

---

## MATERIALS AND METHODS

### 3.1 Bacterial sample collection

The sediment at the bottom of the container having a two year old sample of *Jatropha curcas* biodiesel was collected in a sterile container.

### 3.2 Enumeration and isolation of Bacteria

The collected sample was serially diluted (logarithmically) using 9 ml of sterile distilled water. This is then inoculated on the agar medium by pour plate technique and incubated for 24-48 hours. Petri plates having countable colonies ranging from 30-300 are chosen for enumeration and counted.

### 3.3 DNA extraction, PCR amplification and gene sequencing

#### 3.3.1 Bacterial strain

*E. coli* DH5 $\alpha$  was used as host for transformation experiments.

#### 3.3.2 Antibiotic

Ampicillin (100 mg /ml)

100 mg Ampicillin sodium salt was dissolved in 1ml sterile distilled water and stored at -20 °C.

#### 3.3.3 Stock Solutions

1M Tris-Cl (pH 8.0)

12.11 g Tris was dissolved in 80 ml distilled water, pH adjusted to 8.0 with HCl and the volume was made upto 100 ml.

0.5M EDTA (pH 8.0)

18.61 g Na<sub>2</sub> EDTA. 2H<sub>2</sub>O was added to 80 ml water and stirred vigorously. pH is adjusted to 8.0 with NaOH pellets and the volume was made up to 100 ml.

---

#### 10% Sodium dodecyl sulphate (SDS)

10 g SDS was added to 80 ml sterile distilled water, heated to 65 °C to dissolve and the volume was adjusted to 100 ml with sterile distilled water.

#### 50 X Tris Acetate EDTA (pH 7.8)

242.2 g Tris and 37.2 Na<sub>2</sub> EDTA. 2H<sub>2</sub>O were dissolved in 600 ml distilled water; pH was adjusted to 7.8 with glacial acetic acid (about 57 ml) and the volume was made up to 1 litre with distilled water.

#### Ethidium Bromide (10 mg/ml)

100 mg ethidium bromide was dissolved in 10 ml sterile distilled water by stirring overnight. The container with the solution was wrapped in aluminum foil and stored at 4 °C.

#### 6X Gel Loading dye

4 g (40% w/v) sucrose, 25 mg (0.25%) bromophenol blue and 25 mg (0.25%) Xylene cyanol were added to 8 ml distilled water and dissolved by mixing well. The volume was made up to 10 ml with distilled water, autoclaved and stored at 4°C.

#### X-gal (2 % w/v)

20 mg X-gal (5-Bromo 4-Chloro 3-Indolyl β-D-galactopyranoside) was dissolved in 1 ml Dimethyl sulfoxide (DMSO) and stored in micro centrifuge tube and wrapped in aluminum foil to prevent damage by light and stored at -20°C.

### 3.3.4 Genomic DNA isolation stocks

#### 3M Potassium acetate (pH 4.8)

29.442 g Potassium acetate was dissolved in 20 ml distilled water and the pH was adjusted with glacial acetic acid and the volume was made up to 100 ml.

#### Equilibration of Phenol

The saturated phenol was equilibrated using 0.5M Tris-Cl (pH 8.0). Then 0.1M Tris-Cl (pH 8.0) was added and equilibrated till it reaches pH greater than 7.8.

---

Equilibrated phenol was added with 0.1M Tris-Cl (pH 8.0) containing 0.2%  $\beta$ -mercaptoethanol and stored at 4 °C.

Phenol: Chloroform: Isoamyl alcohol

25 ml phenol, 24 ml chloroform and 1 ml isoamyl alcohol were mixed and stored at 4 °C.

### **3.3.5 Enzyme stocks**

Lysozyme (10 mg/ml)

10 mg lysozyme was dissolved in 1ml 10 mM Tris-Cl (pH 8.0).

Proteinase K (20 mg/ml)

20 mg Proteinase K was dissolved in 1ml solution containing 50 mM Tris-Cl (pH8.0), 1.5 mM Calcium acetate. The stock solution was stored at -20 °C.

RNase (10 mg/ml)

10 mg RNase was dissolved in 1 ml 0.01 M Sodium acetate (pH 5.2) and heated to 100 °C for 15 minutes and allowed to cool slowly to room temperature. 0.1volume of 1M Tris-Cl (pH 7.4) was added to the RNase solution. The stock was stored at -20 °C.

### **3.3.6 Genomic DNA Extraction**

The selected colonies were used to inoculate LB broth and incubated overnight at 37 °C. The culture (1.5 ml) was spun at 7000 rpm for 3 min. The pellet was resuspended in 400  $\mu$ l of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and the solution was incubated for 1h at 37 °C. To the tube, 100  $\mu$ l of 0.5M EDTA (pH 8.0), 60  $\mu$ l of 10 % SDS and 3  $\mu$ l of proteinase K from 20 mg/ml stock were added and incubated at 55 °C overnight. Extracted with equal volume of phenol: chloroform (1:1), centrifuged (10000 rpm; 10 min) and the supernatant was transferred to a sterile tube. The supernatant was extracted twice with phenol: chloroform and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. RNase treatment was done to avoid RNA contamination in the DNA sample. The

---

DNA pellet was resuspended in sterile distilled water and stored at 4 °C for immediate use and at – 20 °C for long-term storage.

### 3.3.7 PCR amplification of 16S rRNA gene

PCR amplification targeting bacterial 16S rRNA gene was performed using the eubacterial 16S rRNA primers.

#### 3.3.7.1 16S rRNA primers sequence (Teske *et al.* 2002)8F primer 5'

AGAGTTTGATCCTGGCTCAG 3' (*E. coli* positions 8 to 27)1490R primer 5'

ACGGCTACCTTGTTACGACTT 3' (*E.coli* positions 1490 to 1509)

PCR amplification was performed in PCR Sprint (Thermo Hybaid). The 50 µl PCR mixture contained 2 µl (10 ng) DNA as a template, 0.5 µM each primer, 50 µM each deoxynucleotide triphosphate, 1X PCR buffer (MBI Fermentas), 1.5 mM MgCl<sub>2</sub> and 1U Taq Polymerase (MBI Fermentas). The following thermal cyclic condition was used for amplification.

#### 3.3.7.2 PCR amplification conditions

Initial denaturation - 95 °C for 5 min

Denaturation - 95 °C for 30 sec

Annealing - 50 °C for 1 min,

Extension - 72 °C for 2 min

Final Extension - 72 °C for 10 min

#### 3.3.7.3 Purification of PCR products

PCR products originating from each nucleic acid sample were purified using Qiaquick PCR purification Kit (Qiagen) according to manufacturer's instruction.

#### 3.3.7.4 Cloning in pDrive Vector

Qiagen PCR cloning kit was used for cloning of 16S rRNA gene. Purified PCR product was ligated into the pDrive cloning vector according to manufacturer's

---

instruction. *E. coli* DH5 $\alpha$  was used as host for transformation experiment. White colonies were randomly picked from recombinant selection plates for further experiments.

### **3.3.7.5 Sequencing of 16S rRNA gene and Database searching**

16S rRNA gene sequencing for all the isolates were carried out with isolated plasmids from the clones using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For sequencing reaction, Big Dye Ready Reaction-Dideoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed. The sequences obtained were searched in GenBank of NCBI (National Centre for Biological Information) using BLAST (**Altschul *et al.* 1995**) to obtain the best homologous sequences.

### **3.3.7.6 Phylogenetic analysis**

Multiple sequence analysis was carried out with 16S rRNA sequences obtained from Genbank using CLUSTALX (**Thompson *et al.* 1997**) and further, NJ plot (**Perrière and Gouy 1996**) and PHYLODRAW (**Choi *et al.* 2000**) were employed for constructing phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

## **3.4 Selection of metals**

Carbon steel, stainless steel, aluminium and copper alloys are commonly used for making storage tanks, tankers, pipes, pumping equipments and diesel engine fuel systems. Hence for the present study Aluminium, Brass, Copper, Mild steels (two types) were chosen. Sheets of commercially available metals in the local market were purchased for the study. Elemental composition of the five selected metals was done in a laboratory SITARC, Coimbatore.

## **3.5 Preparation of test coupons**

For mass loss measurement sheets of commercially available metals were machined into coupons of size (7.5X1.9X0.3cm) as per **ASTM G184**. Holes were drilled on the top centre of the coupons.

---

### 3.6 Biodiesel procurement and characterisation

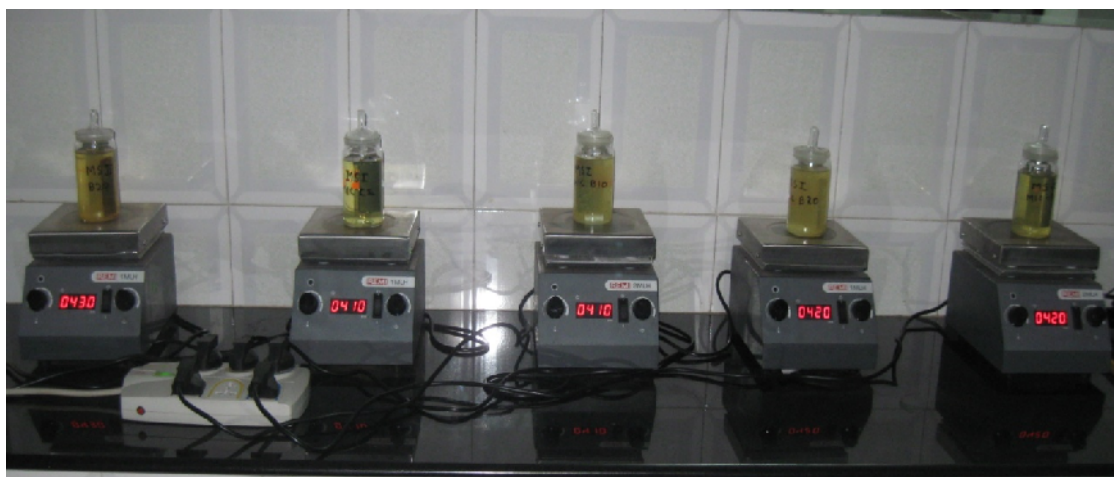
*Jatropha curcas* biodiesel was purchased from a biodiesel exporter in India and characterised in a testing laboratory which follows **ASTM D6751** procedures.

Commercial diesel was purchased from a nearby petrol bunk.

### 3.7 Corrosion rate determination

#### Mass loss method

Coupons of aluminium, brass, copper, mild steel I and mild steel II were polished with 200, 400 and 600 grit emery paper and then degreased using trichloroethylene, dried and stored in a desiccator and used.



**Figure 2. Experimental setup for mass loss measurements**

The following five fuel mixtures were used as test media:

- B100 - 100% JBD
- B5 - 5% JBD and 95% CD (% by volume)
- B10 - 10% JBD and 90% CD (% by volume)
- B20 - 20% JBD and 80% CD(% by volume)
- CD - 100% commercial diesel

**Control system:** 800 ml fuel mixture + 2% (v/v) water (500ppm chloride) to simulate corrosion conditions.

---

**Experimental system:** 800ml fuel mixture + 2% (v/v) water (500ppm chloride) + 0.5% (v/v) of bacterial inoculums (a load of  $1 \times 10^6$  CFU/ml)

The mass loss measurements were carried out using **ASTM G1** standard. Previously weighed metal coupons were immersed in the test matrices and agitated using a magnetic stirrer (**Maruthamuthu et al. 2005**). After 100 h, the coupons were removed and pickled in pickling solutions (**ASTM G3**), washed with water and dried. Final masses of the coupons in each system were taken and the mean corrosion rates (in triplicates) were calculated and expressed in mpy. The corrosion rate was calculated using the following formula.

$$\text{Corrosion rate} = \frac{3.45 \times 10^6 \times \text{mass loss(grams)}}{\text{Density} \left( \frac{\text{g}}{\text{cm}^3} \right) \times \text{Area}(\text{cm}^2) \times \text{Time}(\text{hours})}$$

### 3.8 Electrochemical measurements

Impedance and potentiodynamic polarisation measurements were performed using **GAMRY instrument reference 600** in a three electrode electrochemical cell with a platinum electrode as the counter electrode and a saturated calomel electrode as the reference electrode. Metal coupons embedded in araldite with an exposed surface area of  $1 \text{ cm}^2$  were the working electrodes. For electrochemical studies, the metal coupons were exposed to control and experimental systems same as in the mass loss method for 100 h.



**Figure 3. Electrodes for Electrochemical study**

---

The coupons that were removed from the appropriate systems after 100 h served as the working electrode. The aqueous phase from each system was transferred into the electrochemical cell to serve as the electrolyte. EIS measurements were made after steady state open circuit potential using a 10 mV amplitude sinusoidal signal over frequencies ranging from 100 kHz to 10 mHz. Tafel plots were measured with a scan rate of 10 mVs<sup>-1</sup> and were obtained by scanning from the open circuit potential towards +200 mV anodically and -200 mV cathodically.

### **3.9 Surface morphology**

#### **Optical microscopy**

After the immersion test in various test media for a period of 100 h, the metals were washed with water and then dipped in acetone to remove the oil at the surface of the metal. The corroded surface of aluminium, brass, copper, mild steel I and mild steel II coupons and their polished samples were characterised by inverted metallurgical microscope **KOZO optics model XJM 404T**.

#### **3.10 Scanning electron microscopy (SEM)**

For SEM (**ICON Analytical, Model No: FEI Quanta 200**) studies, the surface of the coupons exposed to the various test matrices for 100 hours was exposed to 2.5% glutaraldehyde for 8 hours and subsequently washed with a graded series (30%, 50%, 70% and 100%) of ethanol for dehydration. The sample was then coated with a gold alloy prior to SEM observations. The entire surface area of the coupon was examined to locate sessile bacteria.

#### **3.11 Laser Profilometry**

A **Zeta-20 True color 3D Optical Profiler** was used to map the surface topography and determine the pit distributions and depths for aluminium, brass, copper, mild steel I and mild steel II metal samples immersed in the control and experimental systems for 100 hours. 35x12 mm<sup>2</sup> areas of both faces (called inside and outside by convention) were examined by taking height measurements at 10 µm intervals in both the longitudinal and traverse directions. A peak count operation was

---

performed to determine the pit depths and densities. During the laser profilometry analysis the highest point on the specimen was assumed to correspond to the original metal surface (prior to immersion) and the pit depths are reported relative to this point.

### **3.12 Statistical analysis**

A two way ANOVA was done for the experimental results of mass loss measurements for the control and experimental systems with JBD, CD and their blends at a 5% level of confidence in order to determine the significance of the two variables viz., metal type and % of blends on the corrosion rate using **AGRES STAT** Software version 7.01