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Biotransformation of Hexavalent Chromium by *Acinetobacter iwofii*

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Abstract: *In the present study, bio reduction of hexavalent Chromium, a transition metal, known for its high solubility and carcinogenicity, using a sewage isolate was tested under controlled laboratory conditions. The objective of the study included determination of the extent of bio degradation of Cr[VI] by the isolate, characterization of the isolate and testing the degradation potential of the isolate under variable conditions of PH and temperature.The bacterial isolate was identified to be a Gram negative coccobacillus, Acinetobacter iwofii which shows the capacity to reduce Cr[VI]at concentration as high as 60 ppm at a temperature of 55 ^{0}C and PH ranging from 6-8 in aerobic conditions. Confluent growth was observed along with reduction of Cr[VI]at 37 ^{0}C; however, reduction was greater at 55 ^{0}C Due to its ability to reduce Cr[VI] the organism shows a considerable potential for use in biotransformation studies involving Cr[VI] contamination.The reduction potential of the isolate was comparable to the reduction potential of the consortia reported previously. However, the relatively low potential to detoxify the environment of Cr[VI] by the isolate, may be enhanced by using it in a consortium with other potential chromate reducers.*

Keywords: *Acinetobacter iwofii, Chromium, Hexavalent, Trivalent*

I. Introduction

Bioremediation is the use of living organisms to reduce, eliminate, immobilize or transform environmental hazards resulting from accumulation of toxic chemicals and other hazardous wastes [1] Initially used only for the eradication of sewage related waste, bioremediation has now been successfully applied to detoxify a range of contaminants such as solvents, explosives, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyl (PCBs). As the name suggests, bioremediation encompasses the use of plants, fungi, algal biomass, microbes and in some cases animals such as fishes, arthropods, filter feeders for the cleanup of the pollutants from the environment. [2]

Contaminants which persist for a long time in the environment are recalcitrant, mobile and toxic to human health. [3,1] Metals and radionuclide's are the examples of such pollutants which unfortunately cannot be degraded to simple inorganic substances even by the use of microorganisms. However, these pollutants can be bio transformed to produce immobile and nontoxic substances which do not affect the environment or the ecosystem directly. This is done by biotransformation of the oxidation states (oxidation/reduction) of metals which render them nontoxic and affect their mobility. Conversely these reactions may also affect the solubility of the transformed metal or radionuclide in such a way that the solubility increases, thus increasing the mobility of the contaminant and allowing it to more easily be flushed from the environment. In either case, biotransformation helps in the removal of metal contamination from the environment. [3,1] The anthropogenic sources of metal contamination can be divided in five main groups: 1) Metalliferous mining and smelting 2) Industry 3) Atmospheric deposition 4) Agriculture and 5) Waste disposal. [3,1] Bacteria and fungi reduce the toxic form of chromium from its +6 state to less toxic +3 state primarily by two mechanisms 1. By the metabolically active enzymatic process and 2. By the passive Biosorption of the Cr [VI] ion onto the cell wall. Several bacteria reduce Cr [VI] through membrane bound reductases such as Flavin reductase, cytochromes and hydrogenases. These enzymes can be a part of electron transport system and use chromate as terminal electron acceptor [3,1,4]. Similarly, the organisms can adsorb Cr [VI] as the cell wall may be regarded as a mosaic of different groups which form coordination complexes with metal ions, a passive process [3,1,5]

In our study, we have targeted chromium, a hard brittle, semi gray heavy metal. It occurs in nature mainly as chromite ($FeCr_2O_4$). This element has been used since the beginning of the industrial age. The most commonly found oxidation states are+3 and +6, with +6 being the most toxic [3,5,6,7]. It has been reported to be teratogen in recent studies. [5] Tanneries are a major source of chromium pollution and release Cr [VI] ranging from 40- 25000 mg/l of waste water. The maximum tolerance of Cr for public water supply has been fixed as 0.05mg/l as per the Indian Standards [7]. According to World Health Organization (WHO) drinking water guidelines, the maximum allowable limit for hexavalent chromium and total chromium (including Cr[III], Cr[VI] and other forms) are 0.05 and 2 mg/l respectively. According to EPA Safe Drinking Water Act,

Maximum Contaminant Level (MCL) is 0.1 mg/l [9]. Biotransformation of Cr[VI] to the less toxic and less mobile form Cr[III] presents an opportunity for bioremediation of hexavalent Cr. [3,4,6,9,10].

II. Materials and Methods

2.1. Sample collection and selection

2.1.1. Collection

Three sewage samples were obtained from Vipanan Industry, Pune and refrigerated until further use. These were named as A, B and C. A 0.1mL of each sample was inoculated in a Bumper tube containing 5 ml of sterile Nutrient Broth and incubated at room temperature for 24 hours with shaking at 100 rpm. 0.1 mL of this culture was plated using the spread plate technique on sterile Nutrient medium agar plates and incubated at room temperature for 24 hours to check for the presence of microorganisms in the samples.

2.1.2. Selection

The three samples were analyzed for their ability to tolerated or reduce Hexavalent chromium by the presence of growth in the form of turbidity and decolourization of the medium from yellow to pale yellow or colorless. 1 ml of each of the sewage samples i.e. A, B and C was inoculated into a 250 ml flask containing 100 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salts (Working Standard Hexavalent chromium solution) to make a final Cr[VI] concentration of 10, 20 and 30 ppm. The flasks were incubated aerobically at room temperature for 48 hours with shaking at 100 rpm. Following incubation, 0.1 ml of the sample was plated using spread plate technique on sterile Davis and Mingioli Medium agar plates supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) to obtain the corresponding concentrations of Cr[VI] present in the respective culture medium. Preliminary Cr[VI] tolerance and reduction experiments revealed that Sample A had the maximum growth and reduction potential in Cr[VI] concentrations of up to 30 ppm. Thus, Sample A was selected for further analysis.

2.2. Standard Hexavalent chromium solution

Hexavalent chromium concentrations were prepared in the manner described in MDHS (reference). 2.828 gm of dried Potassium dichromate ($K_2Cr_2O_7$) was dissolved in deionized water. It was diluted to volume in a 1 Liter volumetric flask and mixed thoroughly to obtain a Standard Hexavalent chromium solution of 1000µg/ml concentration. 10 ml of Standard Hexavalent chromium solution was diluted to volume in a 100 ml volumetric flask with deionized water and mixed thoroughly to obtain the Working Standard Hexavalent chromium solution of 100 µg/ml concentration.

2.3. Enrichment and isolation

2.3.1. Enrichment

Enrichment of Sample A was carried out by inoculating 5 ml of the sample in 100 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) to obtain a final Cr[VI] concentration of 20 ppm in a 250 ml flask. The culture was incubated aerobically at room temperature for 48 hours with shaking at 100 rpm.

2.3.2. Isolation

Following incubation, 0.1 ml of the sample was plated on sterile Davis and Mingioli Medium agar plates supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) to obtain a final Cr[VI] concentration of 20 ppm, to obtain the individual isolates responsible for Cr[VI] reduction.

2.3.3. Sterilization of media

All media and glassware were sterilized by autoclaving at 121^0C at 15 psi for 20 minutes. Glucose solution used in the preparation of Davis and Mingioli Medium broth and plates was sterilized separately by autoclaving at 121^0C at 10 psi for 20 minutes. Pipettes used were sterilized at 180^0C in an oven for 3 hours.

2.4. Chromium reduction experiments

Chromium reduction experiments were conducted aerobically at 37ºC for a period of 10 days. The experiment consisted of six concentrations of Cr[VI](10, 20, 30, 40, 50 and 60 ppm). 2 ml of the isolate suspension was added to 25 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) in a bumper tube, to obtain the above mentioned Cr[VI]concentration. The amount of Cr[VI]in the medium was checked each day to determine the reduction. Cell free medium was used as controls and Hexavalent chromium standards were used as the baseline for detecting Cr[VI]reduction. Reduction was detected using the Diphenyl carbazide assay.Percentage reduction was calculated using the formula:

$$\% \text{ Reduction} = \text{Initial concentration} - \text{Final concentration} / \text{Initial concentration} \times 100$$

2.5. Effect of Temperature on Hexavalent chromium reduction

Experiments to check the effect of temperature on Cr[VI]reduction by the isolate was performed for a period of 10 days. Reduction was checked at two temperatures (4^0C and 55^0C). 2 ml of the isolate suspension was inoculated in a bumper tube, containing 25 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) to obtain the Cr[VI]concentration of 10, 20, 30, 40, 50 and 60 ppm. Cell free medium was used as controls.
Reduction was detected using the Diphenyl carbazide assay daily.

2.6. Effect of pH on Hexavalent chromium reduction

Experiments to check the effect of pH on Cr[VI]reduction potential of the isolate were performed for a period of 12 days. Six different pH (2, 4, 6, 8, 10 and 12) were considered for each of the six Cr[VI]concentrations of 10, 20, 30, 40, 50 and 60 ppm. 2 ml of the isolate suspension was inoculated in 25 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) in a bumper tube. Cell free medium was used as controls. Reduction was detected using the Diphenyl carbazide assay after every 48 hours for a period of 12 days.

2.7. Hexavalent chromium adsorption experiment

To confirm that the observed decrease in the Cr[VI]concentrations were due to reduction and not Biosorption, two separate adsorption studies were conducted using killed cells and metabolically inhibited cells. (2) Cells were killed by autoclaving and metabolically inhibited by incubation at -5°C. For the killed cells, 2 ml of the isolate suspension was inoculated in 25 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working Standard Hexavalent chromium solution) and autoclaved, followed by incubation at 37^0C. For metabolically inhibited cells, 2 ml of the isolate suspension was inoculated in 25 ml of sterile Davis and Mingioli Medium broth supplemented with Potassium dichromate salt (Working StandardHexavalent chromium solution) and incubated at -5^0C in the freezer. Both these experiments were carried out for a period of 48 hours and reduction was detected by decolourization of the medium and by using the Diphenyl carbazide assay.

2.8. Diphenyl carbazide assay

The diphenyl carbazide assay for the measurement of Cr[VI]was performed according to Standard Methods for the Examination of Waters and Wastewaters (reference book) with the modification that the dilutions used for making the standard graph was prepared from the Working Standard Hexavalent chromium solution (100ppm).

2.8.1. Preparation of diphenyl carbazide reagent

The diphenyl carbazide reagent was obtained from Merck Specialties Private Limited and was prepared by dissolving 250 mg of 1, 5 diphenyl carbazide (1, 5 diphenylcarbohydrazide) in 50 mL Acetone. It was stored in a brown bottle and discarded when the solution turned pink.

2.8.2. Preparation of standard graph

To detect decrease in Cr[VI]concentrations, a standard curve was prepared in the following manner. Working Standard Hexavalent chromium solution (100ppm) was used for the preparation of the following dilutions. The diluent used was Distilled water.

Table1: Preparation of working standard of hexavalent chromium

Sr. No	Cr[VI]Concentration (inppm)	Volume of Working Standard Hexavalent chromium solution (100ppm) (in ml)	Diluent (Distilled Water) (ml)	Total Volume (ml)
1	10	1	9	10
2	20	2	8	10
3	30	3	7	10
4	40	4	6	10
5	50	5	5	10
6	60	6	4	10
7	70	7	3	10
8	80	8	2	10
9	90	9	1	10
10	100	10	0	10

2ml of each of the concentrations were taken and 0.2 N H_2SO_4 was used to adjust the solution to a pH of 1.0 ± 0.3. The solution was transferred to a volumetric flask and diluted to 100 mL using Distilled water. It was mixed thoroughly. 2.0 mL of diphenyl carbazide solution was added, mixed and allowed to stand for 5 to 10 minutes for full color development. An appropriate portion of the solution was transferred to a 1 cm cuvette and its absorption was measured at 545 nm. Distilled water was used as reference blank. The diphenyl carbazide solution was used as substrate blank. The final reading was obtained by subtracting the reading of the substrate blank from that of the Test. The standard graph was prepared by plotting the Cr[VI]concentrations in the unit of ppm on the X-axis and the Absorbance in the unit of nm on the Y-axis.

2.9. Identification

The isolate was subjected to various biochemical tests to identify the Genus using Bergey's Manual of Determinative Bacteriology. The tests performed were as follows: -

1. Indole Test
2. Methyl Red Test
3. Voges-Proskauer Test
4. Citrate Utilization Test
5. Sugar Utilization Test
6. Sucrose Test
7. Mannitol Test
8. Arabinose Test
9. Xylulose Test
10. Oxidase Production Test

III. Results

3.1. Presence of organisms in the samples

The presence of organisms in the three samples A, B and C was confirmed by the growth of a large number of colonies on Nutrient Agar plates after an incubation period of 24 hours.

3.2. Selection

The ability to tolerate and reduce Cr[VI]concentration of 10, 20 and 30 ppm was shown by the presence of growth in the form of turbidity and decolourization of the medium from yellow to pale yellow or colorless. Sample A showed maximum turbidity and Sample C showed the minimum turbidity and decolourization of the medium after an incubation period of 48 hours for each of the above mentioned concentrations of Cr[VI]. No turbidity or decolourization was seen in the control flasks.

Table 2: Growth of isolates in presence of Cr[VI]

Sr. No.	Sample	Low Turbidity	Medium Turbidity	High Turbidity
1	A	-	-	+
2	B	-	+	-
3	C	+	-	-

The plated samples showed maximum number of colonies of Sample A and minimum number of colonies of Sample C at Cr[VI]concentrations of 10 ppm. Sample B showed intermediate number of colonies. A large number of colonies were observed up to Cr[VI]concentrations of 30 ppm in case of Sample A. Sample B showed growth up to 20 ppm but not at 30 ppm. Sample C showed the presence of growth in the form of colonies only at 10 ppm. no growth was seen in plates used as control.

Table 3: Growth of isolates at different concentrations of Cr[VI]

Sr. No.	Sample	10 ppm (Cr[VI])	20 ppm (Cr[VI])	30ppm (Cr[VI])
1	A	+	+	+
2	B	+	+	-
3	C	+	-	-

On the basis of these results, Sample A was selected for Cr[VI]reduction experiments as it had the maximum growth and reduction potential for Cr[VI].

3.3. Enrichment and Isolation

3.3.1. Enrichment

Enrichment with Cr[VI]concentration of 20 ppm showed growth in the form of turbidity at room temperature. Decolourization of the medium from yellow to pale yellow and the formation of white precipitate was also seen.

3.3.2. Isolation

The plated enriched culture showed the presence of a single isolate. This isolate was responsible for the reduction of Cr[VI]in the medium.

Table 4: Colony Characters

Sr. No.	Colony Character	Observation
1	Shape	Circular
2	Size	1 mm
3	Colour	White
4	Margin	Entire
5	Elevation	Slightly raised
6	Consistency	Butyrus
7	Opacity	Opaque
8	Gram Character	Gram positive
9	Motility	Non-Motile

3.4. Chromium reduction experiment

The Cr[VI]reduction potential of the isolate was analyzed for a period of 10 days. The amount of Cr[VI] remaining after every 24 hours was detected by the diphenyl carbazide assay. The isolate showed evidence of Cr[VI]reduction by decolourization of the medium and measurement of the decreasing Cr[VI]concentrations by the diphenyl carbazide assay. The results are as follows: -The results showed a complete reduction of Cr[VI]concentration of 10 ppm by Day 2 and up to 30 ppm within a period of 9 days. 60 ppm of Cr[VI]was reduced to 37 ppm by Day 10.

TABLE 5: Reduction in Cr[VI] concentration on day 1 and day 10

Day 1

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	05.30
2	20	12.60
3	30	21.30
4	40	27.30
5	50	40.30
6	60	51.30

Day 10

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	00.00
2	20	00.00
3	30	00.00
4	40	04.00
5	50	26.00
6	60	37.00

3.5 Effect of Temperature on Hexavalent chromium reduction

The effect of Temperature on Cr[VI]reduction was checked by the decolourization of the medium and measurement of the decreasing Cr[VI]concentrations by the diphenyl carbazide assay. The results are as follows: -

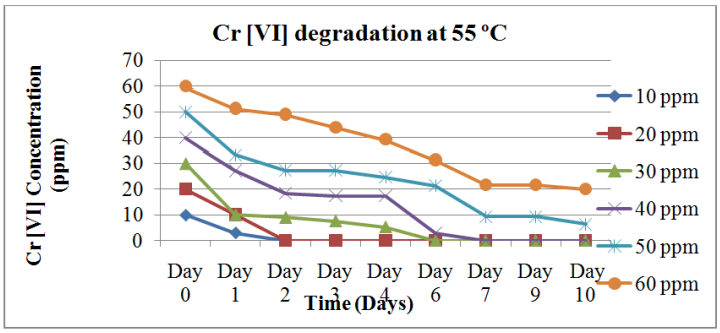
Table 6: Reduction at 55ºC

Day 1

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	03.00
2	20	10.00
3	30	10.00
4	40	27.30
5	50	33.30
6	60	51.30

Day 10

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	00.00
2	20	00.00
3	30	00.00
4	40	00.00
5	50	06.30
6	60	20.00



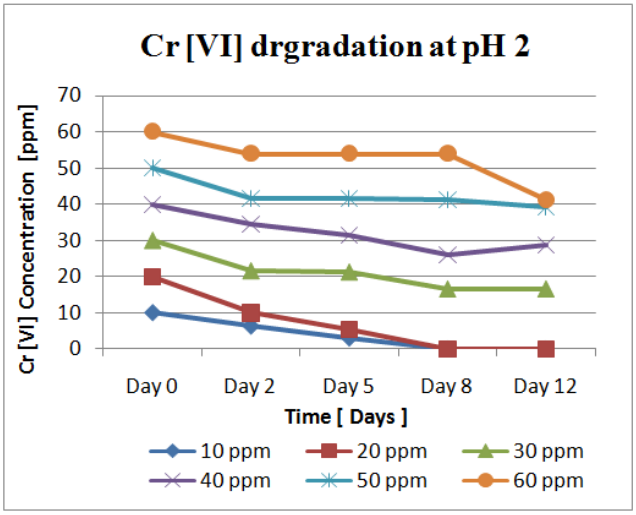
The above results showed a complete reduction of 10 ppm of Cr[VI]by Day 2 and 30 ppm by Day 6. Also, 60 ppm of Cr[VI]was reduced to about 20 ppm by Day 10.Hence, it was concluded that a temperature of 55^0C increases the rate of Cr[VI]reduction as compared to 37^0C.No growth or decolourization was seen in any of the tubes incubated at 4^0C. The diphenyl assay also did not show any reduction in Cr[VI]concentration. Thus, the experiment was discontinued after Day 2.

3.6 Effect of pH on Hexavalent chromium reduction

The effect of pH on Cr[VI]reduction was checked by the decolourization of the medium and measurement of the decreasing Cr [VI] concentrations by the diphenyl carbazide assay. The results for each of the pH are as follows:-

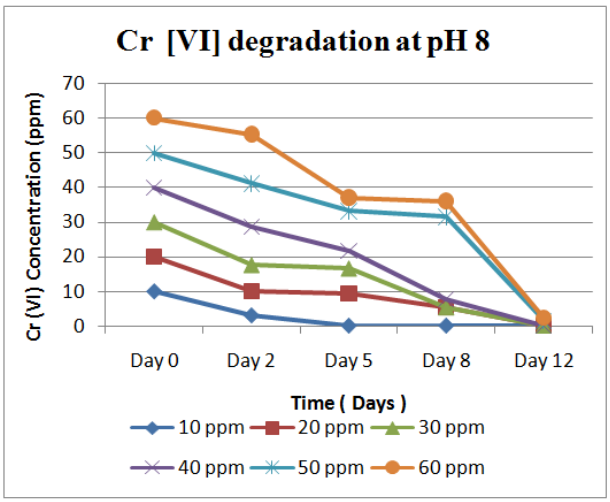
3.6.1. pH 2

Sr. No.	Initial concentration of Cr [VI] on Day 0	Final concentration of Cr [VI] on Day 12
	In ppm	In ppm
1	10	00.00
2	20	00.00
3	30	16.60
4	40	28.60
5	50	39.30
6	60	41.30



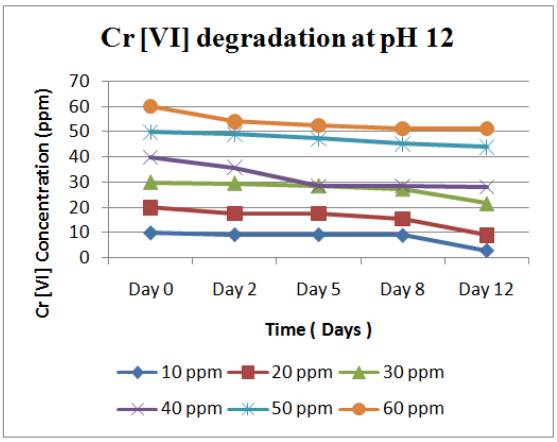
3.6.2 pH 8

Sr. No.	Initial concentration of Cr [VI] on Day 0	Final concentration of Cr [VI] on Day 12
	In ppm	In ppm
1	10	00.00
2	20	00.00
3	30	00.00
4	40	00.00
5	50	01.60
6	60	02.40



3.6.3 pH 12

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	03.00
2	20	09.00
3	30	21.60
4	40	28.30
5	50	44.00
6	60	51.30



3.7 Hexavalent chromium adsorption experiment

Cr[VI]reduction was not evident in adsorption experiments. concentrations of Cr[VI]remained unchanged in case of both, killed cells and metabolically inhibited cells. Decolourization of the medium was not observed and no reduction was detected by the diphenyl carbazide assay.

The results are as follows: -

3.7.1. Killed (Autoclaved cells)

Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	09.75
2	20	19.70
3	30	29.97
4	40	40.00
5	50	49.79
6	60	60.00

3.7.2Metabolically inhibited cells

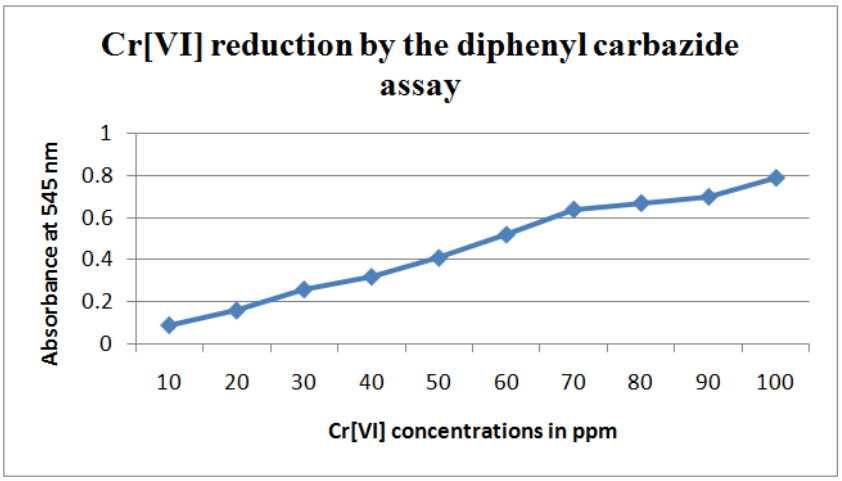
Sr. No.	Initial concentration of Cr [VI]	Final concentration of Cr [VI]
	In ppm	In ppm
1	10	09.90
2	20	20.00
3	30	29.97
4	40	40.00
5	50	49.79
6	60	60.00

3.8 Preparation of standard graph

The standard graph was prepared by plotting the Cr[VI]concentrations in the unit of ppm on the X-axis and the Absorbance in the unit of nm on the Y-axis.

The Absorbance readings for obtained were as follows: -

Sr. No.	Cr[VI]concentration in ppm	Reagent Blank	Absorbance (nm)	Corrected Absorbance (nm)
1	10	0.01	0.10	0.09
2	20	0.01	0.17	0.16
3	30	0.01	0.27	0.26
4	40	0.01	0.33	0.32
5	50	0.01	0.42	0.41
6	60	0.01	0.53	0.52
7	70	0.01	0.65	0.64
8	80	0.01	0.68	0.67
9	90	0.01	0.71	0.70
10	100	0.01	0.80	0.79



Standard Graph of Cr[VI]Reduction using the diphenyl carbazide assay

3.9. Identification

Sr. No.	Characteristics	Observation
1	Tryptophanase production (Indole Test)	-
2	Mixed acid fermentation (Methyl red Test)	-
3	Voges-Proskauer test	-
4	Citrate Utilisation	-
5	Sucrose	-
6	Mannitol	-
7	Arabinose	-
8	Xylulose	-
9	Oxidase Production	-

From the biochemical tests and 16 S rRNA studies the isolate was identified as***Acinetobacter iwofii.***

IV. Discussion

In this study a single organism was isolated from the sewage sample which individually showed resistance to Cr[VI] up to concentrations greater than 60 ppm. It can reduce concentration as low as 10 ppm and as high as 60 ppm with 100 and 66.66 percent reduction respectively at 55^{0C} in aerobic conditions, after incubation for 10 days. The isolate showed both confluent growth as well as reduction of Cr[VI] at 37 ^{0} C: however, reduction was greater at 55 ^{0} C. Thus a higher temperature enhances the reduction potential of this isolate. This could be attributed to the ability of this isolate to adapt itself to the temperature fluctuations found in sewage throughout the year. Hence the isolate has the ability to reduce Cr[VI] over a wide range of temperatures. Similarly, it was found that a neutral to slightly alkaline pH (7-8) was optimum for maximum reduction of Cr[VI]. This was checked by Diphenyl Carbazide assay which was specific for Cr[VI]. Reduction potential was greatly affected at the two pH extremes tested *wiz* pH 2 and pH 12. Bacteria are known to grow best at pH near neutrality and hence most of the enzymes involved in the metabolism are active in the same range of pH conditions. A number of studies suggest both growth dependent and growth independent chromium reduction. (9) In either case chromium reduction seems to be biomass dependent and increase in the initial concentration of Cr[VI] directly affects the rate of reduction potential. These results are in congruence with other works done previously both with fungi as well as bacteria. (5,9)

Most of the studies on Cr[VI] bioremediation have been conducted on consortia obtained from the site of contamination. These consortia contain indigenous organisms, well adapted to the high concentration of Cr[VI] along with the physical conditions such as pH and temperature at the contamination site. (3,5,9). In our study the organism was isolated from a sewage sample- a habitat not known to be previously contaminated by Cr[VI]·Since Cr[VI] concentration in sewage can be considered to be lower than those found in Cr[VI] contaminated sites, the isolate shows slightly reduced rate of reduction as compared to previously studied organisms. However, the results are comparable with those obtained by Horton et al with their low temperature isolate which showed 50 ppm of Cr[VI] at the end of five days. (9) It has been suggested that low levels of contamination are very difficult to remediate. Lack of induction of enzyme systems at low contaminant concentration and problems with availability of contaminants bound to organics and sequestered in other matrices all contribute to persistence of contaminants in the environment. (9) Hence use of these isolates in the efficient clearance of lower level of contamination can be suggested.

The organism which was isolated from the sewage sample was characterized. It was a Gram negative coccobacillus. The other biochemical tests, referred in Bergey's Manual of Determinative Bacteriology, Edition IXwere carried out and the 16S rRNA sequencing was performed by NCCS, Pune. The above results identified the isolate belonging to the genus *Acinetobacter iwofii*. Since this isolate has relatively lower potential to detoxify the environment of Cr[VI] as compared to indigenous microflora, it may be used in a consortium with other potential chromate reducers.

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