Appendix I

Medium used for the enrichment, isolation and growth of chromate reducing bacteria (EG medium):

Ammonium chloride	0.03 g
Dipotassium hydrogen phosphate	0.03 g
Potassium dihydrogen phosphate	0.05 g
Sodium chloride	0.01 g
Magnesium sulfate, heptahydrate	0.01 g
Sodium acetate	2.00 g
Yeast extract	0.15 g
Peptone	0.50 g
Distilled water	1 litre
pH	7.5

In solid media agar-agar was used at a concentration of 2.5 g%.

Stock Cr^{6+} solution :

1000 mM potassium chromate was prepared by dissolution of 19.420 g potassium chromate in 100 ml distilled water. This stock was sterilized separately and added to sterile medium (autoclaved at 121°C for 15 min) to a desired concentration of Cr^{6+} with minimal dilution of medium.

Sabouraud's medium for the cultivation of fungi :

Glucose	20 g
Peptone	10 g
Distilled water	1 litre
pH	4.5 - 5.0

Medium was sterilized by autoclaving at 121°C for 15 minutes.

In solid medium agar-agar was added at concentration of 2.5 g %

Glucose phosphate peptone water :

Peptone	5 g
Dipotassium hydrogen phosphate	5 g
Distilled water	1 litre
pH	7.6

The medium was sterilized at 121°C for 15 min. Glucose(filter sterilized) was then added at a final concentration of 0.5% and dispensed (5 ml) in tubes.

Davis and Mingioli's minimal medium :

Glucose solution (10%)	20 ml
Dipotassium hydrogen phosphate	7 g
Potassium dihydrogen phosphate	3 g
Sodium citrate	0.5 g
Magnesium sulfate heptahydrate	0.1 g
Ammonium sulfate	1 g
Distilled water	1 litre
Trace element solution	5 ml

Trace element solution

Ferrous sulfate	0.5 g
Zinc sulfate	0.5 g
Magnesium sulfate	0.5 g
Sulfuric acid, 0.1 N	10 ml
Distilled water	1 litre

The medium was sterilized by autoclaving at 121°C for 15 min.

Medium for the utilization of cyanide (Moller's medium) **:**

Peptone	3.0 g
Sodium chloride	5.0 g
Potassium dihydrogen phosphate	0.23 g
Disodium hydrogen phosphate	5.64 g
Distilled water	1 litre
pH	7.6

The medium was sterilized by autoclaving at 121°C for 15 min. Upon cooling add KCN solution at a final concentration of 1 mM, dispensed in serum bottles and sealed.

Peptone water base :

Peptone	10 g
Sodium chloride	5 g
Distilled water	1 litre
pH	7.2-7.3

The medium was sterilized by autoclaving at 121°C for 15 min. and a previously sterilized solution of the carbon source was added at a final concentration of 1%.

Medium for the detection of Urease (Christensen's medium) :

Peptone	1 g
Sodium chloride	5 g
Potassium dihydrogen phosphate	2 g
Phenol red (1:500 aqueous solution)	6 ml
Agar-agar	20 g
Distilled water	1 litre
pH	6.8 - 6.9

Basal medium was sterilized by autoclaving at 121°C/15 min. When it cools to about 50°C a sterile solution of glucose was added to give a final concentration of 0.1% and a 100 ml of a 20% solution of filter sterilized urea solution was added and slants were prepared.

Medium used for reduction of Mn^{4+} and Fe^{3+} :

Glucose	20.0 g
Ammonium sulfate	1.0 g
Magnesium sulfate, heptahydrate	0.05 g
Potassium dihydrogen phosphate	0.50 g
Yeast Extract	0.15 g
Distilled water	1 litre
pH	7.2

Mn^{4+} and Fe^{3+} were added as MnO_2 and Fe_2O_3 at concentrations 1 mg/ml after separate sterilization of the solid powders at 121°C for 15 min.

Medium used for reduction of Mo^{6+} (Ghani *et al*, 1993):

Glucose	10.0 g
Ammonium sulfate	3.0 g
Magnesium sulfate, heptahydrate	0.5 g
Disodium hydrogen phosphate	0.5 g
Yeast extract	0.5 g
Sodium chloride	5.0 g
Sodium molybdate, dihydrate	2.42 g
Distilled water	1.0 l
pH	7.0

The medium was sterilized by autoclaving at 121°C for 15 min.

Note: The concentration of phosphate (2.9 mM) was critical for formation of blue coloration after reduction.

TYE agar:

Bacto tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Distilled water	1 litre
pH	7.0

The medium was solidified by using 2.5% agar-agar and sterilized by autoclaving at 121°C for15 minutes.

Luria agar :

Tryptone (Difco)	5 g
Yeast extract (Difco)	2.5 g
Sodium chloride	2.5 g
D-glucose	0.5 g
Distilled water	1 litre

The medium was solidified by using 2.5% agar-agar and sterilized by autoclaving at 121°C for15 min.

Appendix II

Method for the estimation of Cr^{6+} by Diphenyl carbazide method (DPC method):

Diphenyl carbazide reacts with hexavalent chromium to form a pink coloured complex which is estimated spectrophotometrically. The original method according to APHA was modified with respect to volume and a standard calibration curve was generated in the range 1-10 ppm Cr^{6+} .

Protocol

1 ml sample (or sample approximately diluted to 1 ml)
+ 0.5 ml sulfuric acid (1:1 diluted)
+ 0.1 ml O-phosphoric acid
+ 8.5 ml distilled water
+ 0.5 ml diphenyl carbazide reagent (0.25 g diphenyl carbazide in 50 ml acetone)

Read the optical density of developed color at 540 nm after 5 min with distilled water in place of sample as reagent blank.

For samples containing <1 mg/l Cr^{6+} 10 ml of such samples were concentrated upto 1 ml and the rest of the protocol was repeated. The lowest concentration that could be detected was 0.3 mg/l Cr^{6+} .

Method for the determination of ferrous iron concentration :

Reagents:

1) Sodium fluoride complexing reagent
Prepare the reagent in polypropylene bottle. Dissolve 2.1 g Sodium fluoride in 98 ml distilled water and add 2 ml concentrated sulfuric acid.
2) O-phenanthroline solution
Dissolve 10 g 1,10 o-phenanthroline monohydrate in 300 ml distilled water add 10 - 12 drops of concentrated hydrochloric acid (till the complete dissolution of o-phenanthroline)
3) Acetate buffer
Dissolve 125 g ammonim acetate in 75 ml distilled water. Add 300 ml glacial acetic acid and make the volume upto 500 ml with distilled water.
4) O-phenanthroline reagent
Mix 1 volume of 0-phenanthroline solution with 1 volume acetate buffer. This reagent is always freshly prepared.
5) Standard ferrous iron solution.
Dissolve 0.4978 g ferrous sulfate heptahydrate in acidified distilled water and make up the volume to 100 ml. This is 100 mg/l Fe^{2+} .

Protocol :

1. To 0.1 ml sample add 1 ml complexing reagent.
2. Add 0.4 ml of o-phenanthroline reagent.
3. Add 1 ml distilled water.
4. Mix well after addition of each reagent.

Read the absorbance at 510 nm after 5 min.

Prepare a standard graph using the standard ferrous solution in the range 10 - 100 mg/l Fe^{2+} .

Estimation of protein content (Lowry *et al*, 1953) **:**

Sample preparation: Appropriate quantities of cell suspensions or broth were centrifuged at 10,000 rpm for 10 min to separate the cells. The pellet was resuspended in 2 N NaOH (1-2 ml depending on the amount of cells) and heated at 70°C for 1 h.

Protein content from samples was determined using the following protocol with Bovine serum albumin as standard. A standard calibration curve in the range 10-200 µg/ml was generated.

Reagents:

Solution A: 2% Sodium potassium tartarate.
Solution B: 1% Copper sulfate pentahydrate.
Carbonate solution: 2% Sodium carbonate in 0.1N NaOH.
Solution C: Add 1 ml of solution A and 1 ml of solution B to 100 ml of carbonate solution. Mix well.
Folin-Ciocalteau phenol reagent (1N): This is prepared by diluting the commercial reagent with distilled water.

Protocol:

1 ml sample (or sample diluted to 1 ml)
+ 5 ml Solution C
+ 0.5 ml Folin-Ciocalteau phenol reagent.

Incubate in dark for 30 minutes.

Read the developed color at 750 nm with distilled water in place of sample as reagent blank.

Estimation of Biochemical Oxygen Demand (BOD)

(Azide modification of Winklers method):

Reagents:

1) Sodium thiosulfate solution (0.025 N)

Dissolve 24.82 g of $Na_2S_2O_3$ 5H2O in boiled distilled water and make up the volume to 1 litre. This is 0.1 N stock. Keep in brown colored glass bottles. Dilute it 4 times just before use.

2) Alkali iodide azide solution

Dissolve 500 g NaOH or 700 g KOH and 150 g KI in distilled water to make volume upto 1 litre.

Dissolve 10 g NaN_3 in 40 ml distilled water.

Mix solution a and b.

3) Manganous sulfate solution

Dissolve 100 g $MnSO_4$ $4H_2O$ in 200 ml of boiled distilled water and filter.

4) Starch solution

Dissolve 1g starch in 100 ml of warm distilled water and add a few drops of formaldehyde solution.

5) Sulfuric acid

H_2SO_4, conc. (sp. gr 1.84)

Protocol

1. Fill the sample in a glass stoppered bottle (BOD bottle) of known volume (100-300 ml) carefully avoiding any kind of bubbling and trapping of the air bubbles in the bottle after placing the stopper.
2. Pour 2 ml each of manganese sulfate and alkali iodide azide solutions well below the surface of the walls. A precipitate will appear.
3. Shake the contents of the bottle by inverting the bottle repeatedly. Keep the bottle for some time for the precipitate to settle.
4. Add 2 ml sulfuric acid to dissolve the precipitate.
5. Remove 50 ml of the contents in a conical flask for titration.
6. Titrate the contents against sodium thiosulfate solution using starch as indicator. At the end point, initial dark blue color changes to colorless.

Calculations:

$$\text{Dissolved oxygen (mg/l)} = \frac{(\text{ml} \times \text{N}) \text{ of titrant} \times 8 \times 1000}{V_2(V_1\text{-}V/V_1)}$$

where, V_1 is the volume of the bottle after placing the stopper

V_2 is the part of the contents titrated

V is the volume of $MnSO_4$ and KI added.

$$BOD\ (mg/l) = \frac{D_1 - D_2}{P}$$

where,

D_1 is the DO of diluted sample immediately after preparation, mg/l

D_2 is the DO of diluted sample after 5 day incubation, mg/l

P is the decimal volumetric fraction of the sample used.

Estimation of Chemical Oxygen Demand (COD) :

Reagents:

1) Potassium dichromate solution, 0.25 N

Dissolve 12.259 g of dried A. R. grade $K_2Cr_2O_7$ in distilled water to make 1 litre of solution.

2) Ferrous ammonium sulfate solution, 0.25 N

Dissolve 98 g ferrous ammonium sulfate in distilled water containing 20 ml concentrated H_2SO_4 and make upto 1 litre with distilled water.

3) Ferroin indicator

Dissolve 1.458 g 1,10 o-phenanthroline and 0.695 g ferrous sulfate in distilled water and make upto 100 ml.

4) Concentrated sulfuric acid

5) Silver sulfate, solid

Protocol

1. Take 20 ml of sample in a 250 ml COD flask.
2. Add 10 ml 0.25 N dichromate solution.
3. Add 0.2 g Silver sulfate.
4. Carefully add 30 ml sulfuric acid.
5. Reflux for at least 2 h on a hot plate at 150°C.
6. After refluxing allow the flasks to cool and add 90 ml distilled water.
7. Mix well and add 2-3 drops of ferroin indicator and titrate against ferrous ammonium sulfate solution till the end point is reached (solution turns brick red).
8. Repeat the procedure with distilled water in place of the sample. This is the reagent blank.
9. In a separate flask take 10 ml distilled water and add all the reagents. Do not reflux. Titrate the contents of this flask along with the samples. This will give the exact normality of the ferrous ammonium sulfate used.

Calculations:

$$\text{COD, mg/l} = \frac{\text{(b-a) x normality of ferrous ammonium sulfate x 1000 x 8}}{\text{ml sample taken}}$$

where,

a is the ml of titrant with sample
b is the ml of titrant with blank

Protocol for the extraction of total DNA :

1. Inoculate 100 ml Luria broth with a single colony of the culture to be used and incubate on a gyratory shaker for 24 h at 30°C.
2. Centrifuge the broth at 4000 x g for 10 min at 4°C.
3. Wash the pellet at least twice with GET (GET= Glucose 50 mM, EDTA 10 mM, 25 mM Tris pH 8.0).
4. Resuspend the pellet in 2.5 ml $T_{10}E_1$ ($T_{10}E_1$= Tris pH 8.0, 10 mM; EDTA 1mM). Dissolve 10 mg lysozyme in 2.5 ml $T_{10}E_1$. Mix lysozyme solution with the above suspension and ensure the dispersion of cells by vortexing.
5. Keep on ice for 30 minutes.
6. Add SDS to the final concentration of 1% and keep at room temperature for 15 minutes.
7. Add equal volume of phenol-chloroform (1:1), gently mix it and centrifuge at 8000 x g for 10 minutes at room temperature.
8. Collect the upper aqueous phase.
9. Repeat such extractions till interphase is completely lost.
10. To the upper phase add equal volume of chloroform- isoamyl alcohol (24:1). Mix gently.
11. Centrifuge at 8000 x g for 10 minutes at room temperature.
12. Collect the aqueous phase in a glass tube and adjust the salt concentration to 0.15 M NaCl with 5M NaCl and precipitate the nucleic acids with double the volume of distilled ethanol.
13. Spool the nucleic acids with a spooling rod and dissolve in 0.1X SSC buffer.
14. Store the sample at 4°C and check for nucleic acids by gel electrophoresis.
15. Add RNase 100 µg/ml and incubate at 37°C for 1 h.
16. Extract with phenol- chloroform followed by chloroform and centrifuge at 8000 x g at room temperature for 10 minutes.
17. Adjust the salt concentration to 0.15 M using 5 M NaCl.
18. Reprecipitate using double volume of ethanol.
19. Spool the nucleic acids with a spooling rod and dissolve in 0.1X SSC.
20. Read the optical density of the sample at 260 and 280 nm and use for the determination of T_m.

Protocol for the isolation of plasmid DNA :

Amplification in rich medium:

1. Inoculate 10 ml LB medium containing 30 mg/l Cr^{6+} with a single colony of *P. mendocina* MCM B-180. Incubate at 30°C overnight with vigourous shaking.
2. The following morning, inoculate 25 ml LB medium in 100 ml flasks with 0.1 ml of the overnight grown culture. Incubate at 37°C on shaker till culture reaches late log phase (O. $D._{600} \sim 0.6$).
3. At this stage add chloramphenicol to the final concentration of 170 µl/ml.
4. Incubate at 30°C for further 12- 16 h.

Harvesting of bacteria:

1. Harvest the bacterial cells by centrifugation at 4000 x g for 10 minutes at 4°C. Discard the supernatent.
2. Wash the cells in 100 ml of ice cold STE (0.1 M NaCl, 10 mM Tris.Cl (pH 7.8) and 1 mM EDTA).

Plasmid isolation by the alkaline lysis method:

1. Inoculate 5 ml of LB medium containing 30 mg/l Cr^{6+} with a single bacterial colony. Incubate at 30°C overnight with vigorous shaking.
2. Pour 1.5 ml of the culture into an Ependorf tube. Centrifuge for 1 minute in an Ependorf centrifuge. Store remainder of the overnight culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the pellet by vortexing in 100 µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris.Cl (pH 8.0).
5. Store for 5 minutes at room temperature.
6. Add 200 µl of a freshly prepared solution of 0.2 N NaOH and 1% SDS.
7. Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store on ice for 5 minutes.
8. Add 150 µl of ice cold solution of potassium acetate (pH 4.8) made up as follows: To 60 ml of 5M potassium acetate add 11.5 ml of glacial acetic acid and 28.5 ml of H_2O.
9. Close the top of the tube and vortex gently by inverting for 10 seconds and store on ice for 5 minutes.
10. Centrifuge in Ependorf centrifuge for 5 minutes at 4°C.
11. Transfer the supernatent to a fresh tube.
12. Add equal volume of chloroform. Mix by vortexing. After centrifugation in Ependorf centrifuge, transfer supernatent to a fresh tube.
13. Add two volumes of ethanol at room temperature. Mix by vortexing. Stand at room temperature for 2 minutes.
14. Centrifuge for 5 minutes in an Ependorf centrifuge at room temperature.

15. Remove the supernatent. Stand the tube in an inverted position on paper towel to allow the fluid to drain away.
16. Add 1 ml of 70% ethanol. Vortex briefly and then recentrifuge.
17. Again remove all the supernatent and dry the pellet in vacuum desiccator.
18. Add 50 µl of TE (pH 8.0) containing DNase free pancreatic RNase (20 µg/ml). Vortex briefly.

This preparation can be used for restriction digestion also.

Protocol used in the transformation studies :

1. Seed 5 ml TYE medium with one isolated colony of the host to be used for transformation. Shake overnight at 37°C.
2. The next morning seed 6 ml TYE medium with 0.1 ml of the overnight culture.
3. Shake at 37°C until the optical density at 600 nm of the culture reaches a value between 0.5 and 0.6. This usually requires 2 to 3 h incubation at 37°C.
4. Centrifuge the culture at 2000 x g for 10 min at 4°C.
5. Resuspend the cell pellet in 2.5 ml 50 mM $CaCl_2$.
6. Let the suspension stand at 4°C for 15 minutes.
7. Spin the suspension at 2000 x g for 10 minutes at 4°C.
8. Resuspend the cells in 500 µl 50 mM $CaCl_2$.
9. In a sterile tube mix 50 µl TCM buffer with 20- 30 µl of the DNA preparation (0.1-100 ng) to be used for transformation.
10. Add to the DNA- TCM mixture 100 µl of the cell suspension obtained in step 8.
11. Incubate for 15 minutes on ice.
12. Transfer to 42°C for 2 min.
13. Let stand for 10 minutes at room temperature.
14. Add 1 ml prewarmed TYE medium to the mixture and incubate without shaking for 30 minutes at 37°C.
15. Spread 0.1 ml of the mixture directly onto agar plates containing the medium for the selection of transformed cells.
16. Mix the remaining 0.9 ml of cells with 3 ml 1% agar and pour the mixture onto agar plates containing the selective medium.
17. Incubate plates at 37°C overnight.
18. Pick colonies and test them for the presence of recombinant DNA.

Buffers and culture media:

TCM buffer	100 ml
10 mM Tris- HCl (pH 7.4)	1 ml 1 M Tris- HCl (pH7.4)
10 mM $CaCl_2$	1 ml 1 M $CaCl_2$
10 mM $MgCl_2$	1 ml 1M $MgCl_2$
Distilled water	97 ml

Protocol used in the conjugation studies :

1. Mix well 2 ml of overnight cultures of donor and recepient cells.
2. Filter the above suspension through a millipore membrane filter.
3. Place the membrane on TYE agar plate. Incubate at 33°C for 24 h.
4. Suspend the growth in 2 ml 0.85% saline.
5. Carry out serial dilutions in 0.85% saline.
6. Spread 0.1 ml of each dilution onto TYE agar containing 50 μg/ml Rifampicin, TYE agar containing 50 μg/ml Cr^{6+} and TYE agar containing both 50 μg/ml Rifampicin and 50 μg/ml Cr^{6+} .
7. Incubate at 33°C for 48-72 h to allow the formation of colonies of transconjugants.

Preparation of 7.5 % Native Polyacrylamide gels :

Solution A:

Tris buffer	36.6 g
1 N HCl	48.0 ml
TEMED	0.46 ml

Make upto 100 ml with distilled water.

Solution B:

Acrylamide	30.0 g
N-N' methylenebisacrylamide	0.8 g

Make upto 100 ml with distilled water.

Solution C :

Ammonium persulfate 1 mg/ml in distilled water

Mix 5 ml each of solution A & B and add 10 ml solution C

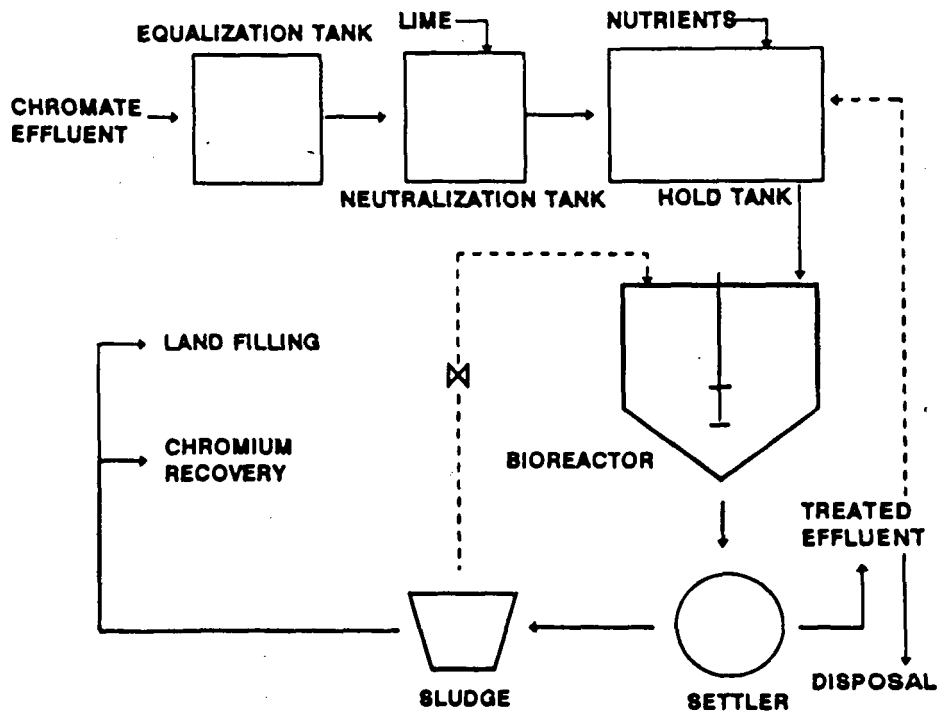
Tank buffer:

Glycine	2.88 g
Tris	0.60 g
Distilled Water	1 litre
pH	8.3

Appendix III

TRANSFER OF TECHNOLOGY TO INDUSTRY

Efforts made for the development of a microbiological process for the removal of chromate from industrial waste waters has been described in the present thesis. The economics worked out for a laboratory-scale process had clearly indicated that the process could be commercialized in India. The possible commercial scale design could be depicted as follows:



As per the above design, the chromate bearing effluent could be pumped to an equalization tank wherein the fluctuations in the actual chromate concentration in the waste water could be taken care of. The equalized stream could then enter a neutralization tank for adjustment of desired pH (if necessary). Such an effluent could then be pumped to a hold tank. Appropriate nutrients could be added to the tank to support microbial growth. The effluent with nutrients could be continuously pumped to a bioreactor containing an actively metabolizing culture of *P. mendocina* MCM B-180. The bioreactor could be agitated at very slow speed of 8-10 rpm so as to achieve chromate reduction with optimum efficiency. The treated effluent (free of Cr^{6+}) from the

bioreactor could be collected in a settler where the chromic hydroxide sludge formed upon reduction could be easily separated. The clarified effluent from the settler could be disposed off. Alternatively, it could be used for dilution of the effluent (feed), if the chromate concentrations encountered were higher than 100 mg/l. The separated sludge from the settler could be disposed off either as a land-fill or utilized for the recovery of chromium metal.

It must be mentioned that the laboratory-scale process developed during the present studies was licensed to a reputed environmental engineering company for upscaling and commercialization in India. Again, it is highly gratifying to mention that the process was successfully demonstrated by the company at pilot scale (2 m^3 per h) for treatment of a cooling tower effluent. It is hoped that the first commercial scale microbiological treatment plant for chromate containing waste waters in India will become operative very soon.