Bio Technology

Modeling of Chromium (VI) Accumulation in *Gordonia polyisoprenivorans* VH2 Using Response Surface Methodology

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Abstract: An experimental design technique was carried out to investigate the bioaccumulation of chromium VI by the rubber degrading nocardioform actinomycete *Gordonia polyisoprenivorans* VH2. Response Surface Methodology (RSM) was adopted to acquire the best level of four variables namely, pH, $FeSO_4$, $NaNO_2$ and Cr (VI) which have been optimized to bring about maximum chromium biosorption percentage. In this respect, the 3-level Box-Behnken design was employed. A polynomial model has been created to correlate the relationship between the four variables and chromium biosorption. The optimal combination of those variables for chromium biosorption evaluated from the non-linear optimization algorithm of EXCEL-Solver was as follows (mg L^{-1}): $FeSO_4$, 18; $NaNO_2$, 3.75; Cr (VI), 41.25 at pH 6.7. Based on the predicted model, an experimental verification of the optimal conditions revealed chromium biosorption percentage of 90%, which is more than 3 folds the basal medium.

Key words: Chromium VI, *Gordonia polyisoprenivorans* VH2, experimental design, heavy metal resistance

INTRODUCTION

Chromium is widely used in the manufacture of metallic alloys for structural and protective purpose, in fabric dyes, wood preservation and in tanning of leather. It is an essential element to animal and human life, however elevated levels of chromium are toxic (Carlo *et al.*, 2003). It is usually found in nature in its trivalent state, but hexavalent chromate form is found generally as a result of human activities causing environmental pollution. Higher concentrations of soluble Cr (VI) are extremely toxic and exhibits mutagenic and carcinogenic effects on biological systems due to its strong oxidizing nature (Losi *et al.*, 1994; McLean *et al.*, 2000; McLean and Beveridge, 2001). Conventional physical and chemical methods for clean up of chromium VI are not recommended for full-scale remediation. However, biotechnological application of Cr-resistant bacteria for detoxification of Cr (VI) has been considered as an economical, effective and safe procedure (Ganguli and Tripathi, 2002; Stern, 1982).

Studies of chromate resistance in microorganism has generally focused on Gram-negative bacteria such as *Alcaligenes*, *Enterobacter* and *Pseudomonas* where resistance is due to reduced intracellular accumulation of chromate (Ji and Silver, 1995). Recently, Gram-positive bacteria resistant to higher concentrations of chromate were isolated and studied. All isolated bacteria belongs mainly to genus *Bacillus* and few to *Corynebacterium* (Carlo *et al.*, 2003; Srinath *et al.*, 2002).

In the last few years, several metal-resistance actinomycetes have been isolated from different environments and well characterized (Amoroso *et al.*, 1998, 2001; Ravel *et al.*, 1998a, 1998b) and many have applications in bioremediation of metal-contaminated sediments.

The application of statistical experimental design in studying heavy metal removal was recently published. Response surface methodology is an efficient, cost effective method to model and optimize bioprocess as it enables research to identify interactions between studied variable, if present, with as few as possible experiments. Studies on the biosorption process applying a modeled based on 2^3 factorial design has been carried out, in which the effects of pH, heavy metal concentration and biomass on biosorption of cadmium from oil field waste waters using *Aspergillus niger* (Barros *et al.*, 2003). Similarly, 2^3 factorial design was applied to study

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the uptake of cadmium and lead. The three operational factors were; temperature, ionic strength and pH (Peternele *et al.*, 1999).

Another group reported how pH, temperature and initial concentration of chromium interacted and ultimately affected chromium removal efficiency from synthetic effluents by *Sargassum* sp. using factorial design (Carmona *et al.*, 2005). They also designed 2^3 scheme to study the removal of Cr (III) and Cr (VI), separately from aqueous solution.

The objective of this study was to optimize the accumulation of chromium VI with the rubber-degrading bacterium *G. polyisoprenivorans* VH2 by applying Box-Behnken factorial design. Special emphasis was given to the impact of initial chromium concentration, pH and the use of other reducing chemicals (Fe^{+2} and NO_2^-) on the efficiency of chromium removal.

MATERIALS AND METHODS

The present study was carried out during January-June, 2005 in Genetic Engineering and Biotechnology Research Institute, Department of Environmental Sciences, Faculty of Science, Alexandria University.

Microorganism: The bacterium used in this study is a rubber-degrading nocardioform actinomycete *Gordonia polyisoprenivorans* VH2 (DSM 44266) that was isolated and characterized as described previously (Linos *et al.*, 1999).

Cultivation: Unless otherwise indicated, 50 mL LB liquid medium was inoculated with 0.5 mL inoculum's of overnight culture. The liquid LB medium (pH 6.5) was supplemented with filter sterilized $K_2Cr_2O_7$ as a source of chromium (VI) to give the desired concentration of metal ion, as indicated in each experiment. The culture was incubated at 30°C with agitation at 200 rpm on a rotary shaker, where growth was quantified by measuring optical density at 660 nm.

Determination of chromium (VI) concentration: The concentration of unaccumulated chromium (VI) ions in the medium was determined spectrophotometrically at 540 nm using 1,5-diphenyl carbazide as the complexing agent. The sample of 1 mL containing free chromium (VI) ions was mixed with 3.3 mL of 0.2 M H_2SO_4 and 1 mL of 1, 5-diphenyl carbazide solution which was prepared by dissolving 0.25 g of 1,5-diphenyl carbazide in 10 mL of absolute alcohol. After 10 minutes the pink-violet colored solution was analyzed for the chromium (VI) ions (Snell and Snell, 1959).

Table 1: Low, moderate and high levels of factors (mg L^{-1}) affecting bioaccumulation of chromium (VI) by *Gordonia polyisoprenivorans* VH2

Factor	Low level (-1)	Moderate level (0)	High level (+1)
pH	6	6.5	7
Fe^{+2}	10	20	30
NO_2	0	5	10
Cr (VI)	25	50	75

Fractional factorial design: In order to optimize chromium bioaccumulation, Box-Behnken design, which is based on response surface methodology was applied (Box and Behnken, 1960). As presented in Table 1, four factors, namely, Cr (VI), pH, Fe^{+2} and NO_2^- were prescribed into three levels, coded −1, 0 and +1 for low, middle and high concentrations (or values), respectively. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response (Cr (VI) accumulation percentage). For the four factors the model used was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 \\ + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 \\ + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2$$

Where, Y is the predicted response, β_0 model constant; X_1, X_2, X_3 and X_4 independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Experiments were performed in triplicate and mean values are given.

Statistical analysis of data: The data of chromium bioaccumulation percentage were subjected to multiple linear regressions using Microsoft Excel 97 to estimate t-values, p-values and confidence levels which is an expression of the p-value in percent. The optimal value of chromium bioaccumulation was estimated using the solver function of Microsoft Excel tools.

RESULTS AND DISCUSSION

Optimization design for Cr (VI) accumulation by *Gordonia polyisoprenivorans* VH2: In order to optimize chromium (VI) bioaccumulation, four variables were studied namely, pH, Fe^{+2}, NO_2^- and Cr (VI) initial concentration. A design matrix based on Box-Behnken experimental design was constructed, where 24 trials plus four replica at the center point (trials 11, 12, 17 and 24)

Table 2: Experimental factorial design results for Cr (VI) uptake by *Gordonia polyisoprenivorans* VH2

Trial	pH	Fe^{+2}	NO_2	Cr (VI)	OD_{660}	Cr (VI) consumption (%)
1	-1	0	-1	0	0.14	40.11
2	1	1	0	0	3.20	91.35
3	0	1	0	1	0.15	22.99
4	0	0	-1	1	0.13	7.16
5	0	1	1	0	3.60	36.57
6	0	1	-1	0	6.00	88.40
7	0	1	0	-1	12.60	98.80
8	0	-1	1	0	3.40	65.98
9	0	0	1	-1	10.00	98.87
10	-1	0	0	-1	0.11	68.11
11	0	0	0	0	5.20	84.23
12	0	0	0	0	6.20	83.79
13	-1	0	0	1	0.12	21.80
14	0	-1	-1	0	5.20	49.33
15	-1	1	0	0	0.14	39.76
16	0	-1	0	-1	6.60	98.94
17	0	0	0	0	5.80	85.40
18	1	0	1	0	4.50	90.93
19	-1	-1	0	0	0.17	25.58
20	1	-1	0	0	7.40	87.90
21	-1	0	1	0	1.43	60.67
22	1	0	0	-1	5.00	97.66
23	1	0	-1	0	2.8	86.54
24	0	0	0	0	6	83.97
25	1	0	0	1	0.18	15.90
26	0	-1	0	1	0.12	13.06
27	0	0	1	1	0.18	27.95
28	0	0	-1	-1	7.4	97.09

Table 3: Regression coefficient of the full polynomial model representing relationships between Cr (VI) consumption % and independent variables (pH, Fe^{+2}, NO_2^- and initial Cr (VI))

Coefficient	Estimate[a]	p-value
β_0	84.35	7.18E-09
β_1	17.85	0.001
β_2	3.09	0.430
β_3	1.03	0.380
β_4	-37.55	2.59E-08
β_{11}	-11.03	0.055
β_{22}	-11.99	0.109
β_{33}	-8.12	0.340
β_{44}	-18.28	0.057
β_{12}	-2.68	0.769
β_{13}	-4.04	0.867
β_{14}	-8.86	0.132
β_{23}	-17.12	0.020
β_{24}	2.52	0.656
β_{34}	4.75	0.405

[a]Estimates are the polynomial model coefficients

were carried out. The design of this experiment is given in Table 2 together with the experimental results. Chromium bioaccumulation % was measured after growing cells for 96 h. Regression analysis was performed to fit the response function (Cr (VI) bioaccumulation %) with the experimental data. The analysis of variance for the four variables indicated that chromium consumption could be well described by a polynomial model with a relatively high coefficient of determination ($R^2 = 0.92$) The statistical analysis of the full model in Table 3 shows that pH and initial chromium concentration had a significant effect on chromium bioaccumulation. Although their positive linear effect, the probability value of the coefficient of linear

effect of Fe^{+2} (β_2) and NO_2^- (β_3) was high (0.43 and 0.38), respectively, which indicates the insignificance of these coefficients. However, the interaction coefficients of these variables was negative significant. The most significant factor in the overall design was the initial chromium concentration either in the linear or the quadratic effect. Reducing the model, by including only the significant terms, does not improve the regression coefficient value and as a result the model could be expressed as follows:

$$\begin{aligned} Cr\ (VI)_{consumption}\ (\%) = {} & 84.35 + 17.85\ X_1 + 3.09\ X_2 \\ & + 1.03\ X_3 - 37.55\ X_4 - 11.03\ X_1^2 \\ & - 11.99\ X_2^2 - 8.12\ X_3^2 - 18.28\ X_4^2 \\ & - 2.68\ X_1 X_2 - 4.04\ X_1 X_3 \\ & - 8.86\ X_1 X_4 - 17.12\ X_2 X_3 \\ & + 2.52\ X_2 X_4 + 4.75\ X_3 X_4 \end{aligned}$$

Where, X_1, X_2, X_3 and X_4 represent codified values for pH, Fe^{+2}, NO_2^- and Cr (VI), respectively. For more description of the relationship between bioprocess variables and the response partial-effects are illustrated in three-dimensional plots.

Finding the optimum point of the variables: The optimum level of each variable has been obtained using the non-linear optimization algorithm of Microsoft Excel. As represented by natural levels, the optimum point was obtained using the following conditions: pH 6.7, Fe^{+2} at 18 mg L^{-1}, NO_2^- at 3.75 mg L^{-1} and initial Cr (VI) at 41.25. Maximum chromium consumption % was attained when pH value was at coded limit (+ 0.38) under experimental constraints. On the other hand, increasing NO_2^-, Fe^{+2} and chromium (VI) concentrations in the medium led to pronounced decrease in chromium consumption.

The main results of this study are presented in Fig. 1, which represents the expected Cr (VI) consumption % response and correlation between variables in three-dimensional plots. Figure 1d shows non-additive effects of Fe^{+2} and NO_2^- due to the significant interaction between them. In the other Fig. 1e and f it is to be seen that the effects of pairs of factors were additive since there are no interactions. By additively of the two-factor effects, it is meant that the effect of one factor on the response does not depend on the level of the other factor. In Fig. 1a, e and f it is obvious that maximum chromium accumulation was attained at low levels of chromium concentration.

Previously, it has been shown that an increase in the removal efficiency occurred with an increase in pH in growing culture of *Aspergillus niger* (Barros *et al.*, 2003). Various authors have evaluated metal removal efficiency as a function of pH variation and have obtained isotherms at different pH values either in living or dead cells (Carmona *et al.*, 2005).
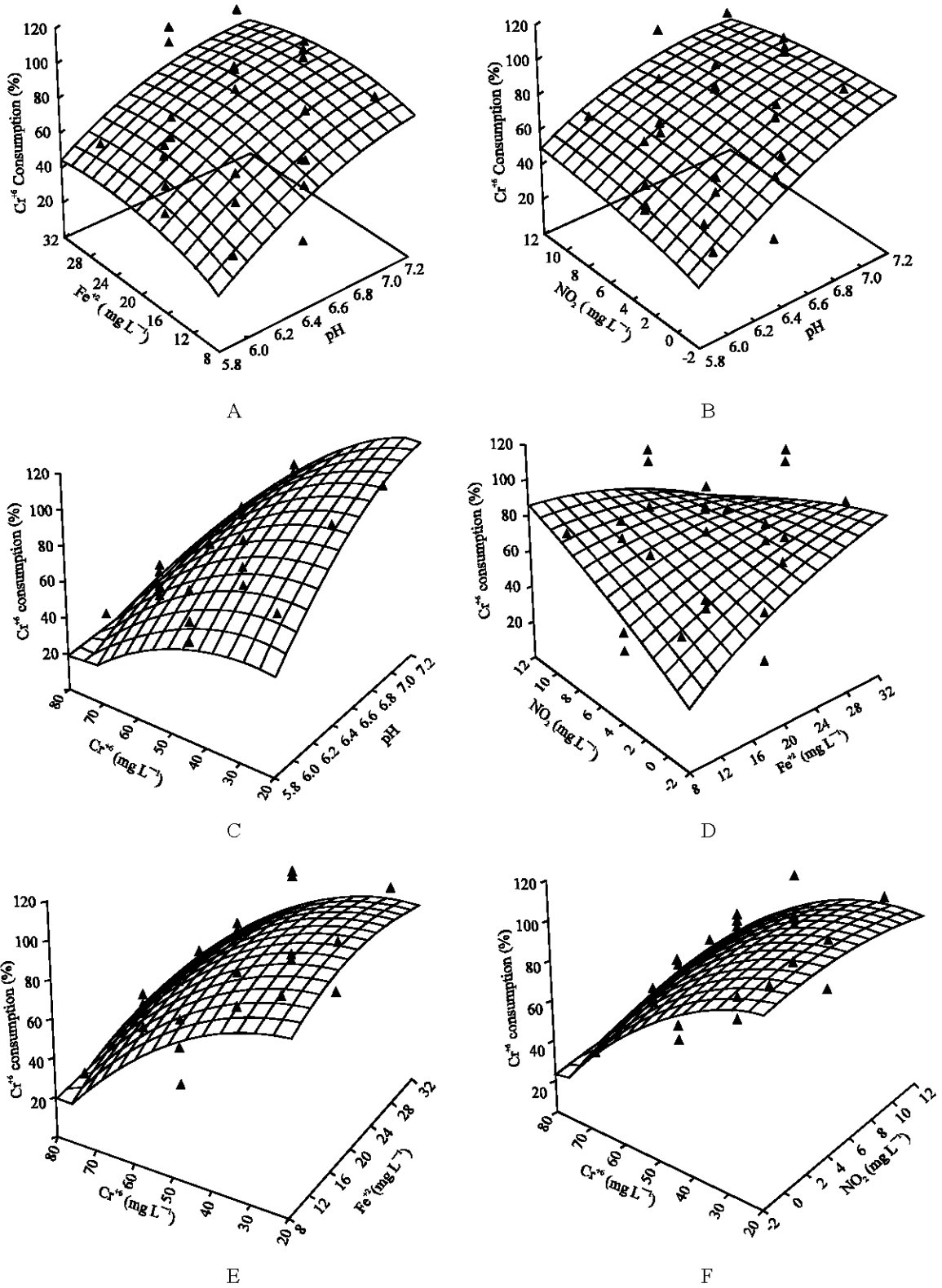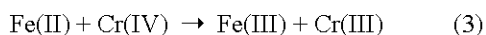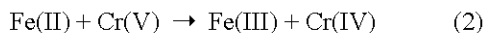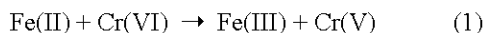
Fig. 1a-f: Cr (VI) consumption (%) response surface from *Gordonia polyisoprenivorans* VH2 as affected by culture conditions

Increasing the Cr (VI) concentration in microbial growth medium results in cell damage producing mutagenic activities (Păs *et al.*, 2004). In accordance to present results, Şahin and Öztürk (2005) reported that initial metal ion concentration remarkably influenced the equilibrium metal uptake, where higher adsorption yields were observed at lower concentrations of metal ions for vegetative and endospores of *Bacillus thuringiensis* (Şahin and Öztürk, 2005). Although, otherwise, *G. polyisoprenivorans* VH2 was capable of growing on higher concentrations of Cr (VI), maximum metal removal efficiency was only attained when metal concentration was 41.25 mg L^{-1}. Higher concentrations of initial Cr (VI) results in dramatic decrease in metal uptake efficiency.

Chemically, numerous observations indicate that ferrous iron could be an important reductant of Cr (VI) in natural waters (Kieber and Helz, 1992). Fe (II) is abundant in many suboxic and anoxic soils and sediments and it is produced through photochemical reactions that occur in sunlight natural waters (McKnight *et al.*, 1988; Voelker and Sedlak, 1995). Results from previous studies, in which excess Fe (II) was added to Cr (VI)-containing solutions, indicate that Cr (VI) rapidly reacts with Fe (II) at pH values between 6.0 and 9.0 (Kieber and Helz, 1992)
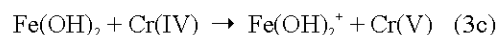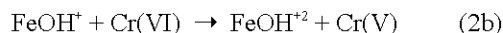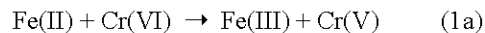
The observed stoichiometry of the reactions suggests that the reduction of Cr (V1) by Fe (II) and Cr (V) actually involves three, one electron-transfer steps:

$$Fe(II) + Cr(VI) \rightarrow Fe(III) + Cr(V) \qquad (1)$$

$$Fe(II) + Cr(V) \rightarrow Fe(III) + Cr(IV) \qquad (2)$$

$$Fe(II) + Cr(IV) \rightarrow Fe(III) + Cr(III) \qquad (3)$$

The observation that a 3:1 ratio of [Fe (II)] : [Cr (VI)] is maintained during the reaction could be explained by two different reaction mechanisms. One possibility, reaction 1 is the rate-determining step. The other, is that a later reaction is the rate determining step and that Cr (V) and Cr (IV) are always present at very low concentrations. Results of previous studies conducted under acidic conditions indicate that reaction 2 is likely to be the rate determining step because of the change in the coordination of chromium; however, it is currently impossible to distinguish between these two mechanisms (Espenson, 1970; Westheimer, 1949). Both of the hypothesized reaction mechanisms should result in first-order kinetics with respect to Cr (VI) and Fe (II).

Over the pH range examined, the concentrations of several different species of Fe (II) and Cr (VI) undergo significant changes (Sedlak and Chan, 1997). The largest changes in the speciation of Cr (VI) occur between pH 5.5 and 7.5, where CrO_4^{2-} increases from approximately 1 to 99% of the total Cr (VI). If $HCrO_4^-$; is responsible for observed increases in the rate coefficient with pH, the rate coefficient would plateau above pH 7.0. In contrast, concentrations of hydroxylated species of Fe(II) [i.e., $FeOH^+$ and $Fe(OH)_2$] increase throughout the entire pH range studied. If the hydroxylated species are more reactive than Fe^{+2}, the rate coefficient would increase throughout the entire pH range studied. The increase in reaction rates at pH values above 4.0 can be explained by increases in the concentrations of $FeOH^+$ and $Fe(OH)_2$. Thermodynamic calculations indicate that the electron-donating OH^- ligands stabilize Fe (III) and make Fe (II) a more reactive reductant (Wehrli, 1990). The high reactivity of hydroxylated species of Fe (II) with oxidants such as oxygen (Millero *et al.*, 1987) and hydrogen peroxide (Moffett and Zika, 1987) also leads to similar increases in reaction rates at pH values above 4.0. If the rate-determining step of the reaction depends upon a one-electron transfer from a Fe (II) species to Cr (VI) (i.e., $HCrO_4^-$ or CrO_4^{2-}) or Cr (V), it should be possible to predict the observed reaction rates by quantifying the contribution of each species of Fe (II) (Sedlak and Chan, 1997):

$$Fe(II) + Cr(VI) \rightarrow Fe(III) + Cr(V) \qquad (1a)$$

$$FeOH^+ + Cr(VI) \rightarrow FeOH^{+2} + Cr(V) \qquad (2b)$$

$$Fe(OH)_2 + Cr(IV) \rightarrow Fe(OH)_2^+ + Cr(V) \qquad (3c)$$

Nitrite was found to be insignificant in response to Cr (VI) accumulation, whereas optimum conditions for Cr (VI) bioaccumulation involves a concentration of nitrite near to null. This could be attributed to the influence of oxygen, where it enhances nitrite oxidation acting as the conjugate reduction reaction of the system. In the case of Cr (VI) reduction, the presence of oxygen led to controversial results. In aerated suspensions, molecular oxygen can compete with Cr (VI) species for the generated electrons, being deterimental for the reduction; this behavior has been reported by (Lin *et al.*, 1993). However, although nitrite is a good OH scavenger, it does not seem to improve and even diminishes the efficiency of Cr (VI) reduction (Navío *et al.*, 1998).

For biomass results, similar trend was estimated as pH and initial chromium concentration were the most significant factors affecting biomass yield. A change in pH value from low to high level results in 11.6% increase in the cellular growth. On the other hand, decreasing initial chromium concentration from high to low level results in 22.7% increase in biomasss (data not shown).

Validation of optimum point: The adequacy of the model was examined by an additional experiment using the derived optimal conditions. The predicted value for chromium (VI) consumption percent was 100% and experimentally the value was 90%, which means that *G. polyisoprenivorans* VH2 capable of utilizing 37.13 mg L^{-1} of chromium (VI) from the optimized medium. This is 90% of the predicted value, which indicates that the generated model is an adequate for prediction of the metal uptake. Therefore, application of such models is of great importance for environmental bioprocess.

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