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Optimization of Growth Factors of Hexa Valent Chromium Reducing Bacterial Strain.

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Abstract: *The aim of this work is to optimize the growth parameters for the reduction of Hexavalent Cr using isolated soil bacteria (SS 31). Different growth conditions like the nitrogen sources, pH, incubation time, temperature, agitation rate and the effect of different additional supplements was studied. The isolated strain shows more than 50% reduction on most of the conditions. But high reduction activity was found to be 0.2g/L Beef extract as nitrogen source in the broth at pH 7, incubation period of 24H at 35^{O}C with agitation rate of 50rpm. The presences of additional supplements like Cystine amino acid, EDTA as metabolic inhibiter, Thiamine vitamin and Streptomycin antibiotic in the culture broth was found enhance the Cr reduction activity. All the optimization studies were studied at Cr concentration of 100µg/ml.*

Key Words: *Bioremediation, Soil bacteria, Cr Reduction, Optimization of growth parameters*

I. Introduction:

Chromium as a toxic heavy metal having large industrial applications and the resultant effluent discharge affect the environment adversely. Conventional chemical, physical methods and activated sludge biological treatment for removal of Chromium are generally efficient in removing the bulk of metal from solution at high or moderate concentrations, where as they may be ineffective or extremely expensive especially when the metals in solution are at low concentration [1, 2].

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 bio reduction based remediation of Chromium contaminated water or soil [3, 4].

Optimization may involve the study of many biochemical and physical parameters, including media formulation and culture parameters for the removal of Cr. Here the main aim of the study was to optimize the growth parameters for the reduction of Cr using the isolated Cr resistant bacteria.

II. Materials and Methods:

2.1 Instrumentation

Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Standard cuvettes of 10mm path length are used for analysis. Standard Chromium was weighed by using Denver Electronic Analytical Balance (SI-234), Autoclave, Laminar air flow , Rotary shaker and Centrifuge were used.

2.2 Chemicals and Reagents:

All the chemicals used for preparation of growth media for isolation and optimization of Bacteriological grade are Merck chemicals limited, Mumbai. Standard $K_2Cr_2O_7$, coloring reagent Diphenyl Carbazide (DPC method) and other chemicals used were of AR grade and were purchased from Loba Chemicals, Mumbai.

2.3 Isolation and Selection of Cr Reducing Bacteria for Optimization:

The microbial strains here used for the study were isolated from different soil samples as per the procedure explained in our previous work [5]. Based on the amount of Chromium found in the broth supernatant percentage of Chromium Reduction was estimated and it was found that the bacterial strains coded SS215, SS31 were capable of high reducing activity (58%, 63% respectively) than other strains. Hence bacteria isolate having high Cr resistant activity (SS 31) was selected and was used for optimization study [5].

2.4 Preparation of Standard Cr Solution:

An amount of $K_2Cr_2O_7$ equivalent of 10mg of Cr weight was weighed accurately and was dissolved in 100ml of double distilled Cr free water. A stock solution concentration of 100µg/ml was obtained. From this stock solution, 10ml was further diluted top 100ml to get a working standard solution of 10µg/ml Cr solution was obtained. This solution was used for construction of calibration curve for the estimation of Cr using Spectrophotometric method.

2.5 Construction of Calibration Curve for Estimation of Cr:

Aliquot of standard drug Cr solution (0.5-5ml; 10µg/ml) was delivered into a series of 10 ml of calibrated tubes. Then 1.0 mL of 0.1N HCl solution was added to each tube mix the solution and then 1ml of Diphenyl Carbazide solution was added. The content was mixed well, wait for 5min to develop purple color. The optical density of the obtained color was measured at 540nm against reagent blank. Calibration curve was constructed by using concentration on x-axis and absorbance on y-axis.

2.6 Estimation of Chromium Reducing Activity by Spectrophotometer:

Diphenyl Carbazide method was followed for the estimation of Cr reducing activity by visible spectrophotometer. To 1ml of Cr solution, 1ml of 1N HCl solution was added and mix the solution and then 1ml of Diphenyl Carbazide solution was added. The content was mixed well, wait for 5min to develop purple color. The optical density of the obtained color was measured at 540nm against reagent blank. The procedure was repeated for different aliquots of standard Cr solution and calibration curve was constructed using concentration against absorbance forum. The calibration curve was used for estimation of Cr in samples.

III. Optimization of Growth parameters:

3.1 Effect of Cr Concentration on Bacterial Activity:

Luria Bertani (LB) Broth was selected for the optimization of study of Cr reducing bacteria. LB broth containing Tryptone (10gm), Yeast extract (5g) and NaCl (10g) with different concentrations (25-1000µg/ml) were prepared and the selected bacteria were inoculated on each of the concentration. The broth was incubated on a rotator orbital shaker. After incubation the broth was centrifuged, supernatant was collected and amount of Cr present in the broth, amount of Cr reduced by the organisms were studied.

3.2 Optimization of Nitrogen Source:

Different Nitrogen sources like Beef extract, Peptone, Yeast extract, Tryptone, Soyabean Meal and Gelatin were optimized in three different concentrations of 75%, 100%, 125% level concentration. After incubation period, the broth was centrifuged and the supernatant was collected. From the supernatant, the amount of Cr present and % of Cr reduced was calculated using Diphenyl Carbazide method.

3.3 Optimization of growth medium pH:

The pH of growth medium was changed in the range of 4.5 to 10.5 using 0.1M NaOH and 0.1M HCl. after the incubation period; the suitable pH for Cr reduction using isolated bacteria was measured using Diphenyl Carbazide method.

3.4 Effect of Incubation Time, Temperature:

LB broth with 100µg/ml Chromium concentration was inoculated with isolated bacteria. The broth was incubated in different incubation temperatures and different incubations times. The % of Cr reduced was calculated using standard DPC reagent.

3.5 Effect of additional supplements (Metabolic Inhibiters, Vitamins, Amino Acids and Antibiotics):

The effect of different amino acids (Alanine, Arginine, Cystine, Glycine, Tyrosine and Tryptophan), Vitamins (Riboflavin, Citric Acid, Ascorbic acid, Folic acid, Nicotinic acid and Thamine HCl), Metabolic Inhibiters (Acetic acid, EDTA, $KMnO_4$, Silver nitrate, NaF and β-Mercaptoethanol) and antibiotics (Penicillin, Streptomycin, Neomycin, Framyicitin and Cephalosporin) were studied for the Cr reduction activity of isolated bacteria (SS 31).

3.6 Effect of inoculums volume and agitation rate on Cr reduction:

Different agitation rates from 25 – 150rpm and different volumes of the inoculums were studied for the reduction activity of Cr.

IV. Results and Discussions:

Microbes can develop a high resistance to heavy metals by a variety of mechanisms to remove ions, such as adsorption to cell surfaces, complexation by exo polysaccharides, intracellular accumulation or precipitation [6, 7]. Hence isolating microbes from polluted environments would represent an appropriate practice to select metal-resistant strains that could be used for heavy metal removal and bioremediation purposes. Hence very high Cr resistant bacteria were isolated from different industrial polluted soils. Among the bacteria isolated, SS 31 found to be very high Cr resistant activity [5].

Hence the optimization of growth parameters for the reduction of Cr was studied using DPC method. Diphenyl Carbazide is the commonly used specific reagent for the Spectrophotometric determination of Chromium [8]. Hexa valent Chromium reacts with 1, 5-diphenylcarbazide to produce a reddish purple color in acidic solution and quantified by measuring its absorbance at its wavelength of maximum absorption.

Standard calibration curve was obtained using standard Cr solution ($K_2Cr_2O_7$) within the Cr concentration range of 0.5-5µg/ml. Standard regression equation was found to be $y = 0.127x + 0.009$ (r^2=0.999). The Cr present in the samples solutions was studied using the standard regression equation. A result of the standard calibration was given in figure 1.

The suitable concentration of Cr used for the optimization of medium composition was determined with a concentration range of 20-1000µg/ml. The isolated organism (SS 31) was found to reduce all the concentrations in the study. But at a concentration of 100µg/ml, the organism was able to reduce 90% Cr in the medium. Hence a concentration of 100µg/ml was found to be suitable concentration for optimization study. Results were given in table 1.

The organism was able to grow and reduce Cr in all the examined Nitrogen sources given in Chart 1. It reduces highest Cr (83.79%) in Beef extract supplemented at a concentration of 0.2g/L medium, whereas lowest reduction was observed in peptone at a concentration of 0.3g/L (39.92%). Other nitrogen sources such as Gelatin, Soyabean Meal and Tryptone at studied concentrations (0.2g/L, 0.25g/L and 0.30g/L) were found to reduce more than 50% Cr in the broth. Hence Beef extract as nitrogen source at a concentration of 0.2g/L was found to be most suitable for the reduction of Cr.

By using 0.1N HCl and 0.1N NaOH solution, the pH of the broth was adjusted within the range of 4.5-10. The effect of pH on Cr reduction was studied after the incubation period. Results given in chart 2 proved that at neutral pH (7) was found to be most suitable for Cr reduction and it reduce 91.5% Cr in the broth. After that very slight base pH of 7.5 reduces (89.81%) more Cr. Extreme high and low pH in the broth shows significantly less Cr reduction activity.

The incubation time that was sufficient for reduction of Cr was studied by incubating the culture broth in different time periods from 12H to 72H. 24H incubation was found to be most suitable and less time consuming incubation period for more reduction of Cr (Chart 3). Different incubation temperatures were also studied and results proved that at room temperature (35^0C) was found to reduce very high Cr (90.436%). The effect of agitation rate of the incubated culture broth was also studied in different agitation rate range of 25-150rpm. More Cr reduction of approximately 90% was achieved at an agitation rate of 50rpm.

The effect of additional supplements in the culture broth like different Metabolic Inhibiters, Vitamins, Amino Acids and Antibiotics were studied. Results proved that the presence of Cystine amino acid, EDTA as metabolic inhibiter, Thiamine vitamin and Streptomycin antibiotic in the culture broth was found to reduce more Cr.

Finally the isolated bacterial strain having 63% Cr reduction activity was selected for optimization study, after the optimization of all the growth parameters, the Cr reduction activity was enhanced more than 90%.

V. Conclusion:

Lesser studies have been conducted by researchers on bioremediation of metals using isolated bacteria. Hence in the present research, a potent bacterium (SS 31) was isolated from locally sourced industrial effluents and its potential to uptake of Chromium was tested. The isolated strain can effectively reduce Hexa valent Chromium under wide range of environmental parameters. The optimization studies were carried at Cr concentration of 100µg/ml. finally the optimized conditions achieved as 0.2g/L Beef extract as nitrogen source in the broth at pH 7, incubation period of 24H at 35^OC with agitation rate of 50rpm. The presences of additional supplements like Cystine Amino acid, EDTA as Metabolic Inhibiter, Thiamine Vitamin and Streptomycin Antibiotic in the culture broth was found enhance the Cr reduction activity.

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List of tables and figures:

S. No.	Concentration Prepared in µg/ml	Amount Found in µg/ml	% Reduction
1	25	14.39	57.56
2	50	34.47	68.94
3	75	56.11	74.81
4	100	89.94	89.94
5	200	128.46	64.23
6	500	289.16	57.832
7	1000	449.98	44.998

Table 1: Results of Determination of Suitable Cr Concentration for Optimization Study.

S. No.	Nitrogen source	Changing conc.	Absorbance found	Amount of Cr. Present µg/ml	%Reducing
1	Beef extract	0.2g/1000ml	0.422	16.212	83.788
2	Beef extract	0.25g/1000ml	1.278	49.886	50.114
3	Beef extract	0.30g/1000ml	0.9975	38.851	61.149
4	peptone	0.2g/1000ml	1.528	59.721	40.279
5	peptone	0.25g/1000ml	1.328	51.853	48.147
6	peptone	0.3g/1000ml	1.537	60.075	39.925
7	Yeast Extract	0.2g/1000ml	0.951	37.022	62.978
8	Yeast Extract	0.25g/1000ml	0.911	35.448	64.551
9	Yeast Extract	0.30g/1000ml	0.898	34.937	65.063
10	Tryptone	0.2g/1000ml	1.121	43.710	56.290
11	Tryptone	0.25g/1000ml	0.793	30.806	69.193
12	Tryptone	0.30g/1000ml	0.711	27.581	72.419
13	Soyabean Meal	0.2g/1000ml	0.789	30.649	69.351
14	Soyabean Meal	0.25g/1000ml	0.779	30.256	69.744
15	Soyabean Meal	0.30g/1000ml	0.858	33.363	66.636
16	Gelatin	0.2g/1000ml	0.993	38.674	61.326
17	Gelatin	0.25g/1000ml	0.789	30.649	69.351
18	Gelatin	0.30g/1000ml	0.852	33.127	66.872

Table 2: Results of Optimization of Nitrogen Source for Cr Reduction

S.NO	incubation period	Absorbance Found	Amount of Cr. Present µg/ml	% Reducing
1	12H	0.716	27.777	72.223
2	18H	0.561	21.680	78.320
3	24H	0.378	14.481	85.519
4	36H	0.389	14.913	85.087
5	48H	0.477	18.375	81.625
6	60H	0.425	16.330	83.670
7	72H	0.398	15.268	84.732

Table 3: Results of Optimization of Incubation for Cr Reduction

Amino acid		Metabolic Inhibiters		Vitamines	
Changed	**%Reducing**	**Changed**	**%Reducing**	**Changed**	**%Reducing**
Alanine	85.637	Acetic acid	77.730	Riboflavin	62.073
Arginine	80.681	EDTA	82.293	Citric Acid	70.138
Cystine	91.381	$KMnO_4$	71.121	Ascorbic acid	70.138
Glycine	90.830	Silver nitrate	69.154	Folic acid	78.674
Tyrosine	66.715	NaF	52.356	Nicotinic acid	75.370
Tryptophan	64.591	β-Mercaptoethanol	61.247	Thamine HCl	81.821

Table 4: Results of Optimization of Nitrogen Source for Cr reduction

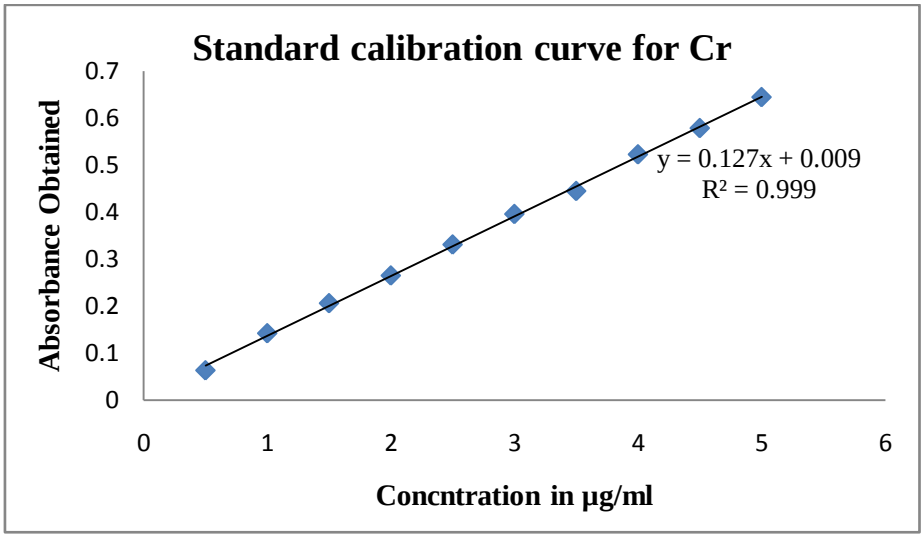

Figure 1: Standard Calibration Curve for Estimation of Cr in Samples.

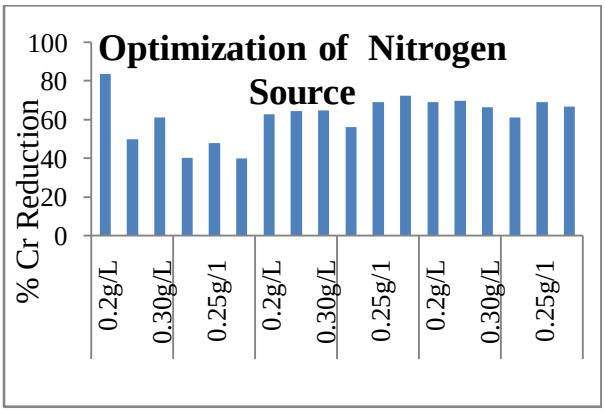

Chart 1: Optimization of Nitrogen Source for Cr Reduction

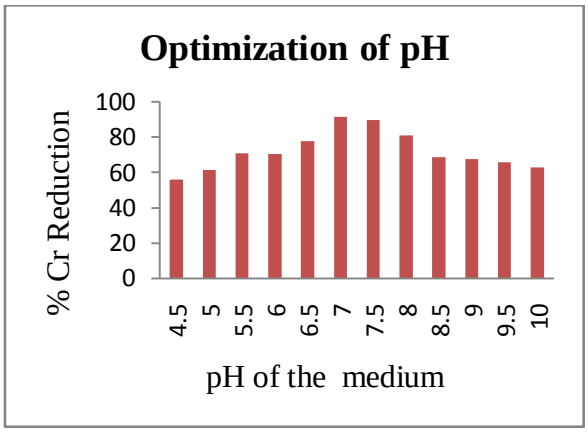

Chart 2: Optimization of pH for Cr Reduction

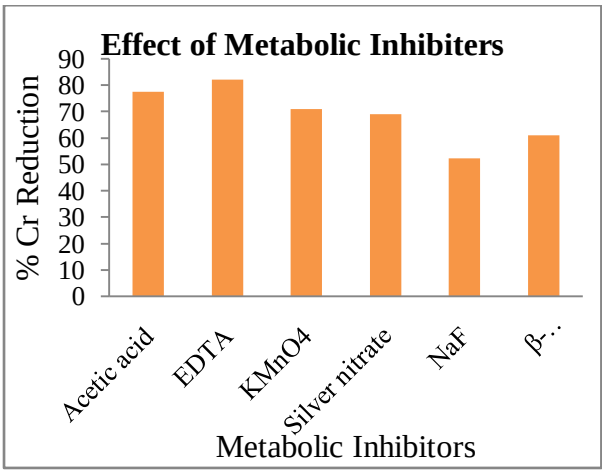

Chart 5: Effect of Metabolic Inhibitors for Cr Reduction

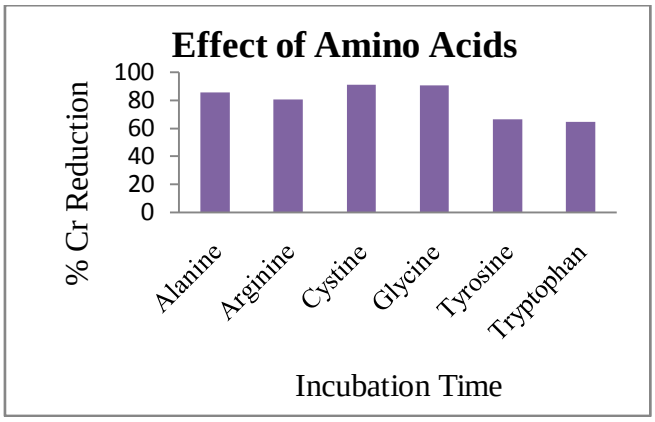

Chart 4: Effect of Amino Acids for Cr Reduction

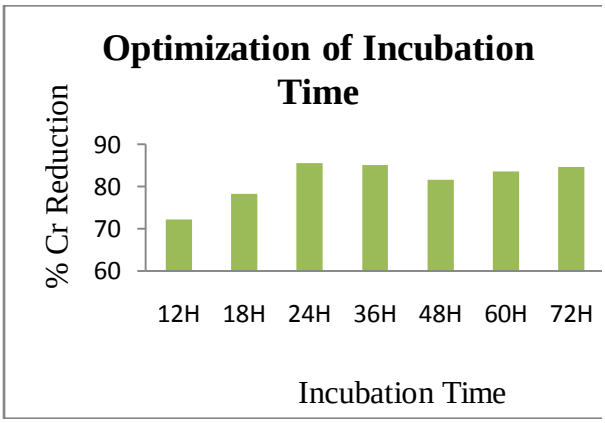

Chart 3: Optimization of Incubation Time for Cr Reduction

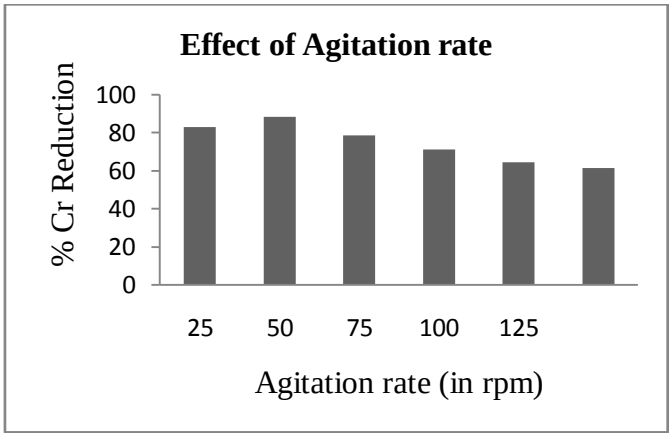

Chart 4: Effect of Agitation rate for Cr Reduction