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# Statistical optimization of medium components for chromate reduction by halophilic *Streptomyces* sp. MS-2

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**Extensive use of hexavalent chromium Cr(VI) in various industrial applications has caused substantial environmental contamination. A marine bacterium, Streptomyces sp. MS-2 showed a high Cr(VI) reduction performance. Streptomyces sp. MS-2 completely reduced 75 mg/l Cr(VI) within 72 h of growth. The effectiveness of the bacterium for reducing Cr(VI) under different conditions was evaluated. Optimum pH and temperature were 7.0 and 37oC, respectively. Statistical screening of medium components for Cr reduction by Streptomyces sp. MS-2 was carried out by Plackett–Burman design. Peptone, yeast extract, inoculum size, and volume of the medium were shown as significant components influencing Cr(VI) reduction. By applying a verification experiment, Streptomyces sp. MS-2 completely reduced 75 mg of Cr(VI) to Cr(III) within 12 h. This optimization strategy led to a 6-fold increase in the reduction rate. This holds great promise for detoxification of Cr(VI) under a wide range of environmental conditions.**

**Key words:** *Streptomyces* MS-*2*; hexavalent chromium; Plackett–Burman design.

## INTRODUCTION

Chromium as a toxic heavy metal often exists in the waste streams from various industries such as mining, metal cleaning, plating, electroplating, leather tanning, metal processing and as corrosion inhibitor in conventional and nuclear power plants (Donmez and Kocberber, 2005; Thacker et al., 2006) . It is well known that chromium exists mainly as two stable oxidation states, Cr(VI) and Cr(III). Almost all hexavalent chromium Cr(VI) contamination is from human activities. The trivalent chromium compounds are less toxic, mobile and available for biological uptake, while hexavalent chromium compounds are 100-fold more toxic than Cr(III) compounds due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Sultan and Hasnain, 2005) . Accordingly, chromium and its compounds can be easily absorbed by living cells (Liu et al., 2006).

Chromates are strong oxidizing agents that can react with nucleic acids. Hence, Cr(VI) poses a greater threat to public health, the environment and ecosystems, compared with Cr(III) (Gibb, 2000; Casadevall and Kortenkamp, 2002; Sedman et al., 2006). The reduction of Cr(VI) to Cr(III) is therefore an attractive and useful process for remediation of Cr(VI) pollution, and the technologies focusing on transformation of Cr(VI) to Cr(III) have accordingly received much more attention. Biological chromium (VI) reduction is more preferable than chemical reduction due to the lower costs, safety and significantly lower quantities of produced sludge (Ganguli and Tripathi 2002; Konovalova et al., 2003; Faisal and Hasnain, 2004).

Microbial reduction of toxic Cr(VI) offers a potential cost-effective bioremediation approach. However, the availability of effective hexavalent chromium-reducing organisms is an essential prerequisite for the bioreduction-based remediation of chromium-contaminated water or soil (Pattanapipitpaisal et al., 2001) . Many bacterial strains have been reported to reduce Cr(VI), indicating an important bioremidial step in detoxification of Cr(VI) - contaminated wastes (Rajkumar et al., 2005; Sultan and Hasnain, 2006). However, there are very few studies on Cr(VI) resistance and bio- reduction by *actinomycetes* . The first report on Cr(VI) reduction by *Streptomyces* was from Das and Chandra (1990). Later, Laxman and More (2002) determined Cr(VI) reduction by *Streptomyces griseus*, whereas Desjardin et al. (2003) used the culture supernatants of *Streptomyces thermo-*

*carboxydus* NH50 to reduce Cr(VI). Recently, Polti et al. (2007) reported Cr(VI) resistance by *Streptomyces* strains.

Process optimization may involve the study of many biochemical and physical parameters, including media formulation and culture parameters. The classical method of changing one medium variable at a time in order to optimize performance is impractical. The need for efficient methods for screening a large number of variables has led to the adoption of statistical experimental designs.

The methodology was based on the Plackett–Burman design (Plackett and Burman, 1946), which provides an efficient way of screening a large number of variables and identifying the most important ones. Such design have already been used in many research projects (Djekrif-Dakhmouche et al., 2006; Gao and Gu, 2007; Liu and Wu, 2007).

Hence, the main objectives of the present study were (i) to investigate the efficiency of the halophilic bacterium *Streptomyces* sp. MS-2 to reduce Cr(VI) under aerobic conditions; and (ii) to optimize the culture conditions by using Plackett–Burman experimental design, while providing useful knowledge for the bioremediation of chromate pollution.

## MATERIALS AND METHODS

### Chemical reagents

Pure and analytical grade chemicals were used in all experiments, including media preparation for growth. Peptone, yeast extract, and diphenylcarbazide were purchased from Sigma Chemical Co, USA. Potassium chromate was obtained from MERCK Chemical Company.

### Organism

*Streptomyces* sp. MS-2 was isolated from marine sediment. The bacterium was identified using the 16S rDNA gene sequence analysis and was submitted to Genbank under the accession number DQ 987870. It was propagated on inorganic salt-starch agar (ISA) (Küster, 1959) slants at 35°C for 7 days and transferred monthly. Stock cultures were maintained as suspensions of spores and hyphal fragments in 25% glycerol at –20°C.

### Stock chromium solution ($K_2CrO_4$)

A stock solution of Cr(VI) was prepared by dissolving 2 g of $K_2CrO_4$ in 20 ml of deionized distilled water and filter-sterilized using a 0.45 µm Whatman filter paper. The sterilized stock Cr(VI) solution was added to sterile medium to a desired concentration of Cr(VI) with minimal dilution of the medium.

### Medium

The Cr(VI) reduction potential of *Streptomyces* sp. MS-2 was assessed in a liquid medium containing (g/l sea water) : glucose 10; peptone 2.5; $KNO_3$ 1.0; $MgSO_4$, $7H_2O$; 0.5; $CaCl_2$ 0.5 and yeast extract, 0.2. For the selection of these factors, Plackett-Burman design was used. The composition of reduction medium varied according to the design matrix.

### Inoculum preparation

The inoculum was prepared by suspending spores from a 1-week old (ISA) culture slant in 5 ml of sterile saline. 1 ml of the homogenous suspension containing $10^4$ - $10^5$ spores was used to inoculate 40 ml of sterile LB medium (tryptone 10 g, yeast extract 5 g/l of sea water) in a 250 ml Erlenmeyer flask and the culture was incubated at 37 ± 2°C for 48 h on a rotary shaker. Cells (grown in form of pellets) were harvested in a sterile centrifuge tube (25 ml) by centrifugation at 9 000 rpm for 10 min. The pellets obtained were resuspended in 20 ml sterile sea water. 5 ml of this prepared inoculum were transferred to 50 ml of reduction medium in 250 ml Erlenmeyer flasks.

### Cultivation

All the experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of reduction medium according to the design matrix. The initial pH was adjusted to 7, using 0.1 M NaOH or 0.1 M HCl. The media were autoclaved at 120°C for 20 min in separate flasks, as complexation of Cr with organic constituents of the medium at an elevated temperature normally reduces toxicity. The culture medium was supplemented with the desirable Cr(VI) concentration. Reduction was carried out aerobically at 37°C on a rotary shaker at 150 rpm. For each experiment, the Cr(VI) concentration was measured.

### Statistical methodology

### Screening of important nutrient components

Plackett–Burman design (Plackett and Burman, 1946) was used to screen and evaluate the important medium components that influence the response. In practice, all the experiments were carried out according to a design matrix, which is based on the number of variables to be studied. The matrix applied to this study is shown in Table 1. Each row represents the 12 different experiments to evaluate their final effects on Cr(VI) reduction and each column represents a different variable. Each independent variable was investigated at a high (+1) and a low (−1) level, which in the present investigation means two different nutrient concentrations. Each column should contain an equal number of positive and negative signs. Nine variables, which were expected to have an effect on Cr(VI) reduction, were identified and their concentrations are shown in Table 2. All experiments were conducted in triplicate and the averages of the results were taken as response values. Uninoculated controls were included to determine the Cr(VI) loss by the components of the culture medium.

### Analytical methods

Samples were aseptically drawn at regular time intervals, centrifuged at 10 000 rpm for 10 min and the supernatant fluid was analyzed for residual Cr(VI). Chromate-reducing activity was determined as decrease of chromate over time using the Cr(VI)-specific colorimetric reagent diphenylcarbazide (APHA, 1989). Spectrophotometric measurements were made immediately at 540 nm. In order to monitor any abiotic Cr(VI) reduction, cell-free controls were also used for each Cr(VI) reduction assay. Total con- centration of chromium (i.e. Cr(VI) + Cr(III) ) in the medium and acid-digested cell pellets was measured by means of atomic absorption (AA) (Perkin Elmer 2380). Cr(VI) reduction efficiency was calculated

**Table 1.** Plackett–Burman matrix for evaluating factors influencing Cr(VI) reduction by *Streptomyces* sp. MS-2.

| Experiment | Factors | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | G | P | Y | Ca | N | Mg | Cr | IS | V |
| **1** | +1 | -1 | +1 | -1 | -1 | -1 | +1 | +1 | +1 |
| **2** | +1 | +1 | -1 | +1 | -1 | -1 | -1 | +1 | +1 |
| **3** | -1 | +1 | +1 | -1 | +1 | -1 | -1 | -1 | +1 |
| **4** | +1 | -1 | +1 | +1 | -1 | +1 | -1 | -1 | -1 |
| **5** | +1 | +1 | -1 | +1 | +1 | -1 | +1 | -1 | -1 |
| **6** | +1 | +1 | +1 | -1 | +1 | +1 | -1 | +1 | -1 |
| **7** | -1 | +1 | +1 | +1 | -1 | +1 | +1 | -1 | +1 |
| **8** | -1 | -1 | +1 | +1 | +1 | -1 | +1 | +1 | -1 |
| **9** | -1 | -1 | -1 | +1 | +1 | +1 | -1 | +1 | +1 |
| **10** | +1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 | +1 |
| **11** | -1 | +1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 |
| **12** | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |

+1: Higher level; -1: Lower level

**Table 2.** Variables showing medium components and test levels used in Plackett–Burman design.

| Variable | Variable code | Low level (-1) | Level (0) | High level (+1) |
|---|---|---|---|---|
| Glucose (g/l) | G | 10 | 20 | 30 |
| Peptone (g/l) | P | 1 | 2.5 | 5 |
| Yeast extract (g/l) | Y | 0 | 0.2 | 1 |
| $CaCl_2$ (g/l) | Ca | 0.25 | 0.5 | 0.75 |
| $KNO_3$ (g/l)) | N | 0.5 | 1 | 1.5 |
| $MgSO_4$ $7H_2O$ (g/l) | Mg | 0.25 | 0.5 | 0.75 |
| $K_2CrO_4$ (mg/l)) | Cr | 50 | 75 | 100 |
| Inoculum size (ml/flask) | IS | 2.5 | 5 | 7.5 |
| Volume of medium (ml/flask) | V | 25 | 50 | 75 |

according to the following equation:

Reduction efficiency % = $(C_i \quad C_f) / C_i \times 100$

Where $C_i$ is initial Cr(VI) concentration (mg/l); $C_f$ is final Cr(VI) concentration (mg/l). Biomass was collected by centrifugation, washed twice with distilled water and dried at 105°C until constant weight.

#### Data analysis

Statistical analyses were performed to identify those medium variables (factors) that had a significant effect, either positively or negatively (Plackett and Burman, 1946) on Cr(VI) reduction. The effect of each variable was determined as the difference between the average value of the response for the six experiments at the high level (+) and the average value for the six experiments at the low level (–) by the following equation:

$$E(X_i) = (\text{R at } (+) \qquad \text{R at } (-)) / 6$$

where E (Xi) is the main effect of the tested variable, and R is the measured response. When the sign is positive, the influence of the variable upon Cr(VI) reduction is greater at a high concentration, and when negative, the influence of the variable is greater at a low concentration. A statistical procedure is used to calculate the limit to which the effects of important independent variables are assigned. The significant level (*p*- value) of each main effect was determined using student's *t*-test:

## RESULTS AND DISCUSSION

### Cr(VI) reduction experiments

Marine bacteria are physiologically more active at 3.5 - 35 g/l of total salt concentration (Calvo and Martinez-Checa, 1998). Since *Streptomyces* sp. MS-2 was isolated from a marine habitat, the nutrient component was dissolved in sea water. Reduction was lower in media prepared with distilled water compared to that with sea water (data not shown). Therefore, the presence of sea water in the culture medium appeared to be a prerequisite for *Streptomyces* sp. MS-2 growth and chromate reduction, indicating the halophilic nature of the strain. The reduction of chromate was accompanied by a change in the color of the medium from yellow to white due to the conversion of hexavalent chromium to trivalent

**Table 3.** Cr(VI) reduction from the results of the Plackett–Burman experiment.

| Experiments | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reduction % | 97.3 | 98.4 | 96.8 | 68.4 | 75.6 | 94.7 | 87.6 | 58.6 | 73.9 | 60 | 78 | 63.7 |

form. Data in Figure 1 show the kinetics of chromate reduction and the effect of chromate on growth of *Streptomyces* sp. MS-2. It is clear that chromate decreased the rate of growth by 12.5% after 72 h. Similar observations were reported previously (Liu et al., 2006; Sultan and Hasnain 2006; Amoozegar et al., 2007). It was also observed that Cr(VI) reduction was growth related (Figure 1). Growth- related chromate reduction was also reported previously (Sultan and Hasnain, 2007).

Complete reduction was achieved within 72 h. Laxman and More (2002) reported that *S. griseus* reduced 75 mg/l chromate within 24 h when chromium was added after 24 h growth. *Bacillus* sp. (XW-4) completely reduced 40 mg/l of Cr(VI) within 66 h (Liu et al., 2006). On the contrary, *B. sphaericus* AND303 failed to cause complete reduction of 10 mg/l Cr(VI) (Pal and Paul, 2004) . Hence, *Streptomyces* sp. MS-2 exhibited signi-ficant Cr(VI) reduction ability as compared to other reported strains.

Mass balance of chromium in batch culture experi-ments showed no chromium accumulation of either Cr(VI) or Cr(III) by the cells, since the total chromium measurements in all experiments were very close to the initial concentration of Cr(VI) added (data not shown). No reduction of chromium was observed in non- inoculated controls included in the experiment even after prolonged incubation up to seven days. The results confirmed that Cr(VI) reduction activity was not associated with sub-stances existing in the medium, and reduction activity required the presence of bacterial cells strictly, which have the potential to detoxify hexavalent chromium. These are in good agreement with previous reports (Liu et al., 2006; Pazouki et al., 2007; Thacker et al., 2007).

pH is one of the most important factors influencing chemical speciation, solubility and bioavailability of Cr in the field (Adriano, 2001). The initial pH of the culture medium was shown to be an effective factor for Cr(VI) reduction. Strain MS-2 could reduce Cr(VI) in a wide range of pH (6 – 9) with maximum Cr(VI) reduction at pH 7.0 (Figure 2). These results are in accordance with the findings of other reports (Wang and Xiao, 1995; Liu et al., 2004). However, since Cr(VI) reduction is enzyme-media-ted, pH changes affects the enzyme ionization rate, changes the protein’s conformation and consequently affects the enzyme activity (Farrell and Ranallo, 2000). The difference in optimum pH value suggests that pH modification is important for different cultures to achieve the maximum Cr(VI) reduction in the bioremediation of chromate.

Cr(VI) reduction by *Streptomyces* sp. MS-2 was evaluated at various temperatures ranging from 20 to 45°C (Figure 3). Reduction was exhibited over the temperature range 30 – 45°C with maximum at 37°C and was negatively affected below 30°C. Losi et al. (1994) reported an optimal temperature of 30 – 37°C for Cr(VI) reduction.

**Evaluation of culture conditions affecting Cr(VI) reduction by *Streptomyces* sp.MS-2**

Plackett–Burman design succeeded in ranking factors from different categories to enable better understanding of the medium effect. The averages of Cr(VI) reduction % for the different trials are shown in Table 3. A wide variation in Cr(VI) reduction (58.6 -98.4%) was clearly observed, which reflects the importance of medium optimization to attain high reduction percentages.

The main effects of the examined factors on Cr(VI) reduction were calculated and are presented graphically in Figure 4. The data showed that peptone, culture volume, yeast extract, inoculum size and glucose within the test range had a positive effect on Cr(VI) reduction, whereas $KNO_3$, $MgSO_4$. $7H_2O$, $CaCl_2$ and chromate contributed negatively.

Some researchers thought that the variables with confidence level above 80% (Pujari and Chandra, 2000) or 85% (Xiong et al., 2004) were significant. The compo-nents were screened at the confidence level of 80% on the basis of their effects (either positive or nega-tive). Table 4 represents the results of Plackett– Burman experiment with respect to the *t*- value, *p-value* and confidence level of each component. A significance at or above the 80% confidence level, indicates that the component was effective in Cr(VI) reduction.

Of the nine culture factors tested, only peptone, yeast extract, inoculum size, and volume of the medium had a significant effect on Cr(VI) reduction at a confidence level above 80% and are thus regarded to be the most significant variables (Table 4). From the results obtained in this experiment, it has observed that the + level of glucose favored Cr(VI) reduction. Glucose was also chosen by other researchers for promoting chromate reduction in *Streptomyces* 3M, *Bacillus* sp (XW-4) and *B. sphaericus* (Das and Chandra, 1990; Pal and Paul, 2004; Liu et al., 2006). Data in Table 4 reveal that $MgSO_4$ and $KNO_3$ had no significant effect on Cr(VI) reduction. This can be due to $NO_3^-$ and $SO_4^{-2}$ not acting as the electron acceptors under aerobic conditions and thus would not compete with Cr(VI) for accepting-electrons, a statement supported by Philip et al. (1998). A similar trend was observed with *Streptomyces griseus* and *Penicillium chrysogenum* PTCC 5037 (Laxman and More 2002;

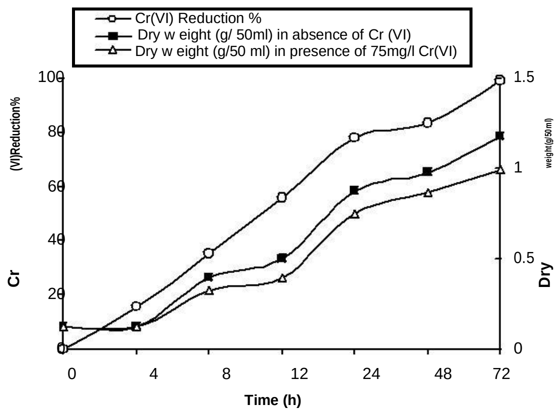


**Figure 1.** Variation of Cr(VI) reduction % and growth curves of *Streptomyces* sp. MS-2 in the absence or presence of 75 mg/l Cr(VI).

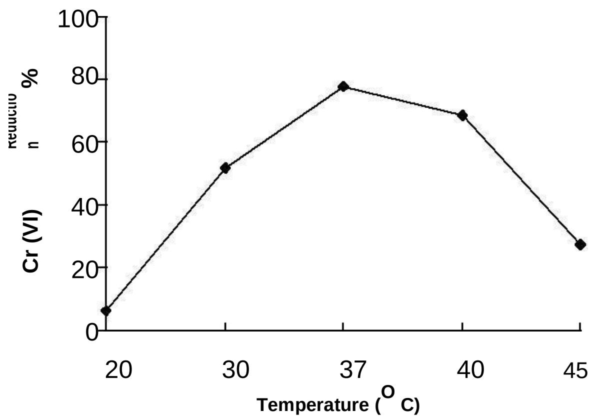


**Figure 3**. Influence of temperature on reduction of Cr(VI) by *Streptomyces* . sp. MS-2 over a period of 24 h. Initial Cr(VI) concentration was 75 mg/l.

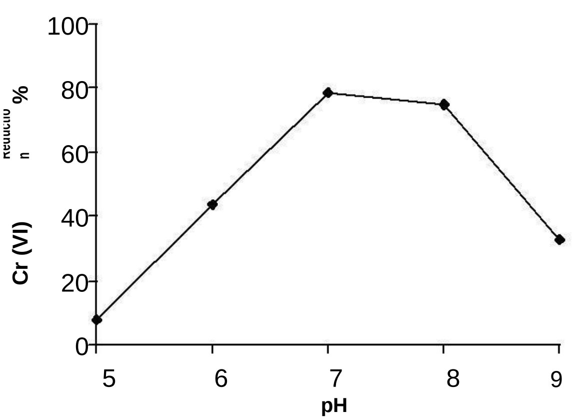


**Figure 2.** Influence of pH on reduction of Cr(VI) by *Streptomyces* sp. MS-2 over a period of 24 h. Initial Cr(VI) concentration was 75 mg/l.

Pazouki et al., 2007). $CaCl_2$ affected Cr(VI) reduction insignificantly, which is in good agreement with a previous report (Laxman and More, 2002). Inoculum size had a profound effect on Cr(VI) reduction in the range studied (5 - 15%), which indicates that an increase in cell mass favors Cr(VI) reduction. McLean et al. (2000) reported the requirement for sufficient biomass to achieve significant Cr (VI) reduction. This finding is consistent with many previous reports on the increase in the reduction percentage with increase in the inoculum size (Pattanapipitpaisal et al., 2001; Pal and Paul, 2004; Sultan and Hasnain, 2007).

The volume of the medium was found to play an important role in Cr(VI) reduction with a confidence level over 90%. Chromate reduction was enhanced by an increase in the volume of the culture medium/flask. Two potential variables (peptone and yeast extract) hadpositive signs and higher confidence levels than the other

other variables (Table 4). Organic sources are considered essential medium supplements for the regeneration of NADH that act as an efficient electron donor for the reduction of Cr(VI). It has been suggested that electrons could be provided to Cr(VI) from peptone and yeast extract, catalyzed by a reductase of *Streptomyces* sp. MS-2 (Laxman and More, 2002; Pal and Paul, 2004).

Cr concentration had a negative effect on reduction. High percentage of Cr(VI) reduction was observed for trials 1, 2, 3 and 6 at 24 h. It is important to note that the amount of Cr in the industrial effluent is usually much less than the amount of Cr used in this study. Increasing or decreasing the level of each respective variable, according to the sign of its main effect, should have a positive consequence with respect to chromate reduction.

## Verification of model and comparison with non-optimized culture conditions

Application of the statistically optimized culture conditions Table 5) for Cr(VI) reduction by *Streptomyces* sp. MS-2 resulted in complete reduction of 75 mg/l within 12 h, and increased the rate of Cr(VI) reduction 6-fold higher than that recorded with the basal medium. The above results indicate that the Plackett–Burman design is a powerful tool for determination of relevant variables, which had a significant influence on Cr(VI) reduction and shows a much higher level of Cr(VI) reduction as compared to basal medium.

In conclusion, the present study provides evidence indicating that *Streptomyces* sp. MS-2 may be used in developing a bioremedial process for chromate-contami-nated saline wastes discharge. Moreover, it represents the first report of chromate reduction by a halophilic actionmycete.

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**Table 4.** Statistical analysis of the explicative factors on Cr(VI) reduction from the results of Plackett–Burman design.

| Variable | *p* -Value | Confidence level (%) | Main effect | *t*- Value |
|---|---|---|---|---|
| Glucose | 0.27 | 73 | 5.99 | 0.6685 |
| Peptone | 0.05 | 95 | 18.18 | 2.5517 |
| Yeast extract | 0.17 | 83 | 8.97 | 1.0321 |
| $CaCl_2$ | 0.45 | 55 | -1.47 | -0.1532 |
| $KNO_3$ | 0.28 | 72 | -5.68 | -0.6321 |
| $MgSO_4.\ 7H_2O$ | 0.31 | 69 | -4.67 | -0.5169 |
| $K_2CrO_4$ | 0.25 | 75 | -6.48 | -0.7266 |
| Inoculum size | 0.19 | 81 | 8.12 | 0.9250 |
| Volume of medium | 0.09 | 91 | 12.52 | 1.5178 |

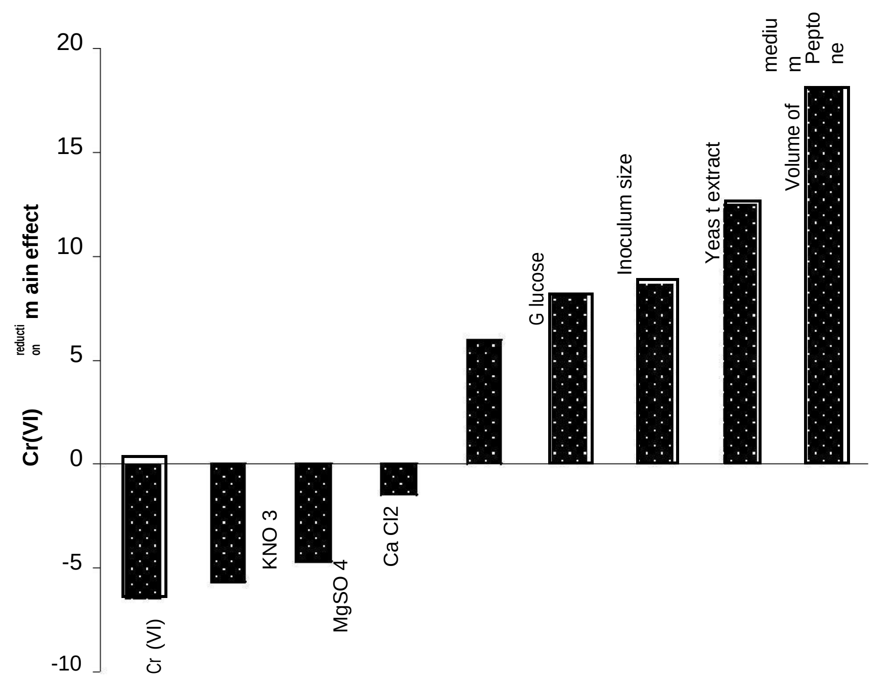


**Figure 4.** Effect of environmental factors on Cr(VI) reduction by *Streptomyces* sp. MS-2 based on the results of Plackett–Burman design.

**Table 5.** Optimal culture conditions for Cr(VI) reduction by *Streptomyces* sp. MS-2.

| Variable | Value |
|---|---|
| Glucose (g/l) | 30 |
| Peptone (g/l) | 5 |
| Yeast extract (g/l) | 1 |
| $CaCl_2$ (g/l) | 0.25 |
| $KNO_3$ ((g/l) | 0.5 |
| $MgSO_4.7H_2O$ (g/l) | 0.25 |
| $K_2CrO_4$ (mg/l) | 75 |
| Inoculum size (ml/flask) | 11.25 |
| Volume of medium (ml/flask) | 75 |

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