemented with 1.5% w/v agar) by the spreading method, and the plates were incubated for 48 hours under anaerobic conditions (AnaeroGen ™ OXOID) at 35^{o}C. Toxicity was estimated in terms of colonies numbers.

2.5. Bioreactor set up

The bioreactor was made of glass and was of 2L working volume. The temperature was kept constant at 35^{o}C by immersing the reactor in a water bath, the operation of which was monitored online. The pH and diluted oxygen (DO≤0.01ppm) were also monitored online. A synthetic wastewater (BS with glucose 2.5g/L as carbon source) with different Cr(VI) concentrations was used as feed, supplied to the reactor by a peristaltic pump. The effluent (1/2 of the working volume) was also removed using a peristaltic pump. The operation of both 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 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 [8]. Since the above 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 ($C_{24}H_{51}OP$), TBP ($C_{12}H_{27}O_4P$) were selected.

Aqueous solutions of initial $K_2Cr_2O_7$ concentration 100ppm (or 1000ppm in the case of Aliquat 336), were mixed with the solvent/extractant mixture in ratio 1:1 for 24h, in 25^{o}C and under constant stirring of 30rpm. 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 0.1mol/L for TOPO. The results of extraction efficiency of the 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.

Table 1. : Cr(VI) removal efficiency from aqueous solutions of $K_2Cr_2O_7$with different solvent/extractants mixturees

Solvent	% of Cr(VI) removal			Interphase formation			$pH_{aq,initial}$.		
	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*					

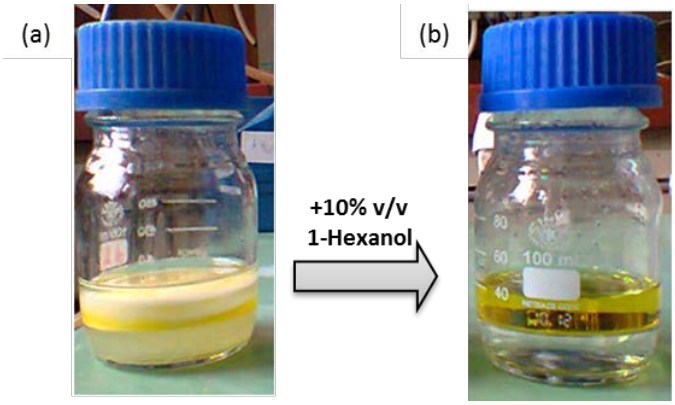
Table 2. : Cr(VI) removal efficiency from aquaeous solutions of $K_2Cr_2O_7$with different solvent/extractants mixtures

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

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 completely inhibit bacterial growth* (Table 2). On the contrary, bacteria exhibited great tolerance in the present of kerosene, suggesting thus 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, preventing thus the separation of distinct organic and aqueous phases. As a result, the formation of an interphase hinders the recovery process and reuse of the organic solvent.

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 [9] and n-decanol [10] can lead to 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 Picture 1.



Picture 1. : Effect of 1-hexanol addition in the separation of organic to aqueous phase during extraction of Cr(VI) with kerosene/Aliquot 336 mixture

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) as well, a batch experiment with kerosene/Aliquat 336/1-hexanol and aqueous solution with 1000ppm 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 extraction results seem to be better for acidic and neutral pH of the aqueous solution. This observation is in agreement with previous studies regarding heavy metals extraction from aqueous solutions using solvent/extractant mixtures [11].

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	4.67	1.28	99.87 ± 0.2
5	5.91	1.27	99.87 ± 0.1
6	7.3	9.74	99.03 ± 0.1
7	7.945	71.84	92.82 ± 0.3
8	8.395	119.90	88.01 ± 4.7
9	8.115	82.39	91.76 ± 3.6
10	8.4	141.55	85.85 ± 0.2
11	8.15	139.76	86.02 ± 0.2

For the further optimization of the process the effect of *ratio of the organic to aquatic phase* to 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 1000ppm 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 4. As shown,a 1:1 ratio seems to be the more effective for both pH values tested.

Table 4. 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	7.24	8.56	99.14
	0.75:1	7.78	15.84	98.42
	0.5:1	7.19	19	98.10
	0.25:1	7.63	50.87	94.91
7	1:1	7.83	61.26	93.87
	0.75:1	8.26	92.81	90.72
	0.5:1	8.35	129.98	87.00
	0.25:1	8.41	427.98	57.20

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

The enriched bacterial culture that was developed as described in the Materials and Methods section, was further used for the inoculation of a bioreactor. The bioreactor was initially loaded with synthetic wastewater of 50ppm Cr(VI) concentration and 2.5g/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 complete removal of Cr(VI), as shown in Figure 1a. During this period the Cr(VI) removal ratio was *13.13ppm.day^{-1}*, whereas the microbial biomass increase estimated in terms of VSS increase was 235%. It should be emphasized that this operation was carried out only with aqueous phase, in order to assess the maximum hexavalent chromium concentration in the acqueous phase that can be effectively reduced by the bacteria. 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 Figure 1b. The Cr(VI) reduction rate during the operation of the reactor as SB was rather diverse, *ranging from 2.8 ppm.day^{-1} to 15.6 ppm.day^{-1}*. This can be attributed to 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 variation of the Cr(VI) reduction rate could further be connected to the gradual appearance of biofilm on the walls of the reactors (Picture 2), which is actually immobilized active microbial biomass.

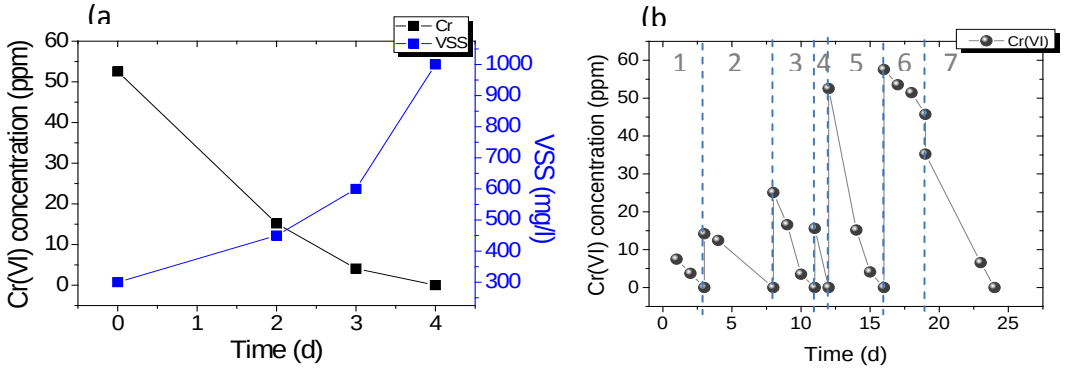


Figure 1. : Cr(VI) uptake and VVS increase (a) and Cr(VI) uptake during operation of the bioreactor at batch and sequential batch mode respectively.

Picture 2. Formation of biofilm on the walls of the bioreactor.

4. Conclusions

The present study aims at the development of a novel two phase liquid:liquid bioreactor for the efficient extraction efficiency of Cr(VI) from wastewaters with high load. 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 complete Cr(VI) removal up to 55ppm. However the system demonstrated rather variable removal rates, which was attributed mainly to the formation of biofilm on the walls of the reactor. The next step is to operate the bioreactor as a two-phase system with high initial chromium (VI) concentrations (of the order of 1000 ppm).

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