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Bioreduction of chromate by immobilized cells of *Halomonas sp*

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Abstract
In this work, the bioreduction of Cr(VI) by immobilized cells of *Halomonas sp* was reported. Ca alginate, acryl amide and agar were tested as the matrices for immobilization. Ca alginate was found to be the suitable matrix among the different matrices studied. Of the various dosages of inoculum studied 2 g/L was found to be the optimum. Glucose at 1 g L^{-1} was completely utilized by the immobilized *Halomonas sp* even in the presence of Cr(VI) at 40 mg L^{-1}. The optimum pH for the bioreduction of Cr(VI) by immobilized *Halomonas sp* was found to be pH 6. The mechanical strength of the beads plays an essential role in the bioreduction process. *Halomonas sp* entrapped in a alginate matrix reported a maximum of 98.9 % of reduction for an initial Cr(VI) concentration of 10 mg L^{-1}. The alginate beads can be reused for 3 times with slight drop in the percentage reduction. The presence of other metals decreased the bioreduction percentage.
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Keywords: Bioreduction; Cr (VI); Halomonas sp; Immobilization; Wastewater treatment.

1. Introduction

Chromium containing wastewaters are generated by tanneries, wood preserving industry, paint industries and electroplating industries [1]. Cr(VI) from these industries pose dangerous threat to humans as well as animals. The world health organization and Environmental protection agency of the USA have set a maximum permissible limit of chromium in the water bodies at 50 µg L^{-1} [1, 2]. The exposure of Cr(VI) in humans have resulted in nasal irritation, skin irritation, ulceration, lung carcinoma and diahhrea [3]. Chromium is soluble under oxidizing conditions [4] Conventional methods yields limited removal of Cr(VI) [5]. The current technologies such as precipitation, ion exchange, reverse osmosis and membrane separation suffer from various limitations. Further they generate metal bearing sludge which is even more dangerous than the effluent itself. They are also costly processes as the requirements for the chemicals are very high. These disadvantages lead to the search for a cost effective and eco-friendly techniques for the treatment of Cr(VI) bearing wastewaters. Biological treatments have great advantage as they have lower impact on the environment as opposed to the other chemical treatment methods [6]. The potential use of the microbes for the reduction of Cr(VI) in the wastewaters is increasingly gaining attention. Many microbes are capable of reducing Cr(VI) from aqueous solutions at neutral pH [7]. The processes by which the microbes interact with the toxic metals to reduce the toxicity are biosorption, bioaccumulation and bioreduction [8]. Metal reduction ability of the microbial cells enhances their capacity for metal resistance which can be applied for detoxification of Cr(VI) available in wastewater.

Bioreduciton of Cr(VI) constitutes an alternative to the conventional methods [2]. Many bacterial cultures are reported for the reduction of Cr(VI) [4, 8-11]. The previous studies on bioreduction of Cr(VI) using free cells have reported Cr(VI) toxicity and damage to cells [1, 12] Cr(VI) inhibits the nucleic acid synthesis there by affecting the growth and metabolism of the microbes [13]. Immobilization of whole cells provides a wide range of advantage, easy to regenerate, immobilized cells are more stable and have the ability to withstand higher Cr(VI) load.

The choice of the immobilization matrix is an important factor in the application of whole cell immobilization. The polymer matrix determines the mechanical and chemical resistance of the microbial cells towards Cr(VI) [14]. Alginates, agar, poly acrylamide are the commonly used matrices for the immobilization of the microbial cells [1, 15]. Biopolymers are non toxic, selective, efficient and inexpensive [16]. Among the different immobilization methods employed entrapment of the microbial cells in a polymer matrix is the common method. The aim of the present study was to investigate the Cr(VI) bioreduction capacity of the immobilized *Halomonas sp*. Various parameters such as the stability of the matrix, the biomass loading rate, pH and reusability of the different matrices were studied.

2. Materials and methods

2.1 Microorganism and culture conditions

Halomonas sp was obtained from the Institute of Microbial Technology, Chandigarh, India and cultivated at 37 ºC in a liquid medium at pH 6 with agitation of 180 rpm on an incubator shaker (Daihan LabTech Co Ltd, Model LSI 3016 –R). The culture was maintained on YEPG- agar media. Subcultures were made on every 15 days of duration and stored at 4 ºC.

2.2 Media

The growth media for bacteria consisted of peptone 5 g L^{-1}, yeast extract 5 g L^{-1}, NaH_2PO_4. The medium for the Cr(VI) reduction consisted of yeast extract 5 g L^{-1}, glucose 1g L^{-1}, K_2HPO_4 0.03 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, $MgSO_4$ 0.01 g L^{-1}, NaCl 0.01 g L^{-1}. The pH of the media was adjusted to 7±0.1 with 0.1 N HCl and/or 0.1 N NaOH. The media was autoclaved at 120 °C for 15 min.

2.3 Preparation of stock solution

Bioreduction of Cr(VI) was studied in an aqueous solution using immobilized cells *Halomonas sp*. The Cr(VI) stock solution was prepared by dissolving 2.82 g of $K_2Cr_2O_7$ in 1000 mL of deionized water. The bioreduction media was prepared by diluting the appropriate quantity of Cr(VI) stock solution in the growth media.

2.4 Immobilization methods

Halomonas sp was entrapped in various matrices such as Ca alginate, polyacryl amide and agar.

2.4.1 Entrapment in Calcium alginate

Overnight cultures of *Halomonas sp* was centrifuged and resuspended in 2% Na alginate solution. The mixture was dropped into 20% $CaCl_2$ solution through burette. The height of the burette was set so as to get a uniform bead size of 4 mm. The water soluble sodium alginate was converted into water insoluble Ca alginate beads on contact with $CaCl_2$. The beads were cured in $CaCl_2$ for 2 h and washed thrice with deionized water.

2.4.2 Preparation of Polyacryl amide gels

A 10% poly acrylamide gel was prepared with varying concentration of inoculums ranging from 0.05 to 2.5 % w/v. The polymerized gel was cut into pieces of approximately 3 X 3 mm^2 [17].The acrylamide gel was stored at 4 ºC for further use.

2.4.3 Agar matrix

2% of agar solution was prepared in boiling water. The polymerization of the agar occurred as the solution was allowed to cool. 0.05 to 2.5 % w/v of inoculums was added to the agar solution under polymerization. The polymer obtained was cut into beads of 3 mm diameter. The agar beads were stored at 4 ºC for further use.

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2.5 Bioreduction studies

The immobilized beads containing the *Halomonas sp* were first transferred to the growth media of 100 mL in a 250 mL flask for activation and incubated in an orbital shaker (Daihan Lab Tech Co Ltd, Model LSI 3016 –R) for at 37 ºC for 52 h. The growth was periodically monitored using a microscope (Zeiss Axiostar, Germany). The metabolically active cultures of the immobilized *Halomonas sp* was transferred from growth media to the bioreduction media containing varying concentration of Cr(VI) ranging from 10 to 40 mg L^{-1} of Cr(VI). For all the experiments carried out with immobilized *Halomonas sp*, bacteria free matrices were used as control to study the effect of the matrices on bioreduction. The effect of media pH, temperature and initial Cr concentration on the bioreduction were also studied. The reusability of the matrices was also studied.

2.6 Analytical procedure

After the bioreduction the supernatants were collected by centrifugation (Remi, R-24) and analyzed for total chromium concentration using Atomic Absorption Spectrophotometer (Varian, AA140). The Cr(VI) concentration was measured spectrophotmetrically (Perkin Elmer, Model Lambda 35) at 540 nm by complexation with 1,5 Diphenyl carbazide.

3. Results and discussion

The substrate utilization of the bacteria during the bioreduction process, temperature and pH on the bioreduction of Cr with immobilized *Halomonas sp* was studied and the results are presented in this section. Various matrices for immobilization like Ca alginate, acryl amide and agar were investigated. The best suitable matrix with high mechanical stability and chemical resistance was optimized. The reusability of the beads was also reported.

3.1 Utilization of glucose during bioreduction of Cr(VI)

Glucose was tested as the sole carbon source for the growth and reduction of Cr(VI) by *Halomonas sp*. 1 g L^{-1} of glucose was added to the bioreduction media and concentration of glucose was analyzed periodically using Dinitro salilic acid (DNS) method. The glucose utilization profile of the *Halomonas sp* immobilized in Ca- alginate was presented in Figure 1. It was observed that the glucose was completely utilized for all the initial concentration of Cr(VI) studied. This was a clear indication that the bacteria were metabolically active throughout the study. Glucose supplemented the energy required for the metabolic activity. The decrease in glucose consumption rate for concentration exceeding 30 mg L^{-1} was due to the inhibitory effect of the Cr(VI) present in the media. The control cultures completely utilized the glucose in less than 24 h of time, the presence of Cr(VI) affected the metabolic activity thereby increasing the time required for the metabolic activity of the organism.

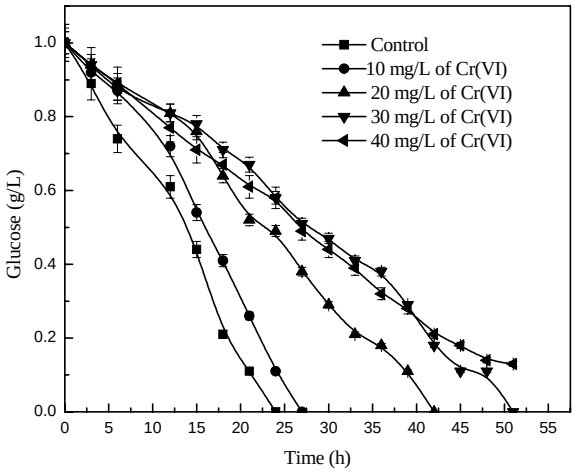

Figure 1. Glucose consumption profile of immobilized *Halomonas sp*

3.2 Effect of pH on bioreduction

The solubility and the ionization state of the Cr(VI) was influenced by the pH of the bioreduction media. The bioreduction was studied at various pH ranging from 2 to 7. Maximum bioreduction was obtained

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with pH 6. A maximum of 96.66, 98.99,91.34, 74.16, 21.66, 18.21, 2 % of bioreduction were reported for pH 7,6,5,4,3,2,1 respectively with an initial Cr(VI) concentration of 10 mg L^{-1} (Figure 2). The increase in bioreduction from pH 4 indicates the physico-chemical interaction between the metal ions and the bacteria. As the pH was increased further it favored the growth of the organism and thereby the bioreduction capacity increased. It was found that pH 6 was the optimum pH for the bioreduction using immobilized beads.

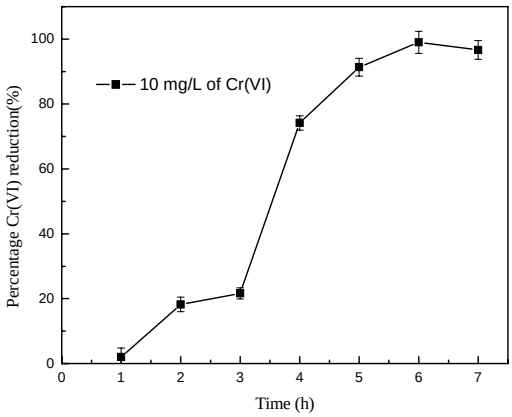

Figure 2. Effect of pH on Cr(VI) bioreduction

3.3 Cr(VI) reduction by Alginate immobilized cell

The Cr(VI) bioreduction by alginate immobilized cells of *Halomonas sp* was reported in Figure 3. It was observed that the efficiency of the immobilized cells in the bioreduction Cr(VI) decreased with the increase in the initial Cr(VI) concentration. It was also observed from the figure that the time taken for the bioreduction also increased with the increase in the initial Cr(VI) concentration. This can be attributed to the fact that the presence of higher concentration of Cr(VI) increased the lag phase of the bacteria. Alginate immobilized cells were able to reduce Cr(VI) completely for an initial Cr(VI) concentration of 9.89 mg L^{-1} 28 h (Figure 3a). It was observed that the Cr(VI) in the range of 18.8 and 28.3 mg L^{-1} were reduced to 2.02 and 3.16 mg L^{-1} respectively within 48 h (Figure 3b and 3c). Ca alginate immobilized *Halomonas sp* reported a minimum of 45.30% bioreduction for an initial Cr(VI) concentration of 38.8 mg L^{-1} (Figure 3d). As discussed earlier the increase in the Cr(VI) affected the metabolic activity of the organism thereby reducing the bioreduction percentage of Cr(VI). The decrease in the bioreduction capacity can also be attributed to the fact, that the mechanical stability of the beads was affected by the presence of the borate and nitrate.

3.4 Effect of cell concentration

The cell concentration in the immobilization matrix was varied from 0.05%(w/v) to 2.5 %(w/v). Figure 4 clearly indicated the increase in the bioreduction capacity of the *Halomonas sp* immobilized in alginate beads. The increase in bioreduction with increase in cell concentration was due to the fact that the bioreduction was dependant on the growth and metabolic activity of the organism. The number of viable colonies increased with increasing inoculums dosage. A maximum of 98.9 % of bioreduction was obtained with an initial Cr concentration of 10 mg L^{-1} for a cell concentration of 0.2 % (w/v). The bioreduction obtained reached a constant value for inoculums dosage exceeding 0.2 % (w/v).

3.5 Screening of different matrices

A maximum of 98.9% reduction of Cr(VI) was reported with alginate matrix for an initial Cr(VI) concentration of 10 mg L^{-1}. The acryl amide and agar matrices yielded a Cr(VI) reduction of 94.14 and 91.2 % of Cr(VI) bioreduction respectively. The acryl amide matrix was found to disintegrate as the study was continued for a period of 52 h. The agar matrix was found to be soluble in the media causing difficulty in analyzing the Cr(VI) concentration. Table 1 showed the percentage reduction of Cr(VI) obtained for different matrices studied with different initial concentrations of Cr(VI) ranging from 10 mg L^{-1} to 40 mg L^{-1}. Addition of borate and nitrate decreased the bioreduction capacity with the alginate. The mechanical stability of the beads was an important parameter in the selection of the matrix. It was

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observed that the alginate beads disintegrated when nitrate and borate was added to the matrix. Similar results were reported by various earlier researchers [1, 18, 19].

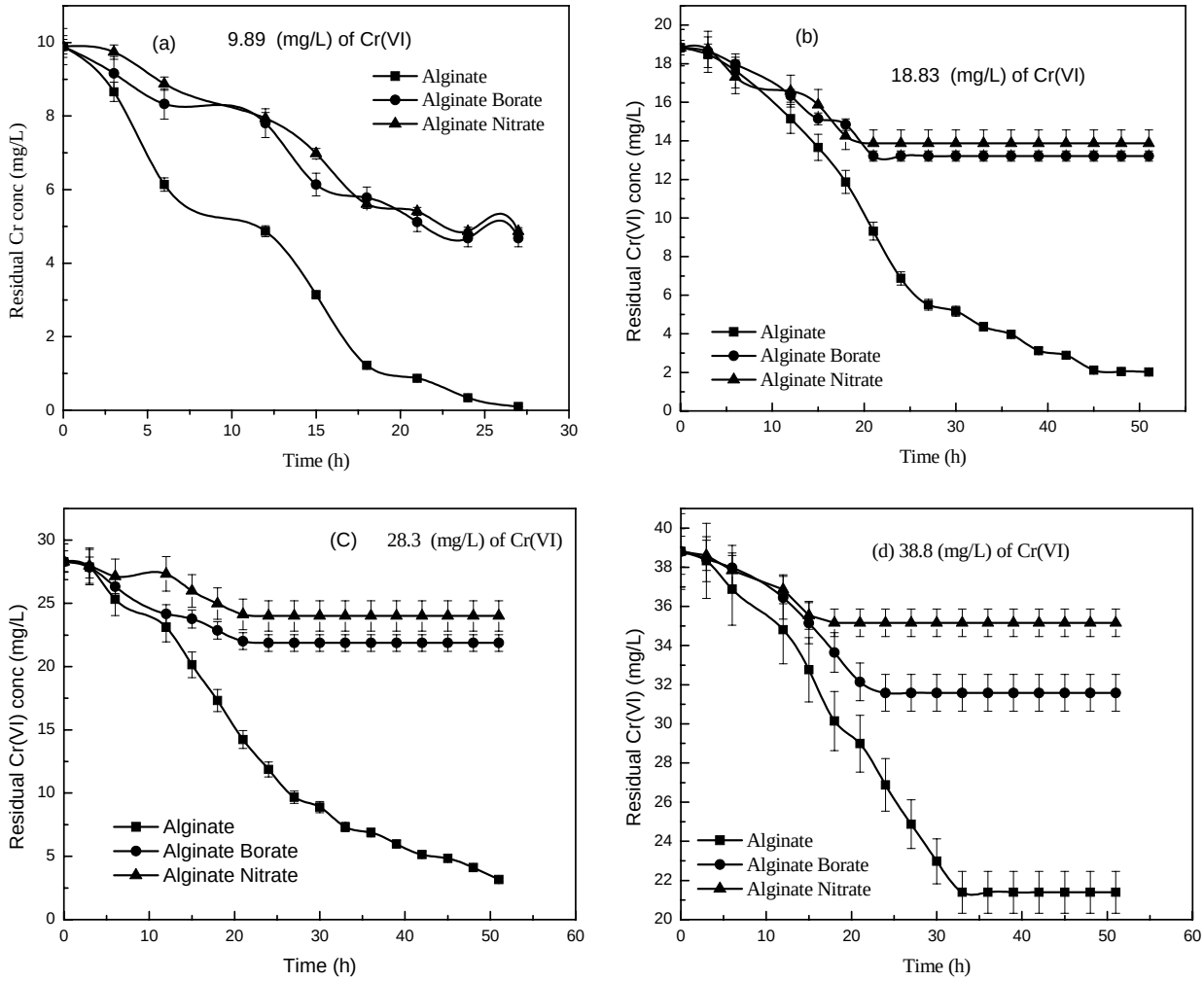

Figure 3. Cr(VI) reduction by *Halomonas* immobilized in Alginate beads : (a) Initial Cr(VI) concentration 9.89 (mg/L); (b) Initial Cr(VI) concentration 18.83 (mg/L); (C) Initial Cr(VI) concentration 28.3 (mg/L); Initial Cr(VI) concentration 38.8 (mg/L)

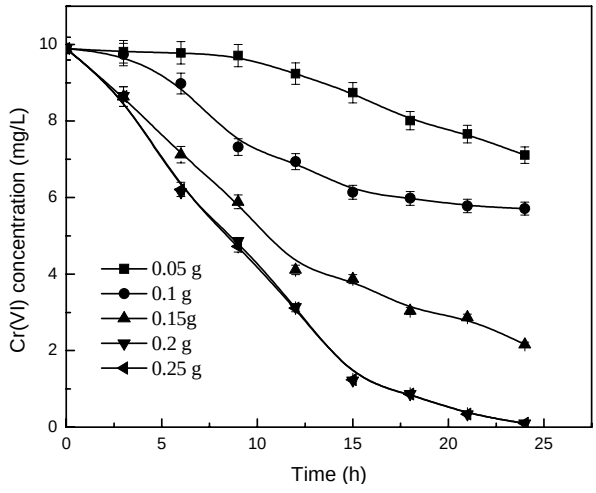

Figure 4. Effect of cell concentration on bioreduction of Cr(VI)

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Table 1. Screening of matrices

Immobilization matrix	Initial (0 h)	Residual (24 h)	Reduction (%)	Bead integrity
Calcium alginate	38.8	21.4	45.30	Retained
Agarose	38.4	31.6	17.70	Disintegrated
Agar	38.4	33.2	13.54	Disintegrated
Calcium alginate	28.3	3.16	88.83	Retained
Agarose	28.3	8.14	71.24	Disintegrated
Agar	28.3	8.84	68.76	Disintegrated
Calcium alginate	18.83	2.04	89.17	Retained
Agarose	18.83	2.87	84.76	Disintegrated
Agar	18.83	3.14	83.32	Disintegrated
Calcium alginate	9.89	0.1	98.99	Retained
Agarose	9.89	0.58	94.14	Disintegrated
Agar	9.89	0.87	91.20	Disintegrated
Alginate borate	9.98	4.68	53.11	Disintegrated
Alginate nitrate	9.98	4.87	51.20	Disintegrated

3.6 Cr(VI) reduction in the presence of other metals

Cr(VI) reduction of the alginate immobilized *Halomonas sp* was studied in the presence of the other metals. Cd was more toxic to the organism as the bioreduction was drastically affected the bioreduction of Cr(VI). It was observed that only 12. 59 % of bioreduction was obtained for an intial Cr(VI) concentration of 10 mg L^{-1} in the presence of Cd. The presence of Mn, Pb and Cu also affected the bioreduction capacity of the immobilized *Halomonas sp* as depicted in Figure 5.

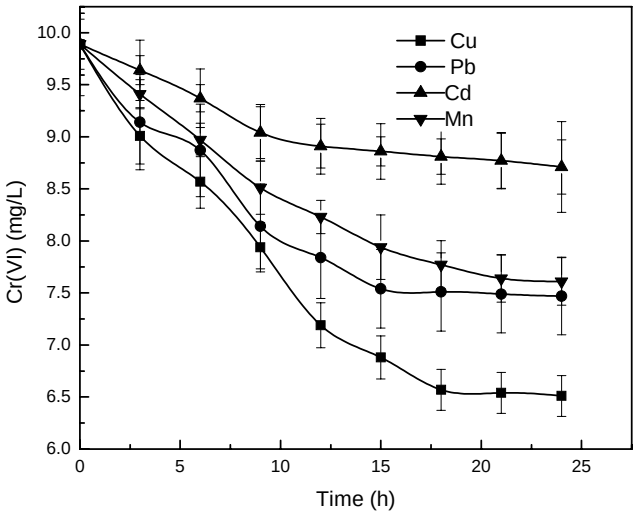

Figure 5. Effect of other metals on bioreduction of Cr(VI) by *Halomonas sp*

3.7 Effect of initial Cr(VI) on bioreduction

Effect of the initial Cr(VI) on the bioreduction was reported in Figure 6. It was observed that the bioreduction time prolonged as the initial Cr(VI) concentration was increased from 10 mg L^{-1} to 40 mg L^{-1} (Figure 6). Increase in Cr(VI) concentration decreased the percentage reduction. A maximum of 98.9% was reported for an initial Cr(VI) concentration of 10 mg L^{-1}. A minimum of 45.30 % of Cr(VI) was reported for an initial Cr(VI) concentration of 40 mg L^{-1}, It can be attributed to the fact that the Cr(VI) at concentration exceeding 20 mg L^{-1} had an inhibitory effect on the growth 3.8 Bioreduction.

The bioreduction of Cr(VI) was studied for 27 h. It was observed that the bioreduction of the Cr increased with time. A maximum of bioreduction 98.9% of Cr(VI) was reported at 27 h for an initial concentration of 10 mg L^{-1} with *Halomonas sp* entrapped in Ca alginate (Figure 7). The Cr(VI) reduction profile clearly indicated the bioreduction capacity of the Ca alginate immobilized cells of *Halomonas sp.* It was observed that the concentration of the Cr(III) increased with time. The simultaneous reduction of

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Cr(VI) and production of Cr(III) is a clear indication of the metabolic activity of the live and active immobilized cells. Figure 7. Clearly showed that the increase in the Cr(III) concentration was proportionate to the decrease in the Cr(VI) concentration.

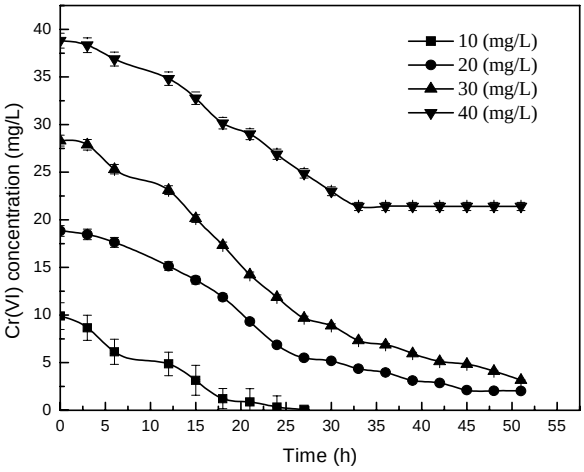

Figure 6. Effect of initial Cr(VI) on bioreduction

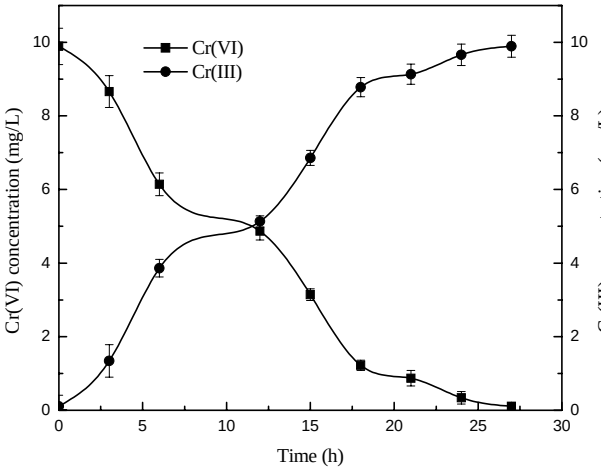

Figure 7. Cr(VI) bioreduction profile

3.9 Reusability

Ca alginate immobilized cells can be reused for 5 times. The percentage reduction with the number of reuses was found to decrease. It was observed that a maximum of 98.99 % of bioreduction was obtained with an initial concentration of 10 mg L^{-1}. The percentage reduction dropped to 88.64 in the second use (Figure 8). The mechanical strength of the beads were completely affected with the reuse. The number of viable colonies decreased with the number of uses.

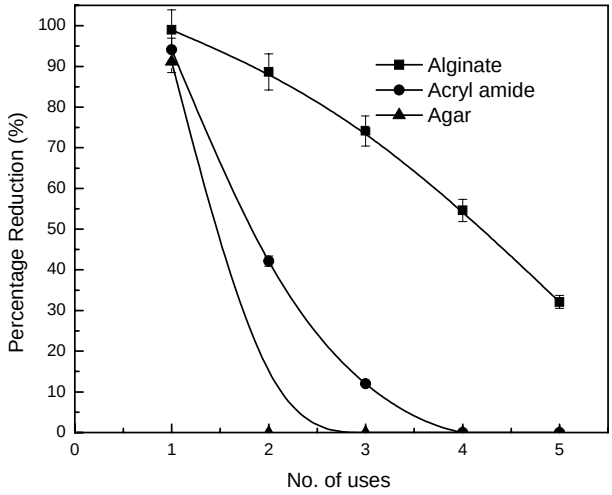

Figure 8. Reusability of beads on Cr(VI) bioreduction

4. Conclusion

Bioreduction of Cr(VI) using the immobilized *Halomonas sp* was dependant on the pH, initial Cr(VI) concentration and metabolic activity of the organism. A maximum of 98.9 % of bioreduction was reported for 10 mg L^{-1} of initial Cr(VI) concentration. Results indicated that the stability of the beads was necessary for significant reduction of Cr(VI). The optimum reduction was observed at pH 6. Ca alginate was found to be the best suitable matrix for the immobilization of the fungus. Cd was found to be more toxic for the bioreduction of Cr(VI) by *Halomonas sp*. Ca alginate immobilized cells of *Halomonas sp* can be effectively used for the treatment of water bodies containing lower concentration Cr(VI), which cannot be treated by the chemical methods. The immobilization provides advantage as the separation of the cells is easier and the cells can be reused.

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