CHAPTER 3

MATERIAL AND METHODS
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This chapter documented describes the methodology of isolation, characterization and optimization of chromium biotransformation using bacterial strains under synthetic solution as well as in electroplating effluent. It also includes the study of bacterial protein profile in controlled and under Cr(VI) stress conditions.

Chemicals used:

Pure and analytical grade chemicals were used in all experiments including media preparation for growth of bacterial strains. Peptone, yeast extract, beef extract and nutrient agar and nutrient broth were supplied by M/s Hi Media chemicals, India. Potassium dichromate and 1,5-Diphenylcarbazide was procured from Merck, Chemical, India. Stock solution for Cr (VI) solution was prepared following standard procedure.

Site Description:

Soil and effluent samples were collected from Luxmi Precision Screw Ltd., Rohtak district, Haryana, India in the month of February, 2011. Rohtak district is located between 76°25' and 76°94' East longitudes and 28°80' North latitude, lying at 219.84m above sea level in south-eastern part of Haryana state. The climate of the study area is semi-arid with extremes of heat in summer and extremely cold in winters. Annual rainfall of Rohtak is around 455mm. The soil samples were collected from random spots around Effluent Treatment Plant in sealed plastic bags and stored at 4°C. All precautions were taken during and after collection. Electroplating industry discharge chromium in wastewaters due to the chrome plating which is one of the step in their manufacturing process.

Materials used:

All glassware used for experimental purposes were acid-washed with nitric acid (10%, v/v) and subsequently rinsed with double-distilled deionized (DDI) water to avoid metal contamination. Sterilization of glassware was carried out as when necessary by autoclaving at 121°C, 115 kPa for 15 minutes. Table 3.1 shows the list of instruments used during the study.
3.1 Sampling and Physicochemical characteristics of soil and Industrial effluent

The industry utilizes chromium, nickel and zinc ions for metal plating process. For characterization of industrial effluent, Color, Temperature(°C), pH, Conductivity, Oil and grease, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solids (TDS), Total Suspended Solids (TSS), Sulfate, Phosphate, Total chromium, Hexavalent chromium parameters were determined. Presence of other heavy metals such as Cadmium, Copper, Iron, Nickel, Lead, Zinc were estimated by Atomic Absorption Spectroscopy (ASS) methods. The physical & chemical properties of soil such as Soil organic matter, Cr (VI), total chromium, Potassium, Sodium of the soil samples obtained from Chromium contaminated sites were characterized. Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) of soil samples was evaluated to understand the morphology, elemental composition and particle density of the chromium contaminated soil sample. The SEM-EDX analyses were carried out with the help of a computer controlled field emission SEM equipped with a EDX detection system located at All India Institute of Medical Sciences, New Delhi.

**Oil and Grease**: Oil and Grease were determined by solvent extraction method using petroleum ether as the solvent (Gupta et al., 2000). 50 ml of petroleum ether, 10 ml of 33% H₂SO₄ and a little volume of ethyl alcohol were added to 250 ml of effluent. After shaking well, the mixture was transferred to a separating funnel. After half an hour water layer was discarded and upper petroleum ether was taken in a pre-weighed beaker. Petroleum ether was evaporated by keeping the beaker in a hot water bath. Final weight of beaker with residue was recorded.

**Calculation**

\[ \text{Oil and grease (mg/g)} = \frac{W_f - W_i}{V} \times 1000 \]

Where,

- \(W_f\) - initial weight of beaker (g)
- \(W_i\) - final weight of beaker (g), and
Table 3.1 : List of Instruments used during the present study

<table>
<thead>
<tr>
<th>INSTRUMENTS</th>
<th>FUNCTION</th>
<th>MODEL No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertical Autoclave</td>
<td>Sterilization</td>
<td>PI110P6 SANCO</td>
</tr>
<tr>
<td>Analytical Balance</td>
<td>Weight measurement</td>
<td>CPA225D SARTORIUS</td>
</tr>
<tr>
<td>Laminar airflow</td>
<td>Aseptic conditions</td>
<td>MICROSIL</td>
</tr>
<tr>
<td>pH</td>
<td>Measurement of pH</td>
<td>Digitl pH meter</td>
</tr>
<tr>
<td>BOD Incubator</td>
<td>Incubation of cultures</td>
<td>NSW-152 CALTON</td>
</tr>
<tr>
<td>Ultra Low Temperature freezer</td>
<td>Preservation of cultures</td>
<td>RQFV-265 REMI</td>
</tr>
<tr>
<td>Double Distillation Unit</td>
<td>Preparation of the stock solution, throughout the experiment etc.</td>
<td>MQD2PQ Bhanu Scientific</td>
</tr>
<tr>
<td>Spectrophotometer (UV/Vis)</td>
<td>Estimation of Biomass and Cr (VI) degradation</td>
<td>UV-2450 SHIMADZU</td>
</tr>
<tr>
<td>BOD Incubator Shaker</td>
<td>Batch degradation kinetics of Cr (VI)</td>
<td>LETTD ORBITEK</td>
</tr>
<tr>
<td>Ultra Centrifuge</td>
<td>Collection of pellet and Cr (VI) estimation</td>
<td>0191 LOGIC CONTROLS PVT</td>
</tr>
<tr>
<td>Atomic Absorption Spectrophotometer</td>
<td>Estimation of total chromium</td>
<td>Z-6100 HITACHI</td>
</tr>
<tr>
<td>Electrophoresis Unit</td>
<td>Protein profile study</td>
<td>Model-192 BIORAD</td>
</tr>
</tbody>
</table>
V – volume of sample.

**Total Dissolved Solids**: Total Dissolved Solids were determined by method as outlined by Gupta *et al.*, (2000). Filter 250 ml of the sample through Whatmann filter paper No. 4 in a pre-weight evaporating dish. Evaporate the sample on the hot water bath until whole water was evaporated. Noted the weight of evaporating dish after cooling it in a dessicator and calculated total dissolved solids as per the following relationship:

\[
\text{Total Dissolved Solids (g/L)} = \frac{\text{Final weight} - \text{Initial weight of the evaporating dish}}{\text{Volume of sample taken}} \times 1000
\]

**Hydrogen ion concentration**: Hydrogen ion concentration was measured by pH meter as per the procedure outlined by Subramaniam (1994). Warming up of instrument was done for 15 minutes. After calibrating the instrument with the known buffer solutions, the electrode was immersed in unknown sample and stirred for three minutes and pH was measured.

**Electrical conductivity**: Electrical conductivity was measured with the help of electrical conductivity meter following the procedure of Gupta *et al.*, (2000). Warming up of instrument was done for 20 minutes. Rinsed the conductivity cell with distilled water and then with the sample. Temperature and cell constant corrections were adjusted on the conductivity meter. Connected the conductivity cell to the meter and dipped in sample. Passed the current and adjusted the current by rotating the dial in such a way that maximum sensitivity was obtained. Conductivity was measured in $\mu$hmhos/cm.

**Biological oxygen demand (B.O.D.)**: B.O.D. was measured according to Gupta, 2000. The pH of the effluent was adjusted to 7 with the help of $1N \text{H}_2\text{SO}_4$ and $1N \text{NaOH}$. The sample was filled in 250 ml B.O.D. bottles and to it 1 ml of allylthiourea solution. Air bubbles were avoided in B.O.D. bottles. The dissolved oxygen of one set was determined immediately following method of oxygen estimation and the bottles of another set were incubated for 5 days. After 5 days the bottles were took out and their dissolved oxygen was determined immediately.

Calculation:

\[
\text{BOD}_5 \text{(mg/L)} = (\text{DO} - \text{D}_0) \times \text{Dilution factor}
\]
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Where, DO = initial dissolved oxygen in the sample (mg/L) and DO₅ = dissolved oxygen left out in sample after 5 days of incubation (mg/L).

**Chemical Oxygen demand:** To 20 ml sample taken in flask of reflux unit, 10 ml of potassium dichromate was added. A pinch, each of silver sulfate and mercuric sulfate was added followed by 30 ml of sulfuric acid. After that Liebig condenser was attached to the mouth of flask and the flask was heated on a hot water bath for 2 hours. After that flask was cooled and it was detached from the unit and diluted up to 150 ml by adding distilled water. After that, 2-3 drops of ferroin indicator solution was added and titrated against ferrous ammonium sulfate solution. End point is reached when blue color of contents was changed to reddish blue. The distilled water was used as blank which was also run simultaneously.

Calculation:

\[ \text{COD (mg/L)} = \frac{(B-T) \times N \times 1000 \times 8}{\text{Volume of sample (ml)}} \]

Where, 
- \( T \) = volume of titrant (FAS) used against sample (ml)
- \( B \) = volume of titrant (FAS) used against blank (ml)
- \( N \) = normality of titrant (FAS) (0.25)

**Sulphate:** Sulphate was measured according to the procedure described by Subramanian (1994). In 50 ml of sample, 10 ml of NaCl-HCl solution was added followed by 10 ml of glycerol-ethanol solution. Contents were kept on a stirrer for mixing. 0.12 g of barium chloride was added to the contents kept on stirrer and left for mixing for another 60 seconds. Immediately absorbance was measured at 420 nm against a suitable blank. Run similar experiments with standard sulfate solution and the standard curve was plotted. Amount of sulfate was measured in mg/l from the standard curve drawn with sodium sulfate.

**Metals estimation:**

**Digestion** – After proper mixing, suitable volume (50 to 100 ml) of sample was taken in 250 ml flask/beaker. 5 ml of concentrated HNO₃ was added. Few glass beads were also added to avoid bumping of the contents. Contents were evaporated on a hot plate to the
lowest volume before the initiation of precipitation. Continue heating and adding Conc. HNO₃ until digestion is completed shown by light colored clear solution. The digested solution was transferred to 100 ml volumetric flask with proper rinsing of the solution to volumetric flask using minimum amount of water. Contents were cooled, diluted up to the mark. Suitable portions of this solution were used for metal estimation.

**Scanning electron microscopy - Energy dispersive X-ray (SEM-EDX) of the soil sample** – The facility for SEM-EDX analysis of soil samples for estimation of chromium was availed from All India Institute of Medical Sciences, New Delhi.

### 3.2 Isolation of pure culture

#### 3.2.1 Source of inoculation

Bacterial strains were isolated from industrial effluent collected from within the premises of Electroplating industry located in Rohtak (Haryana).

#### 3.2.2 Preparation of culture medium

Bacteria were isolated from industrial effluent sample collected from Electroplating industry by using culture medium. All the glassware were cleaned thoroughly with water, dried and sterilized in hot air oven at 180°C for 4-6 hours before use. Composition of Nutrient Broth(NB) (Jeyasingh and Philip, 2005) used is as follows:

**Composition of Nutrient Broth (NB) (g/L):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>2 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 gm</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 gm</td>
</tr>
<tr>
<td>pH</td>
<td>7</td>
</tr>
</tbody>
</table>
Double distilled water was used for the preparation of the medium.

**Composition of Minimal Salt Medium (g/L):**

- $\text{K}_2\text{HPO}_4$: 1.06 gm
- $\text{KH}_2\text{PO}_4$: 0.2 gm
- $\text{MgSO}_4\cdot7\text{H}_2\text{O}$: 0.5 gm
- $\text{CaCl}_2$: 0.05 gm
- $\text{KNO}_3$: 2 gm
- $\text{NaCl}$: 1 gm
- Peptone (Carbon source): 10 gm

**3.2.3 Preparation of agar plates**

Bacterial isolation was done using solidified culture medium (2% agar). The medium was sterilized in autoclave. It was then cooled down to 40-45°C to avoid condensation of moisture during solidification of agar. Thirty ml of medium was poured in petridish, presterilized in oven at 180°C. To avoid contamination, moisture around the flasks was whipped off and mouth was sterilized in flame while pouring the medium. After pouring the medium, petriplates were kept undisturbed for a few minutes so that the medium got solidified.

**3.2.4 Preparation of pure culture**

Bacterial strains were isolated from the soil and sediment sample together with liquid effluent (1:10 w/v) of Electroplating industry collected from three different sites located within the industry. The bacterial strains were isolated from the supernatant of the sample by centrifugation at 900 rpm for 5 min and were used for enrichment in Nutrient
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Broth (NB) media composition of which is already mentioned above. Samples of the culture were collected under aseptic conditions. The serial dilution technique was adopted for the isolation of bacterial strains from the Electroplating effluent. About 1 ml of effluent sample was added to 100 ml of nutrient media and incubated for 24 h in facultative conditions. The shake flask cultures were closed using cotton plugs. Then, 1 ml of this was transferred to 9 ml sterilized distilled water taken in test tube and labeled as $10^{1}$ dilution. From this 1ml was transferred to 9 ml water blank and shaken thoroughly to mix, and labeled as $10^{2}$ dilution. Likewise $10^{3}$ dilution series was prepared by transferring 1 ml of $10^{2}$ dilution to 9 ml water blank up to $10^{6}$ dilution series. Half the ml was taken from each dilution and inoculated into petriplates containing solidified agar medium. These inoculated petriplates were incubated at 30±2°C with their bottom side up for 2-3 days. After 2-3 days, bacterial colonies developed. Through repeated streaking on basal agar medium, purification of species was done.

### 3.4 Screening of Chromium (VI) resistant bacterial strains

The isolates were tested for their resistance to chromate by growing in NB agar plates containing various concentrations of Cr(VI) (25, 50, 75, 100 mg/L) until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained. Significant growth and rapid Cr (VI) degradation kinetics of the specific bacterial species in the presence of 25, 50, 75 and 100 mg/L Cr (VI) during 24 hours of incubation at 30°C, were considered as Cr (VI) resistant. The bacterial strains capable of growing at this condition were selected for further experiments. Growth of the isolates was also evaluated in NB broth containing 25, 50, 75mg/L of Cr(VI). Growth of the isolated in NB broth was determined by measuring the optical density at 600 nm using uninoculated broth as the blank. Relative growth of the isolates was expressed as the percentage of those obtained in untreated control which was taken as 100%.
3.5 Characterization of the Cr (VI) degrading bacterial strains

3.5.1 Morphological characterization:

Morphological characterization of the bacterial isolates was based on the light and electron microscopy. The detailed characterization of configuration was conducted through various procedures for detection of margin, elevation, surface, pigment, opacity. Gram’s reaction of all the isolates was also conducted. Beside these properties such as cell shape, size (µm), arrangement, spore(s) formation, bacterial motility was also studied.

Morphological characteristics of the isolates were studied by culturing the organisms on nutrient agar slants and plates and in nutrient broth. On nutrient agar slants, characteristic like opacity was studied. Optical characteristics were evaluated by amount of light transmitted through the growth. On nutrient agar plate, properties like cell size, pigment, shape, margin and elevation were studied.

**Gram staining** – Twenty four hours nutrient agar slant cultures were used for studying gram staining. Bacterial smear was gently flooded with crystal violet and kept for 1 min on the glass slide. Slide was then washed with tap water. After that smear was flooded with Gram’s iodine mordant and kept for one minute followed by washing with tap water. After that, decolorisation was done with 95% ethyl alcohol followed by washing with tap water. After that counter staining was done with safranin for 15 seconds followed by washing with tap water. Excess water was then removed using blotting paper and slide was then examined under microscope to study the properties like cell shape, arrangement, color and gram reaction.

3.5.2 Physiological characterization:

Physiological characterization included determination of optimum pH, temperature for growth on NaCl (%) and Growth under anaerobic condition.
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**pH profile:** To determine the pH optima, LB medium meant for the growth of the bacterial isolates were adjusted to different pH ranging from 5 to 9 and was seeded with 1% inoculums. Post overnight growth at 30°C under shaking condition at 180 rpm, growth was measured in terms of optical density at 600 nm on UV-Visible spectrophotometer.

**Temperature profile:** For determining optimum temperature, 1% inoculation was provided into LB medium and overnight incubation was done at different temperatures like 4°C, 10°C, 25°C, 30°C, 37°C, 42°C and 55°C. Growth pattern of bacterial isolates were studied using 2.0, 4.0, 6.0, 8.0, 10.0 and 12% NaCl.

**Growth on NaCl (%):** Growth pattern of bacterial isolates were studied using 2.0, 4.0, 6.0, 8.0, 10.0 and 12% NaCl.

**Growth under anaerobic condition:** All the bacterial isolates were also grown under anaerobic condition in the absence of oxygen.

**3.5.3 Biochemical characterization:**

**Starch Hydrolysis:** Starch agar plate cultures were flooded with Gram's iodine solution and iodine was allowed to remain in contact with medium for 30 seconds after which excess of iodine was pour off. Cultures were then examined for presence/absence of a blue-black color surrounding the growth of each test organism.

**Nitrate reduction:** Using aseptic conditions, each organism was inoculated into appropriate labeled tube containing medium using loop. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. Add 5 drops of Solution A (Sulfanilic acid) and then 5 drops of Solution B (α-naphthylamine) to all nitrate broth cultures. Observation was recorded whether or not red coloration develops in each culture. Added minute quantities of zinc powder to the cultures in which red color did not developed. Observation was recorded whether or not red coloration develops in each culture. On the basis of observations, it was recorded whether each organism was capable of nitrate reduction. Identified the end product (NO\(_2^-\) or NH\(_3^+\)/ N\(_2\)), if any that is present.
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**Carbohydrate test:** Each isolate was inoculated in fermentation tube using aseptic conditions and incubated for 24 hours at 37°C. After that all cultures were examined for color and presence/absence of gas bubble. Based on it, each organism was determined whether it fermented carbohydrate with production of acid/acid and gas.

**Indole production Test:** Using aseptic conditions, each organism was inoculated into appropriate labeled tube. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. Added 10 drops of Kovac's reagent to all tube cultures and agitated the cultures gently. In each culture, color of the reagent layer was examined. Based on the color, observations were made whether or not each organism was capable of hydrolyzing the tryptophan.

**Methyl red test:** Using aseptic conditions, each organism was inoculated into appropriate labeled tube containing medium using loop. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. One-third of all the cultures were transferred into empty test tubes for Voges-Proskauer test. 5 drops of methyl red indicator was added to the remaining aliquot of each culture. Examined the color of all cultures and based on that observation was made whether microbe was capable of fermenting glucose with production and maintenance of high concentration of acid.

**Voges-Proskauer test:** To the aliquot of each broth culture separated during methyl-red test, added 10 drops of Barritt’s reagent A and shaked the cultures. Immediately 10 drops of Barritt’s reagent B was added and shaked. Reshaking was done every 3 to 4 minutes. Color of the cultures was examined 15 minutes after the addition of Barritt’s reagent. Based on the color, observation was made whether or not microbe was capable of fermenting glucose with ultimate production of acetyl methyl carbinol.

**Citrate Utilization test:** Using aseptic conditions, each organism was inoculated into appropriate labeled tube. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. All agar slant cultures were examined for presence/absence of growth...
and coloration of the medium. Based on the color, observation was made whether or not each organism was capable of using citrate as its sole source of carbon.

**Hydrogen Sulphide test**: Using aseptic conditions, each organism was inoculated into appropriate labeled tube by means of stab inoculation. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. All SIM cultures were examined for presence/absence of black coloration along the line of the stab inoculation. Observations were made whether or not each organism was capable of producing hydrogen sulphide. Observed all cultures for presence (+) or absence (-) of motility.

**Urease test**: Using aseptic conditions, each organism was inoculated into appropriate labeled tube by means of loop inoculation. One tube served as control. All the tubes were incubated for 24 to 48 hours at 37°C. All Urea broth cultures were examined for color. Based on color, observation was made whether each organism was capable of hydrolyzing substrate urea.

**Oxidase test**: Isolated single colonies from plate were picked up using tooth pick and were gently scratched on the oxidase disks. Oxidase disks acts as an electron donator to cytochrpme oxidase. If the bacteria oxidize the disk (remove electron) the disk will turn purple (observations noted within 60 secs), indicating positive test.

**Catalase test**: On isolated single colonies, 1% H₂O₂ was dropped using a glass capillary tube and appearance of effervescence demonstrates the presence of enzyme. The enzyme catalyses the breakdown of hydrogen peroxide (H₂O₂) with the release of free oxygen. The evolution of gas causes bubbles to form and its indicative of a positive test.

Few of the biochemical characterization tests were performed in Indian Institute of Microbial Technology, Chandigadh for confirmation of the results.

**3.6 Minimum inhibitory concentration (MIC)**:

MIC in microbiology is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC of dichromate for each
isolate was determined by measuring the optical density of bacterial strains at 540 nm. Flasks containing NB medium supplemented with different concentrations (25, 50, 75, 100 mg/L) of K$_2$Cr$_2$O$_7$, were inoculated with 0.5 ml of a fresh overnight culture grown in nutrient broth medium. All the flasks were incubated with shaking at 30°C for 24 hours. The growth of bacteria was monitored by measuring the optical density at 600 nm.

3.7 Study of Bacterial growth profile in complex and defined media

The medium (50 mL) was inoculated with 10% of starter culture and incubated at 30°C with shaking at 180 rpm. At regular intervals, the turbidity of the culture was determined using spectrophotometer at 600 nm. Growth profile was obtained by plotting OD$_{600nm}$ versus time. The bacteria growth profile was also studied in defined medium.

3.8 Preparation of synthetic solutions of chromium (VI) and and Chromium (III) their estimation

3.8.1 Preparation of reagents and synthetic solution of chromium (VI) and Chromium (III)

Potassium dichromate (K$_2$Cr$_2$O$_7$) and Chromium trichloride (CrCl$_3$.6H$_2$O) were used for preparation of synthetic solution of Cr (VI) and Cr(III) respectively.

Stock Cr (VI) solution (100 ppm) – 282.8 mg of potassium dichromate was dissolved in 1 litre of double distilled water. Further dilutions were made by using stock solution.

Stock Cr (III) solution (1000 ppm) – 5.124 g of CrCl$_3$.6H$_2$O was dissolved in 1 litre of double distilled water. Further dilutions were made by using stock solution.

Reagent (s) Required:

5-Diphenylcarbazide Reagent – 200 mg of 5-diphenylcarbazide was dissolved in 100 ml of 90 % ethyl alcohol and added with mixing, an acid solution prepared from 40 ml of concentrated sulfuric acid and 360 ml distilled water. The solution was kept in refrigerator.
3.8.2 Estimation of hexavalent chromium

In acidic medium, hexavalent chromium reacts with diphenylcarbazide to produce a reddish purple complex, which is estimated spectrophotometrically at 540 nm. Some samples were also detected for chromium on Atomic Absorption Spectrophotometer.

3.8.3 Preparation of standard curve for hexavalent chromium

0.2 to 1 ppm of chromium solution was prepared and 100 ml of this was taken in each flask. Now to this, 2.5 ml of diphenylcarbazide solution was added. Contents were allowed to stand for 10 minutes for full color development. Absorbance was noted at 540 nm and standard curve was plotted for Cr (VI). Fig 3.1 shows the standard curve drawn for Cr(VI) estimation.

3.8.4 Estimation of total chromium

For estimation of total chromium, samples were subjected for analysis on Atomic Absorption Spectrophotometer.

3.8.4 Estimation of trivalent chromium

Difference between total chromium and hexavalent chromium gives the measure of trivalent chromium.

3.9 Optimization studies

3.9.1 Effect of pH

The batch experiments were conducted to find out the effects of varying pH on Cr (VI) removal from their respective synthetic solutions of 20 ppm concentration at constant contact time. pH was adjusted using 0.1 N HCl and 0.1 N NaOH. After agitation, solutions
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were centrifuged and supernatants was analyzed for Cr (VI) concentration. From this, optimum value of pH was obtained in each case.

3.9.2 Effect of initial concentration

These studies were conducted by agitating the Cr (VI) solutions at optimized pH for different concentrations ranging from 10 – 60 ppm. The samples were analyzed for residual Cr (VI) concentration.

3.9.3 Effect of temperature

These studies were conducted by agitating the Cr (VI) solutions at optimized pH and optimized initial concentration for different temperature ranging from 25°C to 40°C.

3.9.4 Effect of Contact Time

In order to assess the required time to reach an equilibrium state, the batch dynamics of Cr(VI) biotransformation was studied for each of the native bacterial strains. The Cr(VI) transformation was monitored around 0-96 hours. In this experiment, initial pH was adjusted to 7 and initial Cr(VI) concentration 20 mgL\(^{-1}\) was used. In this study, several flasks with the same mixture of metal solution and bacterial biomass were prepared and each Erlenmeyer flask was removed periodically. After shaking, an aliquot of the mixture (5 mL) was centrifuged. The supernatant was analysed for Cr(VI) concentration. The metal solution at pH 8 without the bacterial biomass was used as control.

3.9.5 Effect of inoculum volume and inoculum age

The effect of inoculum volume and inoculum age on Cr (VI) biotransformation was also monitored. The study was conducted using initial Cr(VI) of 10 mg/l of 100ml volume, optimised pH and optimised incubation temperature 30\(^\circ\)C and speed 150 rpm using a culture flask in an incubator shaker. The inoculum volume of 0.5, 1.0, 1.5 and 2.0
Standard Curve for Cr (VI)

Fig 3.1 Standard curve for Cr(VI)
ml was taken and incubated for 24 hours and Cr(VI) biotransformation was observed in all the flasks.

In order to study the effect of inoculum age, the flasks were inoculated with optimized inoculum volume with 6h, 12h, 18h, and 24h old culture and were incubated at 30°C, at 150 rpm for 24 hours to achieve the maximum Cr (VI) transformation activity.

The % Cr(VI) biotransformation was determined by measuring the concentration of Cr(VI) ion in the aqueous phase before and after contact with bacterial biomass and expressed according to following equation:-

% Cr(VI) biotransformation = \( \frac{(C_0 - C_f) \times 100}{C_0} \)

Where, \( C_0 \) is the initial concentration of C(VI) ion (mg/L)

\( C_f \) 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.

3.10 Industrial applicability

To study the industrial applicability of bacteria, the experiments were carried out in batch mode at optimized conditions obtained from experiments using synthetic solutions for removal of Cr(VI) from electroplating effluent. Sample from each experiment flask(s) was drawn at specific time interval. Sample was centrifuged at 10,000 rpm for 10 min at 4°C. The presence of chromium was determined in supernatant and bacterial cell. Supernatant (50 ml of sample) was mixed with concentrated HNO\textsubscript{3} (5 ml) and boiling chips. The content was boiled and evaporated to 16–20 ml on hot plate. Concentrated HCl (5 ml) was added and boiled again. The solution was boiled till sample become clear and brownish fumes were evident. Finally it was cooled and diluted up to 50 ml with distilled water. Aliquot of this solution was used for determination of the concentration of total chromium with the help of Atomic Absorption Spectrophotometer and chromium (VI) by UV-Visible spectrophotometer.
3.11 Polyacrylamide Gel Electrophoresis

The SDS-PAGE was performed on vertical slab (10 cm × 10 cm × 1mm) gel electrophoresis chamber from BIORAD by the method given by Laemmli (1970).

Reagents used:

1. **Acrylamide- Bisacrylamide stock solution**
   - Acrylamide -30 g
   - Bisacrylamide – 0.8 g
   - Dissolved in Distilled water and made final volume to 100ml. Filtered through Whatmann filter paper No. 1 and stored in brown bottle at 0 – 4°C.

2. **Stacking gel buffer stock – (0.5 M Tris-HCl, pH- 6.8)**
   - Tris -6g
   - 1 M HCl -48.0 ml
   - pH was adjusted 6.8 and final volume was raised up to 100 ml with distilled water.
   - Filtered it through Whatmann filter paper No. 1 and stored at 0 – 4°C.

3. **Resolving gel buffer stock –(1.5 M Tris- HCl, pH -8.8)**
   - Tris -36.3g
   - 1 M HCl-48.0 ml
   - pH was adjusted 8.8 and final volume was raised up to 100 ml with distilled water.
   - Filtered it through Whatmann filter paper No. 1 and stored at 0 – 4°C.

4. **1.5 % (w/v) Ammonium Persulphate (APS) in Distilled water**
   - 0.15 g of APS was dissolved in 10 ml water. This reagent was prepared just before use.

5. **TEMED (N,N,N,N-Tetramethyl ethylene diamine)**
   - It was used as supplied by the manufacturer.

6. **Resolving buffer – (Tris-glycine, pH -8.3) for 1000 ml**
   - Tris – 3 g
   - Glycine -14.4
   - SDS – 1.0 g
pH was adjusted 8.8 and final volume was raised up to 1000 ml with distilled water.

7. **Staining solution**
   Coomassie Brilliant Blue R-250- 1.25 g  
   Methanol-200 ml  
   Glacial Acetic Acid -35 ml  
   final volume was made up to 500 ml with distilled water, filtered and stored at room temperature.

8. **Destaining solution**
   Glacial acetic acid- 75 ml  
   Methanol-50 ml  
   Mixed and distilled water was added to make final volume 1L.

9. **SDS (10% w/v)**
   1 g of Sodium Do-decyl sulphate was dissolved in 10 ml of distilled water.

10. **Sample Buffer**
    1M Tris-HCl, pH -6.8 -12.5 ml  
    SDS -4.0 g  
    B-mercaptoethanol -10.0 ml  
    Glycerol -20.0 ml  
    1% Bromophenol Blue – 4.0 ml  
    Then, distilled water was added to make final volume 100 ml.

11. **Standard molecular weight marker proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K.D)</td>
<td></td>
</tr>
<tr>
<td>β- Galactosidase (from <em>E.coli</em>)</td>
<td>120</td>
</tr>
<tr>
<td>Bovine Serum Albumin (Bovine Plasma)</td>
<td>85</td>
</tr>
<tr>
<td>Carbonic anhydrase (Bovine erythrocyte)</td>
<td>50</td>
</tr>
<tr>
<td>β- Lactoglobin (from Bovine milk)</td>
<td>25</td>
</tr>
<tr>
<td>Lysozyme (from Chicken egg white)</td>
<td>20</td>
</tr>
</tbody>
</table>
12. Preparation of Stacking gel (2.5 %)
Acrylamide solution -2.5 ml
Stacking gel buffer (Tris-HCl, pH -6.8 ) – 5.0 ml
10 % SDS- 0.20 ml
1.5 % APS – 1.00 ml
Distilled water- 11.30 ml
TEMED -0.015 ml

13. Preparation of Resolving gel (12.5%)
Acrylamide solution -12.5 ml
Resolving gel buffer (Tris-HCl, pH -8.8 ) – 3.75 ml
10% SDS -0.30 ml
1.5% APS-1.50 ml
Distilled water- 11.95 ml
TEMED -0.015 ml

Sample preparation

Whole cell lysates were prepared by method adapted from Hitchcock and Brown (1983). A colony of bacteria was picked from NB agar, inoculated into 100 ml of NB Broth and incubated overnight at 30°C. Subsequently, the broth was centrifuged at 3000 rpm × 5 min at room temperature, washed at least three times with and resuspended in 10 ml of PBS (0.9 g NaCl, 0.02 g KCl, 0.02 g KH2PO4, 0.29 g Na2HPO4, distilled water to 100 ml, pH 7.2). Cells from 1.5 ml of washed culture were harvested in a microfuge tube, resuspended in 100 μl of SDS-PAGE sample lysis buffer (62.5 mM Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.1 % bromophenol blue, pH 6.8) and heated at 100°C for 10 min. The lysed samples were then centrifuged (5000 rpm × 10 min) at room temperature (RT) and 20 μl of the filterates with equal volume of loading buffer were loaded on a SDS-12% PAGE gel and electrophoresed using Tris-Glycine-SDS buffer system (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH -8.3) developed by Laemmli (1970).
MATERIAL AND METHODS

Electrophoresis

The SDS-PAGE was carried out using the method of Laemmli (1970) using 12% (w/v) separating and 4% (w/v) stacking gels. Five μl of samples were electrophoresed on 12% acrylamide gel for 3 h at 70 mA using a small electrophoresis chamber. In each gel a wide range molecular weight marker was included. Molecular weights were determined by regression analysis of a standard molecular weight marker (Broad range markers) separated on the same gel parallel to the samples. Gels were stained for 1 h in 0.25% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid, 40% distilled water and destained overnight in 5% methanol, 10% acetic acid, 85% distilled water.

Statistical Analysis

The data were subjected to two way analysis of variance (ANOVA) for test of significance towards Cr(VI) treatment given to the bacterial strains and time required for Cr(VI) biotransformation by using software Graphpad prism 5.0 demo. The present study also employs the use of Box behnken design matrix for optimization of various parameters using Response surface methodology with the help of design expert software. The data was also analysed graphically on Microsoft Excel.