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OPTIMIZATION OF CR(VI) BIOTRANSFORMATION BY RESPONSE SURFACE METHODOLOGY USING *MICROBACTERIUM MARITYPICUM*

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ABSTRACT

In the present study, a novel indigenous bacterium was isolated from the effluent of Electroplating industry having capability of biotransforming Cr(VI) ions into Cr(III) ions. Morphological, Physiological and biochemical characterization of the bacterial strain was carried out and later sequence analysis of 16S rRNA gene of the strain identified it as *Microbacterium maritypicum.* By utilizing this bacterial strain, Response surface methodology was used to study the interactive effect of three crucial operating parameters viz pH, temperature and initial Cr(VI) ion concentration on the process of Cr(VI) Biotransformation. Total seventeen experiments designed by Box-Behnken design matrix were carried out for the bacterial strain for the construction of quadratic model. Analysis of variance (ANOVA) was calculated. The coefficient of determination (R^2) value 0.9509, model F- value 15.06 and its low P-value ($F < 0.0009$) ensured that second-order regression model got well fitted with the experimental data. The regression equation coefficients were also calculated. All the experimental observations were in agreement with the model values.

KEYWORDS: ***Microbacterium maritypicum*, Cr (VI), Response surface methodology, Box-Behnken model, bacteria.**

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INTRODUCTION

The industrial activities have intensified environmental pollution problems leading to emissions which have been adversely affecting the environment, leading to large scale destruction of agricultural land and water bodies world-wide.[1] Heavy metal pollution is one of the most important problems today. Metal pollutants are non-biodegradable and can only be transformed to less toxic oxidation states. Chromium (Cr) is the seventh most abundant element in the earth's crust.[2] Its oxidation state ranges from (-II) to (+ VI), the trivalent and hexavalent states being the most stable.[3] Hexavalent chromium Cr (VI) is discharged into the environment as a result of the wide use of chromium compounds in industrial activities. Its high solubility in aqueous systems, its permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids [4] together with carcinogenic and mutagenic effects on living organisms make industrial effluents needs specific treatments for Cr(VI) elimination. Almost every regulatory agency has listed Cr(VI) as a priority toxic chemical for control, with the maximum allowable level in drinking water of 50-100 µg L^{-1}.[5] The conventional methods for removal of Cr(VI) suffers from several disadvantages and hence the search for new and innovative technology has drawn the attention in biotransformation of metals by utilising microbes. Biological treatments provide benefits in terms of environment, economics and energy. The efficiency of Cr(VI) biotransformation process can be enhanced by optimizing the factors like pH, temperature and initial metal ion concentration. The conventional optimizing approach "one factor at a time" was time consuming.This drawback was overcome by Response surface methodology (RSM) which is a set of mathematical and statistical methods for experimental design and evaluating the effects of variables and searching optimum conditions of variables to predict targeted responses.[6] The present study involves isolation, molecular characterization along with 16S rRNA gene sequencing of chromium resistant bacterial strain and optimization of three variables - pH, temperature and initial Cr(VI) ions concentration for effective Cr(VI) biotransformation.

2. MATERIALS AND METHODS

2.1Physico-chemical characterization of effluent samples

Effluent samples were collected from Electroplating industry Lakshmi Precision Screws Ltd., Rohtak District, Haryana, India. Various physico-chemical parameters of the wastewater sample were analyzed such as color, temperature, Ph, conductivity, oil and grease, TDS, TSS, BOD, COD, Sulfate, Phosphate, total chromium, hexavalent chromium and other metals. The concentration of Cr (VI) and total chromium in the electroplating effluent was observed to be 25 mg/L and 48 mg/L respectively. The maximum permissible limit for Cr(VI) and total chromium in the effluent is 0.1 and 2 mg/L respectively.[7] Hence the effluent sample showed many folds increase in Cr(VI) and total chromium concentration.[8]

2.2 Isolation and characterization of chromium resistant bacterial strains

Microbacterium maritypicum was isolated from the effluent of electroplating industry. The bacterial strain was isolated on nutrient agar medium[9] comprising (g/L): Peptone- 10 g, Beef extract- 2 g, Yeast extract -1 g, sodium chloride- 5 g. The pH of the culture medium was maintained at 7. Purification of the bacterial strain was done through repeated streaking on basal agar medium using serial dilution technique. The growth of bacterial cells was observed after incubation of 48 h at 30^0C. The isolated bacterial strain was characterized morphologically, biochemically and physiologically.

2.3 16S rRNA sequencing and phylogenetic analysis

The taxonomic identity of the strain was further confirmed by 16S rRNA gene sequencing. For 16SrRNA sequencing, the bacterial culture was sent to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology,

Chandigarh. Sequence similarity was searched by National Center for Biotechnology Information BLAST and calculated by pairwise alignment obtained from Ez Taxon database.[10] Analysis of 16S rRNA gene sequence data was performed by using software MEGA version 4.0. The phylogenetic tree was inferred using the multiple sequence alignment with different species of bacteria through neighbor joining method.[11]

2.4 Response Surface Methodology

Present study involves the use of Box-Behnken design and Response surface methodology. The optimization study was conducted in batch mode using nutrient broth media. The Box-Behnken factorial design, which is standard RSM, was established on the basis of Design Expert Factorial design (Stat Ease, 8.0 trial version). Three independent variables viz pH (4 – 8), incubation temperature (25^0C – 35^0C) and initial Cr(VI) ions concentration (10- 40 mg/L) were studied at three different levels to obtain response i.e. biotransformation of Cr(VI) ions. The experiment design was obtained after the selection of three independent variables (maximum and minimum values). The present study involves three- level, three-factorial Box-Behnken experimental design which constituted of 17 experiments. The Cr(VI) biotransformation percentage was calculated using the equation as follows: % Cr(VI) biotransformation = $(C_0 – C_f)*100/ C_0$

Where, C_0 is the initial concentration of C(VI) ions (mg/L) and C_f is the final concentration of metal ion (mg/L) To confirm the phenomenon of biotransformation, the initial and final concentration of total chromium and Cr(III) was also recorded in all the bacterial batch cultures.

2.5 Statistical Analysis

Statistical testing of the model was performed with F-test to obtain the mathematical relationship between response i.e. Cr(VI) biotransformation and the process variables. In order to ensure a good model, the test for significance of regression model was performed by applying the analysis of variance (ANOVA). Following quadratic equation was obtained by varying three parameters:

$Y_i = a_o + \sum a_i X_i + \sum a_{ii} X^2_{ii} + \sum a_{ij} X_i X_j + e$

Where, Y_i (i = 3) is predicted response i.e. biotransformation of Cr(VI) ions using bacterial strain, a_o is the constant coefficient, $a_i$is $_i$th linear coefficient or slope, $a_{ii}$is the $_i$th quadratic coefficient and a_{ij} is different interaction coefficients of the model; X_i, $X_j$are the independent variables and e is the residual error of the model. The independent variables are coded as A, B and C in the present study. The second order polynomial function was fitted to correlate the relationship between independent variables and the response for prediction of the optimum point conditions.

$Y = a_o + a_iA + a_iB + a_iC + a_iA^2 + a_iB^2 + a_iC^2 + a_{ij}A*B + a_{ij}A*C + a_{ij}B*C$

The quality of polynomial model equation was expressed statistically by the coefficient of determination (r^2) and its statistical significance was determined by using F-test. Each experimental design was carried out in triplicates. T-test was used to find the significance of the regression coefficients. The residual error, pure error and lack of fit were calculated from repeated measurements.[12, 13] The desirable response was selected as maximum % Cr(VI) biotransformation at optimum pH, temperature and initial Cr(VI) ions concentration. The relationship between response and experimental levels for each of the factors could be observed as fitted polynomial equation in form of surface plots.

2.6 Quantification of total Chromium and Cr(VI)

Hexavalent chromium was determined spectrophotometrically using s-diphenylcarbazide method.[14] The absorbance was measured at 540 nm using UV-Visible spectrophotometer UV-2450 Shimadzu. Total chromium was quantified using Atomic absorption spectrophotometer after digestion of microbial culture samples with sulfuric acid and nitric acid on hot plate.

3 RESULTS AND DISCUSSION

3.1 Isolation and characterisation of chromium resistant bacterial strain

Bacterial strain was isolated from the electroplating effluent. The morphological, physiological and biochemical characteristics of the isolated specie are shown in table 1.

Based on the studied characteristics, the *Microbacterium sp*. isolated bacterial specie was identified as

Table 1
Morphological, physiological and biochemical characteristics of Microbacterium maritypicum

Morphological tests

Tests	***Microbacterium maritypicum***
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Convex
Surface	Smooth
Pigment	Creamish
Opacity	Transparent
Gram's reaction	**+ ve**
Cell shape	Short rods
Size (μm)	1.5
Arrangement	Scattered
Spore(s)	**- ve**
Motility	+

Physiological tests

Physiological tests:	
Growth at temperatures	
4° C	+
10° C	+
25° C	+
30° C	+
37° C	+
42° C	+
55° C	-
Growth at pH	
pH 5.0	+
pH 6.0	+
pH 8.0	+
pH 9.0	+
Growth on NaCl (%)	
2.0	+
4.0	+
6.0	+
8.0	+
10.0	-
12.0	-
Growth under anaerobic condition	+

Biochemical test

Tests	
Growth on MacConkey	lf
Indole test	-
Methyl red test	-
VogesProskauer test	-
Citrate utilization	+
H_2S production	-
Gas production from glucose	-
Starch hydrolysis	-
Nitrate reduction	+
Catalase test	+
Oxidase test	-
Urea hydrolysis	+
Esculin hydrolysis	+
Arginine dihydrolase	+
Tween 20 hydrolysis	-
Tween 40 hydrolysis	-
Tween 60 hydrolysis	-
Tween 80 hydrolysis	-
Acid Production from	
Galactose	-
Mannose	-
Maltose	+
Sucrose	+
Fructose	(+)
Lactose	+

+: Positive; -: Negative ;(+): Weak positive; lf: lactose fermenting;

3.2 Molecular identification of bacterial strain

The phylogenetic analysis based on 16S rRNA gene sequencing revealed that the bacterial strain formed a distinct branch within the genus *Microbacterium*. The nucleotide sequence of a 1421bp 16S rRNA was obtained, which aligned closely with the sequence of *Microbacterium sp. EP32.* Figure 1 depicts the phylogenetic tree derived from 16S rRNA sequence of *Microbacterium maritypicum.*

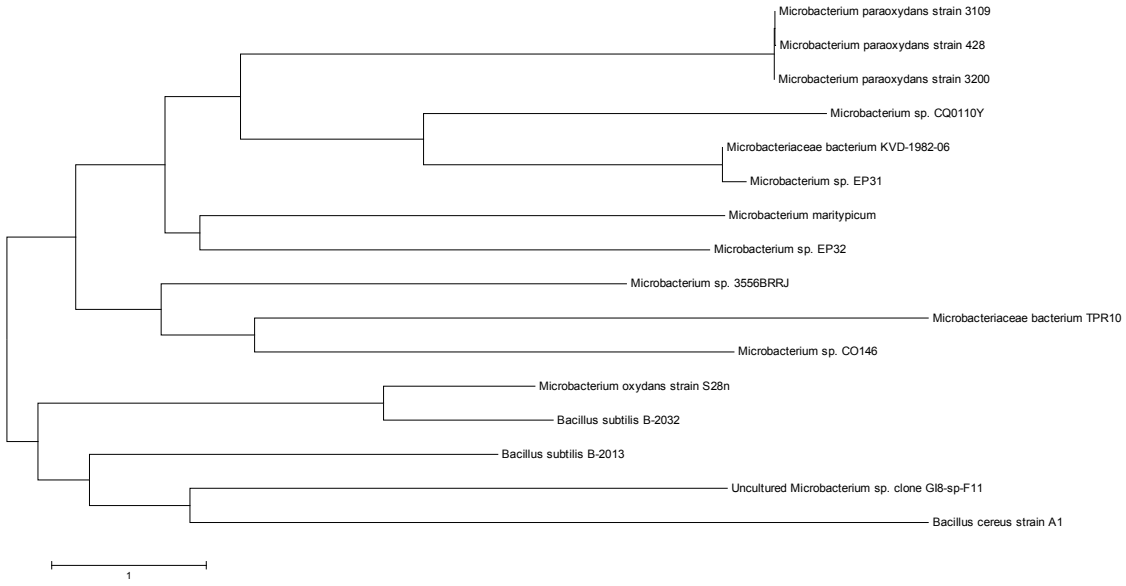

Figure 1

Neighbor-joining tree showing the phylogenetic positions of Microbacterium maritypicum and related taxa based on 16S rRNA gene sequences.

3.3 Response surface methodology and statistical analysis

Box–Behnken design matrix and RSM experiments were used to optimise the process of biotransformation of Cr (VI) ions using *Microbacterium maritypicum.* Three significant parameters were studied pH, temperature and initial concentration of Cr (VI) ions. The domain factor and level selected for designing the box-behnken design is presented in table 2.

Table 2

The experimental domain factor and level for the Box-Behnken design

Code	Name of factor	Range and levels (coded)		
		-1	0	+1
A	pH	4	6	8
B	temperature (^{0}C)	25	30	35
C	Initial concentration (mg/L)	10	25	40

Experiments were performed according to box-behnken design and observed response for biotransformation of Cr(VI) ions by *Microbacterium maritypicum* is summarised in table 3.

Table 3
The Box-Behnken design matrix for experimental design and observed response for Cr(VI) biotransformation using Microbacterium maritypicum.

Experimental Run	pH (A)	Temp. (^{o}C) (B)	Initial Cr(VI) ion conc. (C)	%Cr(VI) Biotransfomation
1	4	30	40	17
2	4	25	25	26
3	6	25	40	44
4	4	30	10	22
5	6	25	10	72
6	6	30	25	73
7	6	30	25	72
8	6	30	25	72
9	8	30	10	68
10	4	35	25	20
11	8	30	40	47
12	6	35	40	39
13	6	35	10	55
14	8	25	25	43
15	8	35	25	70
16	6	30	25	64
17	6	30	25	73

The relationship between independent variables and response was drawn by second-order polynomial equations.The regression equation coefficients were calculated and the result revealed that the response i.e. Cr(VI) biotransformation fitted to the second-order polynomial equation as shown below :- % Biotransformation of Cr(VI) by*Microbacterium maritypicum*= +70.80 + 18.38 *A - 0.25*B - 9.38 *C + 8.25*A*B - 5.00*A*C + 3.25*B *C - 22.15*A^2 - 8.90*B^2 - 9.15*C^2(1)
Significance of each coefficient was determined by Student's t-test and P values. Table 4 shows the result of ANOVA for biotransformation of Cr (VI) ions by the studied bacterial strains.

Table 4
Analysis of variance for RSM variables fitted to quadratic model

Bacterial strain	Source	Sum of squares	d.f.	Mean square	F-value	P-value	Prob>F
Microbacterium maritypicum	Model	6813.71	9	757.08	15.05	0.0009	Significant
	Residual	352.05	7	50.29			
	Lack-of-fit	291.25	3	97.08	6.39	0.0526	Not Significant
	Pure error	60.80	4	15.20			
	r^2	0.9509					
	r^2_{adj}	0.8877					

Values of Prob>F is lower than 0.0500 indicate that model is significant for Cr(VI) biotransformation. The non-significant lack-of-fit (more than 0.05) showed that quadratic model is valid for present study. Non-significant lack-of-fit is good for data fitness in the model. The correlation coefficient (r^2) provides a measure of the model's variability in the observed response values. [15] The predicted r^2 0.9509 and adjusted r^2 of 0.8877 for Cr (VI) ion biotransformation is quiet in agreement with the value of r^2, which is closer to 1.0 indicated that experimental data fits in the studied model. High value of parameter estimated for variables A, B, C, AB, AC, BC, A^2, B^2, C^2 showed a high level of significance indicated the importance of these variables in Cr(VI) biotransformation process. The variable A (pH) showed positive relationship whereas the variables B (Temperature) and C (initial concentration of metal ions) have negative relationship in biotransformation of Cr (VI) ions in *Microbacterium maritypicum*.

Optimization of variables for biotransformation of Cr (VI)

The effect of three interactive parameters: pH, temperature and initial concentration of Cr(VI) ions on the biotransformation of Cr (VI) ions on the basis of quadratic polynomial equation of response surface methodology were analysed as shown in Eq. 1.

Individual effect of pH, temperature and initial Cr(VI) ion concentration:

It could be analysed from equation 1 that individually pH, temperature and initial Cr(VI) ions concentration had a significant effect on biotransformation of Cr(VI) ions (P>0.0009). pH showed positive effect in Cr(VI) biotransformation process. With increase in pH, rate of Cr(VI) biotransformation got increased. However, temperature and Initial Cr(VI) ion concentration (P>0.0009) had a negative effect on biotransformation of Cr (VI) ions since the process of biotransformation decreased with increase in temperature and initial concentration of Cr(VI) to a certain limit.

Effect of pH and temperature

Figure2 shows the interactive effect of two variables pH (A) 4.0–8.0 and temperature (B) 25–35˚C on biotransformation of Cr (VI) ions. The figurealso revealed that biotransformation of Cr (VI) ions were increased with increased pH from 4.0 to 7.0 and temperature from 25˚C to 31˚C and after that biotransformation of Cr (VI) ions decreased with increased pH and temperature. Thus decrease in solution pH causes the formation of more polymerised chromium oxide species. As pH increased, the number of negatively charged sites increased and the number of positively charged sites decreased. A negatively charged surface site on bacteria does not favour the formation of anions due to electrostatic repulsion. In the present study, chromium cations at pH 7 was found to be interacted more strongly with the negatively charged binding sites of the bacterial strain due to ionic attraction. Maximum biotransformation of Cr (VI) was observed 73% at pH 7.0 and temperature 30^{o}C using *Microbacterium maritypicum*. The results obtained by this study are well in agreement with the results obtained by Das and Mishra [16] who reported that maximum specific chromium uptake occurred at pH 7.0 by *Brevebacterium casei*.

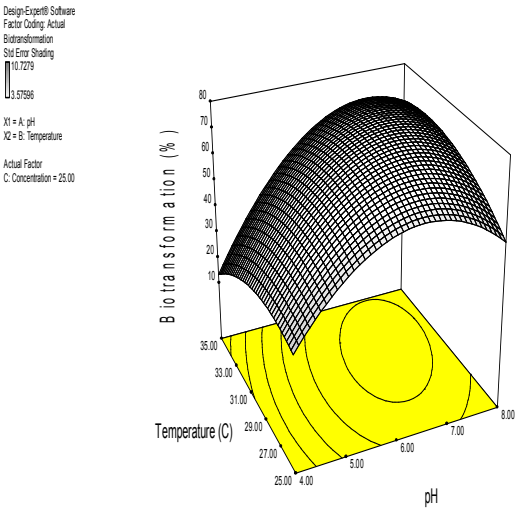

Figure 2

Three dimensional surface plot showing the interactive effect of temperature and pH on Cr(VI) biotransformation by Microbacterium maritypicum.

Effect of pH and initial Cr(VI) ion concentration

Figure 3 shows interactive effects of pH and initial Cr(VI) ion concentration on biotransformation of Cr(VI) using *Microbacterium maritypicum*. It could be analysed from the figure that biotransformation of Cr(VI) ions were increased when pH was increased from 4.0 to 7.0, after that it decreased till pH 8 while the biotransformation of Cr(VI) increased with initial increase of initial concentration of Cr(VI) ions upto 25 mg/L, after that biotransformation of Cr(VI) ions decreased with increased metal ion concentration. Maximum biotransformation was observed 73 % at pH 7 and initial concentration of Cr (VI)

ions at 25 mg/L. Several studies have shown that the presence of Cr(VI) at levels of 0-50 ppm in the microorganism's cells does not interfere with the cell growth of microorganisms (38-40) because besides growth, the microorganism will make side product of hydrogen sulfide (H_2S). Hydrogen sulfide which is produced by the bacteria will react with chromium to form chromium sulfides that are not stable in solution and quickly gets deposited to form $Cr(OH)_3$ i.e. Cr(III) which has lower toxicity than Cr(VI).[17]

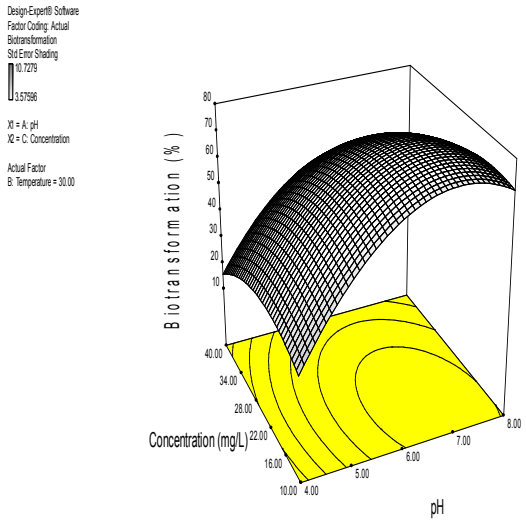

Figure 3

Three-dimensional surface plot showing the interactive effect of initial Cr(VI) ion concentration and pH on Cr (VI) biotransformation by Microbacterium maritypicum.

Effect of temperature and initial Cr(VI) ion concentration

Figure 4 showed interactive effect between temperature and initial concentration on biotransformation of Cr (VI) ions. The biotransformation of Cr (VI) ions increased first with the increase of initial Cr(VI) ions concentration and reached a saturated value and after that removal of Cr (VI) ions were decreased with increase of concentration. However, with increase of temperature from 25°C to 35°C, biotransformation of Cr (VI) ions increased with the increase of concentration up to 30 mg/L and after that transformation of Cr(VI) ions decreased with the increase in concentration of Cr(VI) ions. Maximum biotransformation of Cr(VI) was observed 73 % at 30°C and initial concentration 25mg/L. Similar results were obtained by Essahale *et al.,*[18] who reported that temperature 30^0C constituted favourable temperature for the growth and reduction of Cr(VI) in *Acinetobacter* AB1.

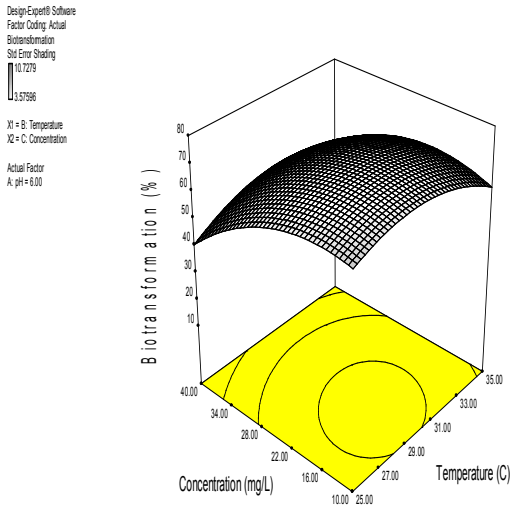

Figure 4

Three-dimensional surface plot showing the interactive effect of initial Cr(VI) ion concentration and temperature on Cr (VI) biotransformation by Microbacterium maritypicum.

3.4 Quantification of total Chromium and Cr(III) ions

During optimisation of pH, initial Cr(VI) ion concentration and temperature, the concentration of total chromium and Cr(III) was observed during 24 hours of incubation time period using *Microbacterium maritypicum* as shown in Figure 5, 6 and 7 respectively. It could be analysed from Fig 5, 6 and 7, that the concentration of total chromium remained almost constant, however the concentration of Cr(III) was found to increase during incubation time period. The initial Cr(III) concentration was observed as 1.08 mg/L, 1.48 mg/L and 0.78 mg/L at time period zero and finally after 24 hrs, Cr(III) concentration rose to 6.22 mg/L, 19.32 mg/L and 6.08 mg/L during optimisation of pH, initial Cr(VI) ion concentration and temperature respectively. Moreover, it was observed that the pH at the end of the experiments was recorded between 7 and 8 that seem to be adequate for the precipitation of the chromium hydroxide.

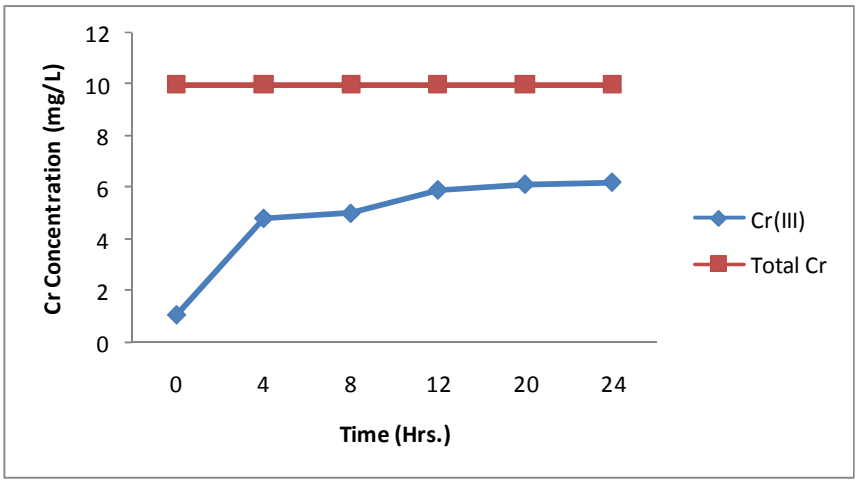

Figure 5

Quantification of total chromium and Cr(III) during optimization of pH

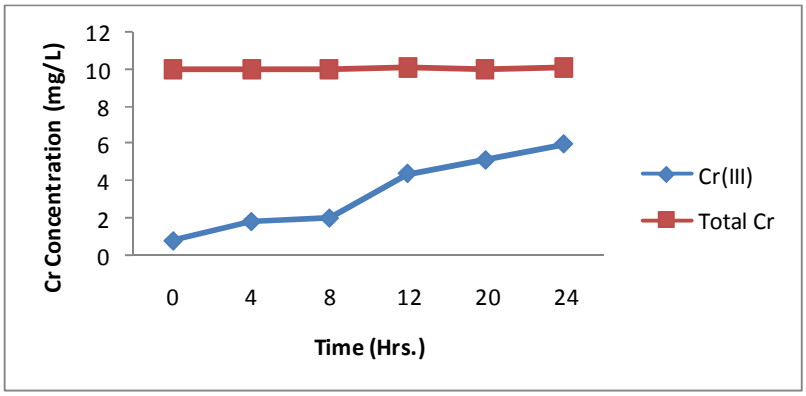

Figure 6
Quantification of total chromium and Cr(III) during optimization of initial concentration

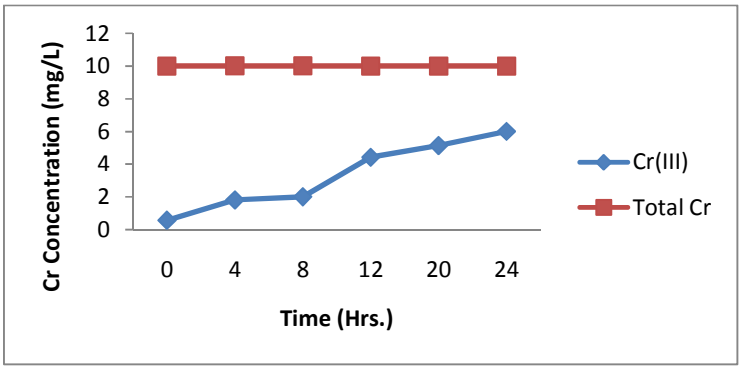

Figure 7
Quantification of total chromium and Cr(III) during optimization of temperature

4. CONCLUSION

The present study involves the use of indigenous bacterial strain which was isolated from electroplating industrial effluents for Cr(VI) biotransformation. Microbial characterization along with 16S rRNA studies revealed this bacterial strain as *Microbacterium maritypicum.* Response surface methodology was employed to observe the interactive influence of three important process variables: pH, temperature and initial concentration of Cr(VI) ions. Values of "Prob>F" less than 0.0500 indicated that model terms have significant effect on biotransformation of Cr(VI) ion using *Microbacterium maritypicum*. Maximum biotransformation of Cr(VI) was observed as 73 % at pH 6, temperature 30˚C and initial concentration of ions 25 mg/L. RSM approach proved useful and accurate for the optimisation process in the present study. Moreover, use of indigenous bacterial strains reduces the dependence on outside resource and technology for Cr(VI) biotransformation process.

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