
*MATERIALS AND
METHODS*

3. MATERIALS AND METHODS

The present study entitled “**Bioremediation of nickel electroplating effluent and its impact on the growth and biochemical constituents of green gram and cat fish**” was conducted under five phases:

PHASE 1

Isolation and identification of metal tolerant bacteria and fungi from the nickel electroplating effluent contaminated soil

PHASE 2

Removal of nickel from the effluent using bacterial and fungal isolates

PHASE 3

Physicochemical characterisation of raw and microbially treated nickel electroplating effluent

PHASE 4

Impact of nickel electroplating effluent (untreated and treated) on the biometric and biochemical parameters of green gram (*Vigna radiata* L.) and on the soil quality

PHASE 5

Impact of nickel electroplating effluent (untreated and treated) on the growth and biochemical constituents of a fresh water cat fish [*Clarias gariepinus* (Burchell)]

PHASE I

3.1. ISOLATION AND IDENTIFICATION OF METAL TOLERANT BACTERIA AND FUNGI FROM THE NICKEL ELECTROPLATING EFFLUENT CONTAMINATED SOIL

3.1.1. Collection of the effluent

Fifty litres of the raw nickel electroplating effluent sample was collected in clean plastic cans from nickel electroplating industry (Plate I A and I B) located at Siddhapudur in Coimbatore district, Tamil Nadu, South India. The effluent collected was stored at 4°C for further studies.

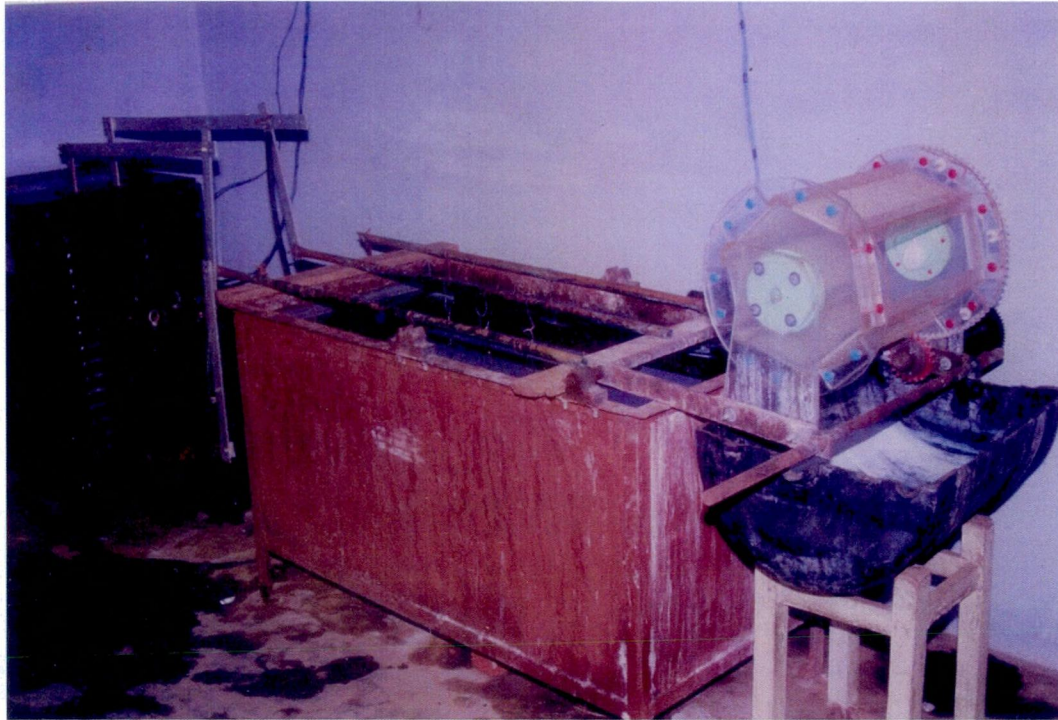
3.1.2. Collection of the soil sample

For the present study, the soil sample was collected from the nickel electroplating effluent discharged area at a depth of about 50 cm from the surface and dried at ambient temperature. The lumps in the soil sample was crushed by using a porcelain mortar and pestle and stored in cloth bags for subsequent analysis. The metal tolerant microorganisms were isolated from the soil sample.

3.1.3. Isolation of bacteria

Ten grams of the effluent contaminated soil sample was weighed and dispersed in 100 ml of sterile distilled water and stirred well. The sample was serially diluted upto 10^{-8} dilution. One milliliter of the sample was drawn from each dilution and was plated on sterile petri plates. Nutrient agar medium was poured onto the petri plates and incubated at 37°C for 24 hrs, for the development of bacterial colonies as described by Jensen (1968). The individual colonies were selected and transferred to nutrient agar slants for further study.

PLATE I



**A. ELECTROPLATING BATH USED IN THE PROCESS OF
NICKEL ELECTROPLATING**



**B. POINT OF DISCHARGE OF NICKEL ELECTROPLATING
EFFLUENT**

Nutrient agar medium prepared with following ingredients was used as culture medium for the experiment:

Peptone	-	5g
Beef extract	-	5g
NaCl	-	5g
Distilled water	-	1000 ml
Agar	-	20g
pH	-	7.0 ± 0.1

3.1.3. a. Screening bacterial species for nickel tolerance

A loopful of the isolated bacterial species was inoculated into sterile nutrient broth and incubated at 37°C for 24 hrs. From this 2% (v/v) of the bacterial culture was inoculated into different concentrations of effluent (25%, 50%, 75% and 100%) separately and incubated at 37°C for 24 hrs. The percentage removal of nickel in different concentrations of effluent was also observed. Three bacterial species that were highly tolerant to nickel were isolated and identified.

3.1.3. b. Identification of bacteria

Based on the morphological (shape, size, Gram staining, motility and nutrient agar) and biochemical characteristics (catalase, oxidase, indole production, methyl red, voges proskauer, citrate utilization, starch and gelatin hydrolysis, urease, nitrate reduction and carbohydrate fermentation tests) the bacterial isolates were identified following the procedures described in Bergey's Manual of Determinative Bacteriology (1994) and by Aneja (1996). The methodologies adopted for identification of bacterial isolates are given in Appendix 1.

3.1.3. c. Maintenance of bacterial stock cultures

The stock cultures were maintained on nutrient agar slants and stored at 4°C in a refrigerator. The cultures were periodically transferred to fresh nutrient agar slants at intervals of one month.

3.1.4. Isolation of fungi

Ten grams of effluent contaminated soil sample was weighed and dispersed in 100 ml of sterile distilled water and stirred well. The sample was serially diluted upto 10^{-8} dilutions and 1ml of the sample was drawn from each dilution and was pour plated on sterile petri plates. Rose bengal chloramphenicol agar medium was poured onto the sample and the plates were then incubated at room temperature (28°C) for 5 days. The well grown individual colonies were isolated and maintained on rose bengal chloramphenicol agar slants at 4°C for further study. The fungal isolates were cultured in rose bengal chloramphenicol agar medium with following composition:

Dextrose	-	10g
Peptone	-	5g
MgSO ₄	-	5g
KH ₂ PO ₄	-	5g
Rose Bengal	-	0.03g
Chloramphenicol	-	0.03g
Distilled water	-	1000 ml
Agar	-	20g
pH	-	5.0 ± 0.1

3.1.4. a. Screening fungal isolates for nickel tolerance

The isolated fungal colonies were inoculated into sterile rose bengal chloramphenicol broth and incubated at 28°C for 5 days. From this 2% (v/v) of the fungal culture was inoculated into different concentrations (25%, 50%, 75% and 100%) of effluent separately and incubated at room temperature (28°C) for 5 days. The percentage removal of nickel in different concentrations of effluent was also observed. Three fungal species which were highly tolerant to nickel were isolated and identified.

3.1.4. b. Identification of fungi

The fungal hyphae and spores were mounted on a clean glass slide and stained with lactophenol cotton blue. The preparation was covered with cover slip and observed under the compound light microscope for the identification of fungal isolates (Appendix 2).

PHASE II

3.2. REMOVAL OF NICKEL FROM THE EFFLUENT USING BACTERIAL AND FUNGAL ISOLATES

3.2.1. Preparation of live and dead biomass

A comparative study on the removal of nickel using live and dead biomass was carried out. A loopful of bacterial culture was inoculated into the nutrient broth separately and was incubated at 35°C for 24 hrs. A loopful of fungal culture was inoculated into rose bengal chloramphenicol broth separately and was incubated at room temperature for 5 days. The microbial biomass was strained through Whatman filter paper No. 1 and the wet biomass was washed with deionized water which served as “live biomass”. The live bacterial and fungal biomasses were autoclaved separately in the final wash

water at 121°C for 20 minutes. Excess water was drained off after sterilization and the wet biomass was dried at 37 - 40°C for 4 - 5 days until moisture content was removed. Flakes of dry bacterial and fungal biomass were crushed in a mortar and pestle and the powdered biomass was considered as “dead biomass” for the present study. The sorption profile was studied by adding 1g of the live and dead biosorbent in 100ml of the nickel electroplating effluent (Muraleedharan *et al.*, 1991).

3.2.2. Removal of nickel from different concentrations of effluent using live and dead bacterial and fungal biomass

A pilot study was carried out with different concentrations (25%, 50%, 75% and 100%) of the nickel electroplating effluent. 100ml of different concentrations of the nickel electroplating effluent was taken in Erlenmeyer flask and sterilized. Each flask was inoculated with 1g of the live and dead bacterial and fungal biomass separately under aseptic conditions and incubated at 37°C for 24 hrs (bacteria) and at room temperature for 5 days (fungi). At the end of the incubation period, the samples were removed, centrifuged at 5000 rpm for 10 minutes at 4°C and the supernatants were analysed for their efficiency in the removal of nickel.

From the above study, it was observed that nickel removal was efficient in 25% concentration of nickel electroplating effluent. The dead biomass was found to remove nickel more effectively from the effluent when compared with live systems. Hence, 25% concentration of nickel electroplating effluent was taken for further studies.

3.2.3. Optimization of pH, temperature and incubation period on the biosorption of nickel

For the uptake of nickel from the effluent, different operational parameters were optimized. One gram of the dead bacterial and fungal biomass was inoculated separately into a series of 250ml conical flasks containing 25% concentration of nickel electroplating effluent. The pH was set at different ranges from 3 to 9 (3, 4, 5, 6, 7, 8, 9) by adjusting with 1N HCl or 1N NaOH. At each pH, the cultures were incubated at different temperatures (20°C, 25°C, 30°C, 35°C and 40°C) and for different incubation periods {bacteria (6, 12, 24, 48, 72, 96, 120 and 144 hrs), fungi (1, 3, 5, 7, 9, 12 and 15 days)} to find out the optimum pH, temperature and incubation time for their activity. At the end of the incubation period the samples were removed, centrifuged at 5000 rpm for 10 minutes at 4°C and the supernatants were analysed for the removal of nickel.

From the above study it was observed that, *Aspergillus niger* (a fungal isolate) was efficient in the removal of nickel at 25% concentration of nickel electroplating effluent when compared with the other bacterial and fungal isolates. Hence, *Aspergillus niger* was used for the present study on bioremediation of nickel electroplating effluent.

3.2.4. Evaluation of nickel absorption by Scanning Electron Microscopy

To determine the effect of nickel on the surface of the *A. niger* (both metal treated and untreated) scanning electron microscopic study was performed to observe the spatial relationship between cells and metals. The cell pellets were washed thrice with phosphate buffer saline, suspended in 1ml of fixative solution (2.5%

glutaraldehyde and 2% formaldehyde) and kept for 6 hrs at 4°C. The fixative solution was removed by centrifugation and the cell pellets were washed again with phosphate buffer saline for five times and finally resuspended in 1ml of sodium phosphate buffer. The cells were coated with 90 Å thick gold palladium for 30 minutes and fixed on the coverslip coated with poly - L - lysine and then mounted on aluminium stubs. The coated cells were viewed at different magnifications to confirm the nickel accumulation in the fungal mycelium (Srivastava and Thakur, 2006).

PHASE III

3.3. PHYSICOCHEMICAL CHARACTERISATION OF RAW AND MICROBIALLY TREATED NICKEL ELECTROPLATING EFFLUENT

Electroplating industry is one of the major contributors of heavy metal pollution in surface water. The effluent may contain toxic components which when deposited on the soil may cause adverse effect on crop production and also on human health. Hence, an attempt has been made in the present study to analyse the physicochemical characteristics of the raw and microbially treated nickel electroplating effluents.

3.3.1. Characterisation of raw and microbially treated nickel electroplating effluent

The raw and microbially treated effluents were subjected to various physicochemical analyses. The physical (colour, odour, turbidity, pH, electrical conductivity, total suspended solids and total dissolved solids) and chemical (Biological oxygen demand, Chemical oxygen demand, total hardness, total alkalinity, chlorides, sulphates, phosphates, ammoniacal nitrogen, calcium, magnesium, sodium,

potassium, nickel, oil and grease) parameters were analysed (Appendix 3) in the effluents. Prior to the physicochemical characterisation of microbially treated effluent, the biomass in the effluent was separated by centrifugation at 12000 x g for 15 minutes and only the supernatant was taken for the analysis (Chandra, 2001).

3.4. IMPACT OF NICKEL ELECTROPLATING EFFLUENT (UNTREATED AND TREATED) ON THE BIOMETRIC AND BIOCHEMICAL PARAMETERS OF GREEN GRAM (*Vigna radiata* L.) AND ON THE SOIL QUALITY

3.4.1. Selection of the plant for pot culture experiment

Green gram (*Vigna radiata* L.co var 6) plant was selected for the present experiment due to its luxurious growth, easy availability, nutritious value and extensive consumption by humans. It is one of the predominant sources of proteins and certain amino acids like lysine and tryptophan in vegetarian diets. It is relatively drought tolerant and well adapted to a range of soil conditions including light soils and can thrive even under limited irrigation. Moreover, it is suitable for crop rotation and crop mixtures (Pandiyan *et al.*, 2006). Further they are adaptable to the prevailing environmental conditions.

3.4.2. Design of the experiment

Red soil and sand were sieved and mixed in equal proportion (1:1) and used for the pot culture experiment. The seeds of green gram were collected from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu. Seeds of uniform size and weight were selected for the experiment. They were surface sterilized with 0.1% mercuric chloride solution for 2 minutes and washed with tap water thoroughly to remove surface contamination.

A pot culture experiment was conducted with three treatments and each treatment was replicated thrice (Plate II). The treatments were:

T₁ - Control (Tap water)

T₂ - Untreated nickel electroplating effluent (25% concentration of nickel electroplating effluent)

T₃ -Treated nickel electroplating effluent (25% concentration of microbially treated nickel electroplating effluent)

Healthy seeds were soaked in each of the above treatments, for overnight and washed thoroughly with distilled water to remove traces of the effluent and in each pot ten seeds were sown. The pots were kept under laboratory conditions for the assessment of various plant growth parameters. The pots with seeds were irrigated with tap water, untreated and treated nickel electroplating effluent regularly.

3.4.3. Biometric parameters of green gram plants

Germination percentage and vigour index were calculated seven days after sowing. Vigour index was calculated using the formula,

$$\text{Vigour index} = \text{germination percentage} \times (\text{root length} + \text{shoot length})$$

(Abdul Baki and Anderson, 1973).

The plants were removed on the 7th, 30th and 60th days after sowing and were washed in running water to remove soil particles and pressed between filter paper folds to remove water droplets. Shoot and root lengths and fresh weight were measured on the respective days. The plants were dried in oven at 60°C ± 1°C and their dry weights were also recorded. The growth, yield and selected biochemical parameters were analysed on 30th and 60th days after sowing. The number and weight of the pods/plant were evaluated on 60th day.

PLATE II



EXPERIMENTAL SET UP FOR PLANT GROWTH STUDIES

T₁ - CONTROL

T₂ - UNTREATED NICKEL ELECTROPLATING EFFLUENT

T₃ - TREATED NICKEL ELECTROPLATING EFFLUENT

3.4.4. Biochemical parameters of green gram plants

Selected biochemical constituents namely total proteins (Appendix 4a), total carbohydrates (Appendix 4b) and nickel (Appendix 3o) were analysed from the green gram plants grown in tap water, untreated and treated nickel electroplating effluent on 30 and 60 days after sowing by adapting standard methods as described in the appendices. Total chlorophyll (Appendix 4c) in the leaves of the test plant was also analysed. At the termination of the experiment (60th day) the pods (Appendix 3p) were analysed for the presence of nickel content.

3.4.5. Physicochemical parameters of untreated and treated soil samples

Soil analysis namely pH, electrical conductivity, organic carbon, available macronutrients (nitrogen, phosphorus, potassium, sodium, calcium and magnesium) and micronutrients { zinc, iron, copper, manganese (Appendix 5) and nickel (Appendix 3p) } was done prior to treatment and after treatment with tap water, untreated and treated nickel electroplating effluent.

3.5. IMPACT OF NICKEL ELECTROPLATING EFFLUENT (UNTREATED AND TREATED) ON THE GROWTH AND BIOCHEMICAL CONSTITUENTS OF A FRESH WATER CAT FISH [*Clarias gariepinus* (Burchell)]

3.5.1. Selection of the fish

The fresh water cat fish, *Clarias gariepinus* popularly known as African cat fish was selected for the present study due to its faster growth, high recuperative, ability to survive in oxygen depleted water, hardy and tough nature and its culture involving simple and low risk methods.

3.5.2. Experimental Design

An attempt was made to assess the impact of nickel electroplating effluent (untreated and treated) on the fresh water fish, *Clarias gariepinus*. Fresh water fish, *Clarias gariepinus* (Plate III) was collected from a fish farm in Coimbatore, Tamil Nadu. The fishes were transported to the laboratory in a polythene bag containing oxygenated water. They were kept in two large aquarium tanks and were acclimatized to laboratory conditions for a period of 15 days in non chlorinated water (Plate IV). Water was changed daily to provide sufficient oxygen and they were fed with fish feed regularly.

Feeding was stopped one day prior to the start of the experiment. Three experimental tubs were setup and each tub was filled with tap water, untreated and treated effluent separately. Each treatment was replicated thrice (Plate V). For each treatment five fishes were introduced and allowed to grow. No mortality was recorded during the experimental period. Length and weight of the fishes were measured on the 60th day of the experiment. At the end of the 60th day, the selected parts (muscle, liver, gills and kidney) were dissected and analysed for the biochemical constituents namely total proteins (Appendix 4a), total carbohydrates (Appendix 4b), lipids (Appendix 4d) and nickel (Appendix 3p).

3.5.3. Histological studies in the selected tissues of the fish (*Clarias gariepinus*)

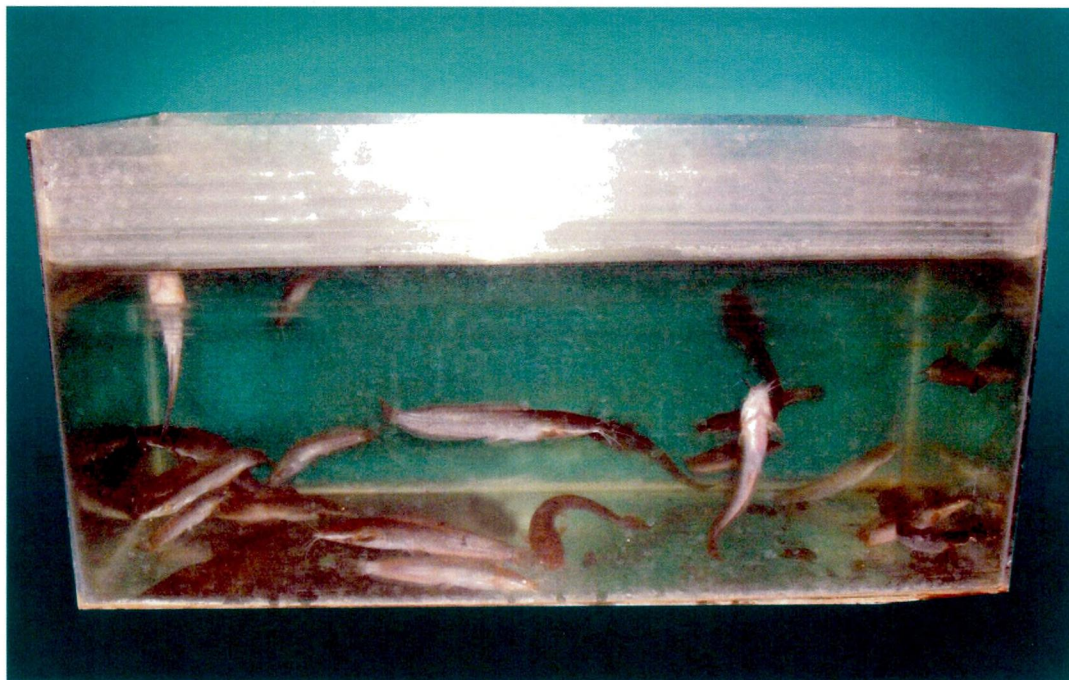
At the termination of the experiment (60th day), the gills, liver and kidney tissues of the control and experimental group of fishes were removed and fixed separately in 10 per cent formalin solution. The fixed tissues were dehydrated in graded series of acetone (50, 70, 90 ml) solution and cleared in xylene.

PLATE III



***CLARIAS GARIEPINUS* – EXPERIMENTAL FISH**

PLATE IV



ACCLIMATIZATION OF *CLARIAS GARIEPINUS*

PLATE V



EXPERIMENTAL SET UP FOR FISH GROWTH STUDIES

T₁ - CONTROL

T₂ - UNTREATED NICKEL ELECTROPLATING EFFLUENT

T₃ - TREATED NICKEL ELECTROPLATING EFFLUENT

The study tissues were embedded in molten paraffin wax (58 - 60°C) separately. The blocks containing the tissues were cooled and the sections were cut using rotary microtome to 8-9 μ thickness. The paraffin sections were carefully mounted on glass slides using Mayer's albumin as adhesive and the sections were immersed in xylene for 2 minutes to clean the paraffin wax. The sections were double stained with eosin and haematoxylin. The stained sections of the tissues were examined and microphotographs were taken to study their histological changes.

3.6. STATISTICAL ANALYSIS

The data obtained were statistically analysed by one way and two way analysis of variance ($P < 0.05$) using statistical software Sigmastat 3.1.