Experimental

3.1. Introduction

The instrumentation, analytical procedure, adopted for the present investigations are well known for fundamental studies in electrochemistry. Hence a general outline of the experimental setup for electrochemical investigations, and all other experimental procedures adopted in this research programme are presented in this chapter.

3.2. Sample collection

Freshwater was collected from Ammon pond, 1 km away from CECRI, Karaikudi and was used for the present investigation. Freshwater samples were collected in sterile stoppered container and brought to the laboratory for biofilm formation. Collections of freshwater were made over a period of two months. Stagnant pond water was rich with organic nutrients and also enriched with numerous microorganisms. Freshwater was therefore considered to be an ideal medium for growing and studying biofilm formation. Electrodes / specimens were exposed in the freshwater where water was changed once in 24 h interval for test duration of 60-days.

3.3. Physico-chemical analysis of freshwater

Freshwater samples were subjected to analysis for Physio-chemical characteristics as per the methods described by APHA and Trivedy. The amount of trace metal present in the freshwater was also measured using Atomic absorption spectrometry (AAS). The sample was dried in an air oven and the residue was digested in 10 ml of concentrated H₂SO₄ by heating in a water bath. The
acid digest was diluted to 50 ml and then filtered through Whatman No.1 filter paper. The filtrate was analyzed by Atomic absorption spectrometry. Natural freshwater analysis was also done by Water Quality Meter (WQC-24), TOADK, Japan, in a few instances.

3.4. Biofilm formation

The freshwater was sterilized with autoclave at 121°C and used as control. The specimens were exposed in to the natural freshwater and also allowed to form 60-days old biofilm. The immersion of specimens was done carefully, so that the water line on the sample was sufficiently below the welded junction to ensure no galvanic interaction between the two metals. The natural freshwater was changed once for every 24 hrs interval, upto 60 days in order to make the biofilm active.

3.5. Bacteriological analysis of natural biofilm

Specimens were exposed to freshwater upto 60 days and biofilm formed on surface. The specimen was washed with sterile distilled water to remove un-attachment of aquatic microorganisms. The attachment of biofilm was scraped by employing sterile knife to collect biofilm in sterilized vial. The biofilm sample made up to 10ml of diluted solution using Milli-Q water. The samples were used for the total viable counts, carbohydrate and protein estimation.

The samples were serially diluted (tenfold) with sterile distilled water and the samples were plated using pour plate technique for the isolation of aerobic bacteria. The nutrient agar medium consists of (grams per liter): peptic digest of animal tissue 5 g, NaCl 5 g, beef extract 1.5 g, yeast extract 1.5 g, and agar 15 g. Acid producer medium (Thiobacillus broth) consists of (grams per liter): (NH₄)₂SO₄ 0.40 g, KHPO₄ 4 g, CaCl₂ 0.25 g, FeSO₄ 0.5 g, MgSO₄ 0.5 g, NaSO₄ 5 g, and agar 15 g. Mn agar base consists of (grams per liter): beef extract 1 g, yeast extract 0.075 g, MnCO₃ 2 g, Fe(NH₄)₂(SO₄)₂ 0.15 g, sodium citrate 0.15 g, and agar 15 g. The medium was used to
enumerate the heterotrophic bacteria (HB), acid producing bacteria (APB) and manganese-oxidizing bacteria (MOB) respectively.

One ml aliquot of appropriate dilutions was pipetted out into a sterile Petri plate and 20 ml of medium such as nutrient agar medium, acid producer medium and manganese base medium were added into each petriplate. The sample was mixed thoroughly by rotating the plate clockwise and anticlockwise direction and allowed to solidify. This procedure was repeated for the taken dilutions and different medium. Duplicate plates were also maintained. Bacterial counts were made after 24-48 hours of incubation, after incubation the plates were counted for bacterial colonies. The bacterial populations were expressed as number of colony forming units (CFU / cm²) of biofilm sample.

3.6. Estimation of protein and carbohydrate in natural biofilm

3.6.1. Carbohydrate estimation by phenol - sulphuric acid method

The biofilm extracted solution was used for the carbohydrate estimation. 1 ml of sample solution was taken in a clean test tube and made up to 1 ml with distilled water to this solution 1 ml of phenol reagent and 5 ml concentrated sulphuric acid was added. At the endpoint, the golden yellow color appeared. The standard carbohydrate was used for the calibration of carbohydrate estimation. The color was measured at 490 nm using UV-VIS-NIR spectrophotometer (CARY 500 scan).

3.6.2. Protein estimation by Bradford’s method

The biofilm extracted solution was used for the protein estimation. 1 ml of sample solution was taken in a clean test tube and made up to 1 ml with 0.1M phosphate buffer (pH 7.2). To this
solution, 0.1ml of Bradford reagent was added. At the endpoint, the blue color was appeared. The standard protein was used for the calibration of protein estimation. The color was measured at 595 nm using UV-VIS-NIR spectrophotometer.

3.7. Epi-fluorescence microscopy

The specimens were removed from the freshwater. Biofilm covered specimens were washed with sterilized distilled water to remove the unattached bacteria. The specimen with biofilm was immersed for 5 min in a 3% glutaraldehyde solution in order to fix the biofilm to the surface. After that electrode was gently rinsed with autoclaved sterile distilled water and then 2 drops of 0.01% aqueous solution of acridine orange were added. It was incubated for 10 min; the excess stain was washed with autoclaved sterile distilled water and examined by the Epi-fluorescence microscope. The bacteria were observed under oil-immersion (100X). The morphology of the isolate was studied by Epi-fluorescent microscope (Nikon E200, Tokyo, Japan). Microscope images were captured by digital camera (Nikon COOLPIX5400).

3.8. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to image the surface topography after exposure. The specimens were searched for surface features with dimensions consistent with the size of the microorganisms colonizing the surface. A Hitachi model S-3000H, Scanning electron microscope with a beam voltage set to 20 KV. Biofilm specimen washed with Milli-Q water to remove unattached bacteria was used. The sample was fixed with 0.1M phosphate buffer (pH 7.2) for 4 hrs at room temperature and then specimens were immersed in 2.5% gluteraldehyde + 4% para formaldehyde in 0.1M phosphate buffer (pH 7.2) overnight, then washed with serial ethanol wash 25%, 50%, 75% to 100% concentration to remove $\text{H}_2\text{O}$ molecules and then acetone.
(100%) wash. Then freeze-drying was maintained in incubator at 36 hrs (37°C). Dried specimens were coated with gold sputter using ion sputtering Jeol model JFC 1100 and ultimately observed SEM.

3.9. Fourier Transform Infrared spectroscopy (FTIR)

The biofilm samples were collected from specimen surface using sterilized knife and used for FTIR analysis. FTIR spectra were recorded using FT-IR spectrometer (Thermo Nicolet Model 670) equipped with a DTGS detector. All spectra were corrected for KBr background using the OMNIC software. The spectrum was taken in the mid IR region of 400-4000 cm\(^{-1}\) with 64-scan speed for all biofilm and the spectrum was recorded in the transmittance mode. Compressed pellets were prepared by mixing 2 mg of the scrapped lyophilized biofilm with 100 mg of KBr pellets were fixed in the sample holder and the analysis was carried out.

3.10. Potential measurements

The open circuit potential (OCP) of electrodes / specimens were measured using a high resistance Multimeter (Rish Multi 18S). The reference electrode, saturated calomel electrodes (SCE) were maintained in the laboratory and inter-calibrated with each other for every prior experiment. Thus, the potentials reported in this paper should be regarded as having accuracy of about \(\pm 1\) mV. OCP measurements were improvised in laboratory condition using natural freshwater. As soon as the samples were exposed in the freshwater, the initial potentials of the samples were recorded and monitored as a function of time until they reached a steady state value. Similar experiments were carried out using control. The average values for six specimens were plotted. All assessments were carried out at temperature of 30\(\pm 1\)°C.

3.11. Polarization studies
Cathodic polarization experiments were conducted in freshwater under air-saturated condition using a computer controlled Potentiostat (Gamry instruments Inc., Warminster, USA, DC105 using EChem Analyst version 5.3 software) in a one-liter electrochemical cell. A three-electrode setup was used which consists of the electrode / specimen as the working electrode, SCE as the reference electrode and a platinum sheet as the auxiliary electrode. The GCE was first exposed in the electrochemical cell for ten minutes to attain equilibrium with the electrolyte. Cathodic and anodic polarization were initiated at open circuit potential and polarized to -1.0 V and +1.2 V vs SCE at a scan rate of 0.166 mV / s respectively. The cathodic and anodic polarizations were carried out on specimen with and without biofilm and each experiment was done at least three times. IR drop compensation was not needed since this was a high conductivity electrolyte. All measurements were also carried out at 30 °C for optimum bacterial growth.

3.12. Cyclic voltammetry studies

All the cyclic voltammetry experiments were carried out using a Potentiostat / Galvanostat BAS100B workstation, USA. The cyclic voltammetry was conducted using a conventional three-electrode cell. All the cyclic voltammetry experiments using SCE. Potential cycling was carried out in the range of -0.5 V to 1.0 V vs SCE at a scan rate of 5 mV/s. Experimental data were recorded in the absence of stirring or gas bubbling into the electrolyte.

3.13. Electrochemical Impedance studies

The AC impedance studies were carried out using Gamry Potentiostat, Gamry Frame Work version 3.2, EIS 300 with lock-in-amplifier model SRB 10 DSP (Stanford research systems). The impedance was conducted using a conventional three-electrode cell. In the AC impedance test,
the specimen was kept at the OCP and a sinusoidal potential with 5 mV amplitude for the frequency region of 0.01 Hz to 30 KHz was applied using a frequency response analyzer.

3.14. Mott-Schottky studies

Mott-Shottky plots are analyzed in a conventional three-electrode electrochemical cell equipped with Gamry potentiostat (EIS 300) measurements were made using lock-in-amplifier and adequate software. The frequency used as the very commonplace value 1580 Hz. The potential scanning range was -1V to +1V vs reference electrode. The amplitude of sinusoidal voltage perturbation signal was equal to 10 mV. The Mott-Shottky plots of the 316L SS, alloy C276 and Nickel 200 were measured at ambient temperature. All the potential were expressed with respect to SCE.