CHAPTER 4

EXPERIMENTAL DETAILS

MATERIALS AND METHODS FOR BACTERIAL IDENTIFICATION

4.1. Sampling of Natural Pond Water

The water sample was collected from Ammon Pond Source at Karaikudi. The collections were made for the period of 100 days. The water sample was collected in sterile stoppered bottle and brought to the laboratory in an ice box to avoid microbial proliferation and contamination during transport. Physico-chemical and biological characteristics of the natural pond water has been analysed and the water was changed for every 24 hours interval upto 100 days in order to make the biofilm in alive conditions.

4.2. MIXED CULTURE SYSTEM

The nutrient broth medium peptone (Hi-media, LR) 5 g/l, yeast extract (Hi-media, LR) 3 g/l, Beaf Extract 1.5 g/l, pH = 7.4 was prepared and sterilised. The co-culture of Pseudomonas Vibrio and Bacillus was inoculated in the system. The composition of nutrient broth was considered as 100%. 3% nutrient broth was made from the stock solution by dilution which was used for electrochemical study.

4.3. ABIOTIC OR CONTROL SYSTEM

1. Natural pond water was sterilized by autoclaving at 120°C for 15 to 20 minutes. This sterilised water was used as a control (without bacteria).

2. 3% nutrient broth was used for the control of mixed culture system (without bacterial inoculation).
4.4. PHYSICO-CHEMICAL ANALYSIS OF NATURAL POND WATER

The samples were analysed by the following methods as given by Trivedy (1984). The chemical characteristics of natural pond water is given in Table 4.1.

**pH measurement**: pH is the negative logarithm of hydrogen ion concentration of the solution and it was measured by using pH meter and standard buffers at room temperature.

**a) Dissolved Oxygen**

The dissolved oxygen was estimated by Winklers iodimetric method.

**b) Total hardness of water**

Hardness of water is generally caused by the calcium and magnesium ions present in water. 50 ml of water sample was taken in a conical flask and one ml of buffer solution was added and titrated against EDTA (0.01M) solution using Eriochrome black-T (100-200 mg) as an indicator. The total hardness of water was calculated using the following formula.

\[
\text{Hardness as mg/l CaCO}_3 = \frac{\text{ml of EDTA} \times 100}{\text{ml of sample}}
\]

Calcium was estimated from the formula

\[
\text{Calcium (mg/l)} = \frac{\text{Vol. of EDTA used} \times 400.8}{\text{ml of sample}}
\]

Magnesium ion concentration was estimated by the formula

\[
\text{Mg mg/l} = \text{Total hardness} - \text{calcium hardness} \times 0.244
\]
c) Chloride Estimation

Silver nitrate reacts with chloride to form very slightly soluble white precipitate of silver chloride. At the end point, the chloride get precipitated. Free silver ions react with chromate to form silver chromate of reddish brown colour. 50 ml of water sample was taken in a conical flask and 2 ml of potassium chromate solution was added and titrated against silver nitrate (0.02N) till the appearance of red tinge.

The chloride was calculated using the formula

\[
\text{Chloride mg/l} = \frac{\text{(ml X N) of AgNO}_3 \times 1000 \times 35.5}{\text{ml of sample}}
\]

d) Inorganic Phosphate Estimation

50 ml of filtered sample was taken in a conical flask and 2 ml of ammonium molybdate was added into this solution and 5 drops of SnCl₂ was also added. A blue colour appeared. The reading was taken at 690 nm on a spectrophotometer using distilled water as a blank. Concentration of inorganic phosphate was found with the help of the standard curve.

4.5. MICROBIOLOGICAL ANALYSIS

The water sample was serially diluted using 99 ml and 9 ml sterile distilled water blanks. The total viable bacterial counts were enumerated using nutrient agar medium by pour plate method. The plating procedure for micro biological analysis should not exceed more than six hours after collecting the sample.

One ml of aliquots of appropriate dilutions were pipetted out into the sterile petriplates and 20 ml nutrient agar medium was added in each petriplates. The
samples were mixed thoroughly by rotating the plates clockwise and anticlockwise direction and allowed to solidify. Then the plates were incubated for 24 to 48 hours at 37°C. Duplicate samples were tested along with control. The plates containing bacterial colonies with 30-300 numbers were selected and total viable counts were made. The bacterial populations were expressed as colony forming units per ml (CFU/ml) of water sample.

4.6. MICROBIOLOGICAL ANALYSIS OF BIOFILM GENERATED ON THE METAL SURFACE

Isolation and characterisation of the microorganisms in the biofilm samples were done in the laboratory. The metallic coupons of mild steel, copper and stainless steel size of 10.5 cm x 5.5 cm were used. The metallic specimens were mirror polished and cleaned by trichloroethylene. Afterwards the coupons were exposed to U.V. light to kill the bacteria and immersed in the natural pond water. The pond water was changed once in every 24 hours interval for 100 days. The microbial growth generated on the exposed materials were scrapped thoroughly using sterile spatula and collected in sterile 1% peptone water. Estimation of total viable counts of the organism from biofilm was done by using nutrient agar medium. The isolated colonies were putrified using appropriate medium by streaking methods.

4.7. IDENTIFICATION OF BACTERIAL ISOLATES

Morphologically dissimilar well isolated colonies were randomly selected and streaked on nutrient agar plates to obtain pure culture. After noting the colony morphology and pigmentation of the colony, the pure cultures were streaked on nutrient agar slant and stored below 4°C to keep the bacterial strains viable. Periodic subculture was done once in a month.
The characterisation of bacteria up to generic level was done according to the key described in the Bergeys manual of systemic bacteriology (1984-89). Mae faddin (1980) and Prokaryotes manual (1984) were also used for the identification procedures.

For characterisation of bacterial strains a loop-ful bacterial cultures was inoculated into the sterile nutrient broth and incubated over night. The fresh overnight broth cultures were subjected to the following microscopic, physiological and biochemical tests for the differentiation, characterisation and identification.

a) **Gram staining**: A loopful of the broth cultures were subjected to gram staining procedure and observed microscopically under oil immersion.

b) **Motility**: A loopful of the culture was tested for motility using hanging drop technique and results were recorded.

c) **Spore staining**: A loopful of old (72 to 124 hrs) culture was subjected to spore staining procedure and observed under oil immersion objectives.

**Test for oxidation - Fermentation**: The oxidation fermentation (OF) basal medium was incorporated with glucose. Test tube with sterile medium and inverted Durham's tube was inoculated with a loopful of test culture and tube was inoculated at 37°C for 24-48 hours. Acid and gas production was observed from the change in the colouration in the test tube and the collection of gas in the Durham's tube respectively. The same test was made using various sugars like mannitol, dextrin, lactose to observe the acid production.

d) **Catalase**: Overnight culture was taken on a glass slide and 1 ml of 3% hydrogen peroxide was added to the culture. Appearance of gas bubbles was recorded as positive result.
e) **Oxidase test**: In a clean glass slide a drop of culture was placed and the oxidase disc was placed on the culture, the purple colour appearance was considered as the positive result.

### 4.8. ELECTRODE / COUPON DESIGN FOR CORROSION STUDIES

Three types of metals have been selected for this study. ie. mild steel, 316 stainless steel and copper. Their compositions are given below.

**Composition of mild steel**

<table>
<thead>
<tr>
<th>Element</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur</td>
<td>0.02 - 0.03%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.40 - 0.50%</td>
</tr>
<tr>
<td>Carbon</td>
<td>2%</td>
</tr>
<tr>
<td>Iron</td>
<td>97.47 - 97.58%</td>
</tr>
</tbody>
</table>

**Composition of 316 stainless steel**

<table>
<thead>
<tr>
<th>Element</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr</td>
<td>17.21%</td>
</tr>
<tr>
<td>Ni</td>
<td>9-13%</td>
</tr>
<tr>
<td>Mo</td>
<td>2.0 - 3.0%</td>
</tr>
<tr>
<td>C</td>
<td>0.03%</td>
</tr>
<tr>
<td>Mn</td>
<td>1.50%</td>
</tr>
<tr>
<td>Si</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

**Composition of Copper**

Copper: 99.9%
4.9. IMMERSION TEST

The studies were carried out by immersion tests as recommended in ASTM standard G 31-86. The mild steel, stainless steel and copper coupons of the size 25 x 20 x 3 mm specimens were polished, degreased, weighed and exposed in natural pond water system, mixed culture system and abiotic system in 1 litre beakers for a period of 100 days. At frequent intervals, weight loss of mild steel, stainless steel and copper coupons were measured. From the weight loss the corrosion rate in mmpy was obtained using the following formula

\[
\text{Corrosion rate (mmpy)} = \frac{87.6 \times \text{wt.loss (mg)}}{\text{Density} \times \text{Area} \times \text{Time}}
\]

\[
\text{Density (gm/cc)} \quad \text{Area (cm}^2\text{)} \quad \text{Time (hours)}
\]

4.10. ELECTROCHEMICAL CELL DESIGN

Mild steel, stainless steel and copper electrodes of 1 cm\(^2\) dimension, with an extended stem of 12 cm length were used in potential measurement, polarisation study and impedance measurement. The connecting rod of the electrode was first wrapped with teflon tape and coated with araldite. The electrodes were machine polished to a mirror finish, degreased with trichloroethylene.

The cell assembly used for polarisation and impedance experiments is shown in Fig 4.1. The cell assembly is a single walled glass cell of 500 ml capacity. At the top, three ground joints were provided to introduce working and auxiliary electrode as well as luggin capillary. The reference electrode (SCE) was connected through the respective culture medium - KCl salt bridge. Studies were carried out after the corrosion potential reached a steady state value.
Fig 4.1 Electrochemical cell with auxiliary, working and reference electrodes of electrochemical measurements.
Fig 4.2 Set-up for potentiodynamic polarisation studies
Fig 4.3 Set-up for Impedance Studies.
4.11. TAFEL POLARISATION STUDIES

Polarisation measurements were carried out potentiodynamically using potentiostat (PAR model 173) in conjunction with potentiocan generator (PAR 175) and XY recorder (Rikadenky Model R206) employing stationary electrode.

The electrode potential was fixed at 200 mV cathodic to open circuit potential and allowed to attain a steady state value. The steady state polarisation was carried out from -200 mV to +200 mV w.r.t the OCP at a scan rate of 0.5 mv/Sec. The \( i_{corr} \) values were obtained from the plot of \( E \) vs log \( i \) curve. Fig 4.2 shows the photograph of the experimental setup.

4.12. IMPEDANCE STUDIES

The impedance studies were carried out using computer controlled EG & G system model M 6310 with software M 398. A three electrode cell assembly was used for impedance measurements. Test specimen as the working electrode, a large platinum foil as counter electrode and saturated calomel electrode (SCE) as reference electrode were used. After attainment of a steady state value an AC signal of 10 mV amplitude was applied and impedance values were measured for frequencies ranging from 0.1 Hz to 100 kHz. The values of \( R_t \) were obtained from the Nyquist plots. Fig 4.3 shows the photograph of the impedance analyser.

4.13. XRD ANALYSIS

The immersed specimens both in natural pond water system, mixed culture system and abiotic system were removed and cut in the size of 1 cm\(^2\). The specimens were dried and the surface film was analysed by x-ray diffraction (XRD). The XRD pattern was recorded using computer - controlled XRD system, JEOL (Japan) model JDX - 8030 with CuK radiation (Ni - filtered = 1.5418 A\(^0\)) at a rating of 40 kV, 20
AM. The "Peak search" and "Search match" programmes (built in software) were used to identify the peak table, and ultimately for identification of the XRD peaks.

4.14. SCANNING ELECTRON MICROSCOPY

The specimens were removed from biotic and abiotic system and placed in sterile filtered cocodylate buffered (0.1 M; pH 7.2) gluteraldehyde solutions (2.5% in distilled water). Before examination under SEM, coupons were rinsed in sterile distilled water, dehydrated through a graded series of acetone, critical point dried in liquid CO₂ and gold sputtered. Observations were made in a JEOL SEM model No.JSM 35 CF operated at 25 KV accelerating voltage.

4.15. MATERIALS AND METHODS FOR STUDYING THE INFLUENCE OF VARIOUS BIOCIDES WITH INHIBITOR ON MILD STEEL, STAINLESS STEEL AND COPPER IN FRESH WATER ENVIRONMENT

i) Biocides and Inhibitors used

Biocides: Cetyl Trimethyl Ammonium Bromide (CTAB)
Cetyl Pyridinium Bromide (CPB) were used as biocides.

Inhibitor: Aminotrimethylene phosphonic acids (20 ppm) + Zinc (5 ppm) was used as inhibitor.

4.16. SOURCE OF BACTERIA FOR BIOCIDAL EVALUATIONS

Pond water was used as bacterial source. 1 ml of pondwater was inoculated in 100 ml nutrient broth and incubated at 37°C for 24 hours. 1 ml of 24 hours old bacterial culture grown in nutrient broth was inoculated in 100 ml of pond water to maintain 1% broth culture. In all the experiments, the bacterial counts was maintained in the range of log 5.01 bacteria per ml as initial count.
4.17. PREPARATION OF STOCK SOLUTIONS

i) **Inhibitor**: (Aminotrimethylene Phosphonic acids) The stock solution of aminotrimethylene phosphonic acid was prepared by dissolving 1 gm in 100 ml of sterile distilled water, which gave 10,000 ppm.

ii) **Zinc chloride solution**: The stock solution of zinc chloride was prepared by dissolving 100 mg of zinc chloride in 100 ml sterile distilled water, which gave 1000 ppm.

iii) **Biocides**: Cetyltrimethyl ammonium bromide (CTAB) and cetylpyridinium bromide (CPB) were used as biocides. Their stock solutions were prepared by 1 gm of each biocides dissolved in 100 ml sterile distilled water separately, which gave 10,000 ppm.

4.18. METHODOLOGY

The 1% nutrient broth culture (24 hrs) was used in evaluation of biocides. Subsequently the biocides / inhibitor were added in various concentrations. Every 24 hours interval, sampling were made to enumerate viable bacterial counts employing pour plate techniques. The efficiency of inhibitor was measured by weight loss and tafel polarisation studies.

4.19. EVALUATION OF BIOCIDES AND INHIBITOR

i) **Biocides**: To find the influence of biocides in corrosion process, Cetyltrimethyl ammonium bromide (CTAB) and Cetyl pyridinium bromide (CPB) were added in pond water inoculated with bacteria (1% broth) and weight loss in mild steel, stainless steel and copper were determined after seven days. Bactericidal efficiency was also noticed in presence of biocides.
ii) **Biocides + Inhibitor:** To find the influence of biocides (CTAB and CPB) on inhibitor (Amino trimethylene phosphonic acids and ZnCl₂) solutions, both the biocide and inhibitor were added in pond water (1% broth), simultaneously. The inhibition efficiency was determined for mild steel, stainless steel and copper by weight loss technique. Subsequently the influence of inhibitor on bacterial efficiency was also noticed.

iii) **Inhibitor addition after killing bacteria by biocide:** To avoid the interference between biocide and inhibitor, biocides were added in pondwater inoculated with bacteria (1% broth) to kill the bacteria. After 24 hrs the inhibitor (ATMP + Zn) was added in same pondwater and subsequently coupons were immersed for 7 days to find the corrosion rates.
Table 4.1

Chemical characteristics of natural pond water

<table>
<thead>
<tr>
<th>Element</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium carbonate</td>
<td>50-100 ppm</td>
</tr>
<tr>
<td>Total hardness</td>
<td>80-150 ppm</td>
</tr>
<tr>
<td>Salinity</td>
<td>283 ppm</td>
</tr>
<tr>
<td>pH</td>
<td>7.1 - 8.1</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>4.2 - 5.1 mg/l</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>0.3 uM</td>
</tr>
<tr>
<td>Nitrate</td>
<td>2.46 uM</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.23 uM.</td>
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</table>
REFERENCES

