Chapter 4

Materials and Methods

4.1 Cultivation of acidophilic microorganisms

The acidophilic microbial consortium selection was done similar to “top down” approach given by Rawlings and Johnson (2007). In the ‘top down’ approach (‘see-who-wins’ approach), a mixture of micro-organisms is used to inoculate the test material (in - laboratory or pilot-scale operations) and an assumption is made that a limited number of these acidophiles will emerge as a stable and effective bioleaching consortium.

Micro-organisms used in this ‘see-who-wins’ approach were derived from natural mining environments. Acidophilic microorganisms were obtained from different ore samples.

4.1.1 Collection of ore samples-

Two ore samples were collected from different mines (Figure 3).

1. Bauxite Mine, Radhanagari, Maharashtra

Bauxite ores are abundant in this area (Plate 1). Bauxite is a naturally occurring, heterogeneous material composed primarily of one or more aluminium hydroxide minerals, plus various mixtures of silica, iron oxide, titanias, aluminosilicate and other impurities in minor or trace amounts (Formula \( \text{Al}_2\text{O}_3 + \text{SiO}_2 + \text{TiO}_2 + \text{Fe}_2\text{O}_3 \)).

2. Pyrite mine, Chitradurga, Karnataka

Pyrite ores are abundant in this area (Plate 2). The mineral pyrite (iron pyrite) is an iron sulfide with the formula \( \text{FeS}_2 \).
Plate 1. Bauxite ore sample collection from Radhanagari mines, A. Site of ore sample collection, B. Closer view of bauxite ore.
Plate 2. Pyrite ore sample collection from Chitradurga mines, A. Site of ore sample collection, B. Closer view of pyrite ore.
Approximately 20 g ore sample from each spot was taken in a polythene bag and was transported to the laboratory. The ore was finely grinded and was used for experiments.

![Map of India showing sites of ore sample collection.](image)

**Figure 3:** Map of India showing sites of ore sample collection.

### 4.1.2 Enrichment of acidophiles

10 g of each ore sample collected from two spots was added in a 500 ml capacity Erlenmeyer flask containing 100 ml of modified -9K medium. Composition of -9K medium (g/L) is given below:

- Ammonium sulfate - 3.0
- Di-potassium phosphate- 0.050
- Magnesium sulfate- 0.050
- Potassium chloride- 0.1
Calcium nitrate- 0.010

The pH was adjusted to 2.45 with 1N H₂SO₄. The flasks were kept for incubation at 30°C in an orbital rotary incubator shaker at a speed of 140 rpm for 20 days for the growth of acidophiles. After incubation 10 ml of culture medium was taken and was added in 100 ml of modified +9K medium. Modified +9K medium has the same composition as -9K medium except that 45 g/L of ferrous sulphate heptahydrate was added. The flask was incubated at 30°C in an orbital rotary incubator shaker at a speed of 140 rpm for 10 days. After incubation, 10 ml of medium was taken and was added in 100 ml of fresh modified +9K medium. The flask was incubated at 30°C in an orbital rotary incubator shaker at 140 rpm for 5 days. The growth of acidophiles in the flask was confirmed by microscopic observations of organisms in the broth (See section 4.1.3). The presence of iron oxidizers among acidophiles was confirmed by determining the ferrous iron concentration using the method mentioned in section 4.5.

The enriched acidophilic cultures obtained from two flasks with different ore samples were mixed as follows. Five ml enriched culture was taken from each flask and was added in a 500 ml Erlenmeyer flask containing 100 ml of modified +9K medium. The flask with mixed culture was incubated at 30°C in an orbital rotary incubator shaker at 140 rpm for 2-3 days. The growth thus obtained was considered as a mixed bacterial consortium (MBC) and was used for further studies.

4.1.3 Microscopic observations

4.1.3.1 Wet Mount
A drop of MBC culture was taken on a clean and grease free slide. A clean cover slip was placed on it. The slide was then observed under high power objective of light microscope. Presence of microorganisms and their morphology was noted.

4.1.3.2 Staining Method

A smear of MBC culture was prepared on a clean and grease free slide. The smear on the slide was treated with 5% ammonium thiocyanate till the red traces disappeared from slide washings. Then the slide was flooded with 1% Z.N.C.F. (Ziehl-Neelsen’s Carbol Fuchsir) and steamed for 15 minutes and care was taken such that the stain did not become dry while heating. The slide was observed under oil immersion objective after drying for detecting the presence of microorganisms. (Paknikar and Agate 1995)

4.1.4 Preservation of mixed bacterial consortium (MBC)

MBC was preserved in liquid modified +9K medium at 4°C in refrigerator. During preservation, the iron oxidizing ability of MBC was assessed for every 1 month.

4.1.5 Determination of iron oxidation activity of mixed bacterial consortium

The growth activity of bacterial consortium was determined by measuring the concentration of ferrous irons in solution during incubation. This was achieved by taking two Erlenmeyer flasks of 500 ml capacity containing 200 ml of sterile +9K medium. MBC (15%) was then added to only one of these flasks. Uninoculated flask was used as a control. Both the flasks were kept on a rotary shaker at 140 rpm at 30°C. The ferrous iron content in both flasks was estimated after every 12 h using the method mentioned in Section 4.5 and result of test flask was compared with control.
4.2 Effect of MBC inoculum concentration on iron oxidation

200 ml of modified +9K medium was added in three Erlenmeyer flasks of 500 ml capacity. Different concentrations of MBC inoculum (5 %, 10% and 15%) was added in a corresponding flask. The flasks were kept for incubation at 30°C on orbital rotary incubator shaker at 140 rpm for 48 h. The iron oxidation was confirmed both by visual observation of medium and by chemically determining the ferrous iron using the method mentioned in section 4.5.

4.3 Effect of initial pH on iron oxidation by MBC

This was achieved by using various initial pH (1.80, 2.00, 2.20, 2.40, 2.60 ±0.02) of the modified +9K medium. 200ml modified +9K medium was added in 500 ml capacity Erlenmeyer flask. pH was adjusted by digital pH meter (Elico LI 127). 15% MBC inoculum was added. The flasks were then incubated on an orbital rotary incubator shaker adjusted to 140 rpm at 30°C for about 2 to 3 days. Sample from each flask was taken for every 12 h up to 2 to 3 days for determination of pH, ferrous iron content. pH was measured using a digital pH meter (Elico LI 127) and ferrous iron content was measured by the method given in section 4.5.

4.4 Effect of initial ferrous iron concentration on iron oxidation by MBC

This was achieved by adding initial concentration of ferrous iron 5g/L, 7g/L, 9g/L, 11g/L and 13g/L in the 200 ml modified +9K medium contained in 500 ml capacity Erlenmeyer flask. pH was adjusted to 2.40. 15 % MBC inoculum was added. The flasks were then incubated on orbital rotary incubator shaker adjusted to 140 rpm at 30°C up to 10 days. Sample from each flask was taken for every 12 h for about 2 to 3 days for determination of pH and ferrous iron content. pH was measured using a
digital pH meter (Elico LI 127) and ferrous iron content was measured by the method given in section 4.5.

4.5 Determination of iron oxidation ability

4.5.1 Visual observation

As the ferrous iron (Fe²⁺) oxidizes to ferric iron (Fe³⁺), the resultant ferric iron gives reddish or brownish colour to medium. This reddening or browning of medium was taken as a measure of iron oxidation. More the reddening or browning of medium, more was the amount of ferrous iron that was oxidized.

4.5.2 Gravimetric method

1 ml of broth sample was taken in 250 ml of Erlenmeyer flask, 2 ml of 10M H₂SO₄ was added in it and then 0.5ml n-phenanthranilic acid reagent was added. Finally 25 ml of distilled water was added. The solution was mixed well and was titrated against 0.025N K₂Cr₂O₇ to obtain the end point at pale green to pink. Amount of K₂Cr₂O₇ required to produce the end point (burette reading) was taken for calculating the amount of ferrous ions. Decrease in the concentration of ferrous ions indicates the oxidation of ferrous ions.

Preparation of n-Phenylanthanilic acid -

0.1 g of n-Phenylanthanilic acid was dissolved in 100 ml of 0.005N NaOH.

1 ml of 0.025N K₂Cr₂O₇ = 1.389 mg of Fe²⁺
4.6 Preparation of printed circuit board powdered sample

Printed circuit board assemblies (PCBA) were collected from scrap market. PCBA was made up of different electronic components such as RAM, PCI slot, condensers, transistors, heat sink etc. attached to printed circuit board (PCB) (Plate 3).

Attached plastic parts viz. RAM, PCI slot, chip slots were removed from printed circuit board. Printed circuit board is made up of metals required for working of electronic equipment and support of non-metals. These printed circuit boards were shredded by using a file. The powder thus obtained was composed of metals as well as nonmetals (composition varies, but mainly contains epoxy or phenolic resin).

The powder was sieved using a sieve of size BSS- 18, ASTM-20 having pore size 0.85 mm to get the powdered sample. The powder without any pretreatment was directly used for bioleaching studies.

4.7 Chemical analysis of printed circuit board powder

Chemical analysis of waste printed circuit board powder was carried out to determine the heavy metal content of PCB powder. It was determined by two different ways -

I. 1g of homogenized powder was added in 100 ml of aqua regia. It was heated for about one hour and filtered through Whatman filter paper no. 1. The total volume was made up to 100 ml using double distilled water. This sample was used for the detection of heavy metals with the help of atomic absorption spectrophotometer.

II. 1 g of homogenized powder was added in a 100 ml of volumetric flask. Few drops of concentrated HNO₃ were added in the flask and volume was made to 100 ml with double distilled water. The sample was then filtered with Whatman filter
Plate 3: Different types of waste printed circuit boards used in the study (A-F).
paper no. 1 prior to heavy metal analysis. Heavy metals were detected using atomic absorption spectrophotometer.

4.8 Determination of copper tolerance of mixed bacterial consortium

As the Printed circuit board contains more amount of copper, it was decided to first check the initial copper tolerance of MBC.

200 ml of modified +9K medium was taken in 500 ml Erlenmeyer flasks. pH was adjusted to 2.42. 15% inoculum (MBC) was added to it. 1M CuSO₄ was prepared and was added in these flasks so as to achieve the final concentrations in the range of 1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM and 20mM. These flasks were kept on orbital rotary incubator shaker with 140 rpm at 30°C for 10 days. Metal tolerance was determined by estimating ferrous iron content of medium for every 24 h. Ferrous iron content was estimated using a method as mentioned in section 4.5.

4.9 Bioleaching of metallic copper

As printed circuit board powder contains more amount of metallic copper, the ability of MBC to solubilize metallic copper was tested.

Copper turnings of length <0.5mm and width <0.05mm were taken in varying concentrations ranging from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 % (w/v) in modified +9K medium (Plate 4A). pH was adjusted to 2.40. 15% MBC inoculum was added to it and the flask was incubated at 30°C on orbital rotary incubator shaker at 140 rpm for 10 days. Metal tolerance was determined by estimating ferrous iron content of medium for every 24 h. Ferrous iron content was estimated using a method as mentioned in section 4.5. Bioleaching of copper was confirmed by visually
observing the presence of copper turnings in the flask. Bioleaching of copper was also confirmed by estimating soluble copper content using atomic absorption spectrophotometer.

4.10 Bioleaching of zinc

As moderate concentration of Zn was present in PCB, it was decided to check the zinc solubilizing ability of MBC.

Zinc dust was taken in varying concentrations ranging from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 % (w/v) and was added in 500 ml Erlenmeyer flask containing 200 ml of modified +9K medium, with addition of 15% inoculum. Flasks were kept for incubation on rotary shaker at 140 rpm at 30°C up to 10 days. Metal tolerance was determined by estimating ferrous iron content of medium for every 24 h. Ferrous iron content was estimated using a method as mentioned in section 4.5.
Bioleaching of zinc was confirmed by estimating soluble zinc content using atomic absorption spectrophotometer.

4.11 Bioleaching of lead from solder

As printed circuit board contains solder (tin + lead), it was decided to check the solubility of lead by MBC.

Solder was purchased from market and was shredded into pieces of 1-2 cm in length. These pieces were used in different concentrations like 0.1% w/v and 0.2% w/v and were added in 500 ml capacity Erlenmeyer flask containing 200 ml of modified +9K medium (Plate 4B). pH was adjusted to 2.42. 15% inoculum was added to that flask and the flasks were incubated on orbital rotary shaker at 30°C at 140 rpm. Ferrous iron content was estimated using a method as mentioned in section 4.5. Bioleaching of lead was observed by atomic absorption spectrophotometer.

4.12 Bioleaching of metals from waste printed circuit boards using powdered sample

1.5 % (w/v) PCB powder was added in 500 ml Erlenmeyer flask containing 200 ml of modified +9K medium previously adjusted to pH 2.40. 15% inoculum was added to it and flasks were kept on rotary shaker at 140 rpm and 30°C. Samples were taken after every 48 h up to 10 days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elico LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.13 Effect of different parameters on bioleaching of metals from PCB powder
4.13.1 Effect of PCB powder concentration

This was achieved by adding PCB powder (10, 20, 30, 40 and 50 g/L) in the 200 ml of modified +9K medium of pH 2.42 taken in 500 ml Erlenmeyer flask. 15% MBC inoculum was added. The flasks were then incubated on rotary shaker adjusted to 140 rpm at 30°C for about 10 days. Samples were taken after every 48 h up to 10 days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elico LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.13.2 Effect of initial pH

This was achieved by using various initial pH (1.80, 2.00, 2.20, 2.40, 2.60 ±0.02) of the modified +9K medium. 200 ml modified +9K medium was taken in 500 ml capacity Erlenmeyer flask. pH was adjusted by digital pH meter (Elico LI 127). 15% MBC inoculum was added. 10 g/L PCB powder was added. The flasks were then incubated on rotary shaker adjusted to 140 rpm at 30°C up to 10 days. Samples were taken for every 48 h up to 10 days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elico LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.13.3 Effect of initial concentration of ferrous iron

This was achieved by adding ferrous iron in concentration of 5 g/L, 7 g/L, 9 g/L, 11 g/L and 13 g/L in the 200 ml modified +9K medium of pH 1.8 contained in
500 ml capacity Erlenmeyer flask. 15 % MBC inoculum was added. 10 g/L PCB powder was added. The flasks were then incubated on orbital rotary incubator shaker adjusted to 140 rpm at 30°C up to 10 days. Samples were taken after every 48 h up to 10 days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elico LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.14 Detection of heavy metals during bioleaching

4.14.1 Detection of heavy metals in leachate by atomic absorption spectrophotometer (AAS)

Heavy metals present in leachate was determined by atomic absorption spectrophotometer (AAS).

Sample preparation for AAS analysis

Sample taken for heavy metal detection was first filtered with Whatman filter paper no. 1 in a sample tube of capacity 25 ml. To the 20 ml of sample, 2 ml concentrated nitric acid was added so as to avoid jarosite formation. The samples were then kept at 4°C till the analysis.

Detection of heavy metals

The samples were directly used for the detection of heavy metals using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300). The samples were analyzed using a standard operating procedure of atomic absorption spectrophotometer. Samples were diluted whenever necessary according to range of
particular metal determination. The metal concentration in sample was determined and final reading was multiplied by dilution factor.

4.14.2 Detection of heavy metals in precipitate by Scanning Electron Micrograph and Energy Dispersive X-ray Analysis (SEM-EDAX)

Heavy metals present in precipitate were determined using SEM-EDAX. The precipitate formed during bioleaching was taken, dried and mounted on carbon-coated copper grids for SEM-EDAX. Micrographs were obtained using a JEOL (6360) JED-2300 analysis station operating at 200 kV. Concentration of particular heavy metal was then determined using micrographs.

4.15 Bioleaching of metals from waste printed circuit boards using large pieces

Sample Preparation

Use of large pieces of printed circuit boards instead of using its powder was attempted. If the PCBs are directly placed in contact with bacterial consortium it results in no leaching. This is due to the chemical coating of the PCBs which may be green, red, blue or orange in colour. The chemical coating doesn’t allow the bacteria to penetrate through it and thus the bacteria fail to reach to the metal. The removal of such chemical coating can solve the problem; hence this was carried out by using chemical method. In chemical method PCBs were dipped overnight in a 10M NaOH. The PCBs were then washed under running tap water. The washed water was replaced by fresh distilled water until the adhered NaOH was removed (approximately 4-5 times). This was checked by determination of pH of washed water. Neutral pH of washed water confirms the complete removal of NaOH (Plate 5).
Plate 5: Waste printed circuit boards, A. Before chemical treatment, B. After chemical treatment.
4.15.1 Bioleaching of printed circuit board pieces (of size 12 × 6 cm) in tray

PCB was cut into two large pieces of size 12×6 cm. Each piece was added in an individual plastic tray. 1 L of modified +9K medium was added in each tray. pH was adjusted to 2.42. 15% inoculum was added and the tray was kept on orbital rotating shaker adjusted at 50 rpm at 30°C for 10 days. Samples were taken for every 48 h up to 10 days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elcito LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.15.2 Bioleaching of printed circuit board pieces (of size 4 ×2.5 cm) in Erlenmeyer flasks

Printed circuit boards were broken into pieces of having approximate size 4 × 2.5 cm. Two pieces were added in the 500 ml Erlenmeyer flasks containing 200 ml modified +9K medium. pH was adjusted to 2.42. MBC inoculum (15%) was added in it and the flasks were kept for incubation on orbital rotary shaker adjusted to 140 rpm at 30°C. Samples were taken for every 48 h up to 10days for determination of pH, ferrous iron content and soluble metal content. pH was measured using a digital pH meter (Elcito LI 127), ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

4.16 Bioleaching of metals from PCB powder using immobilized MBC

4.16.1 Preparation of Immobilized MBC
After cultivation followed by centrifugation at 1500 g for 10 min to remove precipitate, the bacterial cells in the supernatant were harvested by centrifugation at 10 000 g for 15 min. The cell pellets were washed for three times with dilute H₂SO₄ (pH 1.5) and finally resuspended in a small volume of sterilized water.

Sodium alginate was used for the immobilization. 15 ml MBC cell suspension was added in equal volume of sodium alginate (4%). That solution was thoroughly mixed. The mixture was added drop wise in chilled CaCl₂ (1M) with the help of pipette. Formed Beads were kept as it is in chilled CaCl₂ for overnight, for hardening of the beads.

4.16.2 Iron oxidation by immobilized MBC

Immobilized beads were added in a 500 ml capacity Erlenmeyer flask containing 200 ml of modified +9K medium. The flask was then incubated at 30°C in an orbital rotary incubator shaker adjusted to 140 rpm for 48 h. Another flask with non immobilized cells (15% MBC inoculum) was kept as a control to compare the iron oxidizing activity (by determining concentration of ferrous iron using method mentioned in section 4.5) of both.

4.16.3 Bioleaching of PCB powder using immobilized MBC

Immobilized beads were added in a 500ml capacity Erlenmeyer flask containing 200 ml of modified +9K medium. pH was adjusted to 2.42. 10 g/L PCB powder was added to it. The flask was then incubated at 30°C in an orbital rotary shaker adjusted to 140rpm. Another flask with non immobilized cells (15% MBC inoculum) was kept with that flask to compare the bioleaching efficiency of both. Samples were taken for every 48 h up to 10 days for determination of pH, ferrous iron
content and soluble metal content. pH was measured using a digital pH meter, ferrous iron content was measured by the method given in section 4.5 and soluble metal content (Cu, Pb, Zn and Ni) was detected using atomic absorption spectrophotometer (Perkin Elmer A Analyst 300).

### 4.17 Isolation and Identification of acidophiles present in mixed bacterial consortium

Developed microbial consortium at the end of all bioleaching experiments was used for isolation and identification.

### 4.17.1 Isolation of acidophiles using various solid media

**Isolation of chemolithotrophic iron oxidizers by using solid medium**

The obtained mixed bacterial consortium (MBC) was used for the isolation of chemolithotrophic iron oxidizers. The medium was prepared by using three different solidifying agents separately,

1. Agar agar 3.2%
2. Agarose 1% (Sisco medium EEO, type I)
3. Gelrite 1%

50 ml of modified -9K medium (double strength) having pH 2.45 was prepared, to this solidifying agent was added. It was then digested by keeping in heating mantle with intermittent shaking (Do not shake vigorously, as it leads to bubble formation) After digestion of solidifying agent, 50 ml of FeSO₄·7H₂O (double strength) previously adjusted to pH 2.45 was added to it and mixed properly, then the plates were poured.
After solidification 0.1ml of MBC was spreaded on the plates. The plates were kept for incubation at 30°C till the development of colonies (up to 15 days).

After incubation development of dark brown/ reddish brown colonies on medium confirms the presence of iron oxidizers.

**Isolation of acidophilic heterotrophic iron oxidizers by using solid medium**

The obtained mixed bacterial consortium (MBC) was used for the isolation of acidophilic heterotrophic iron oxidizers. As agarose has given better results as a solidifying agent, it was decided to use agarose for preparing the medium. The medium was prepared as follows,

50ml of modified -9K medium (double strength) was prepared with addition of 0.02% yeast extract. 1% agarose (Sisco medium EEO, type l) was added to it and was digested in heating mantle with intermittent shaking (Do not shake vigorously, as it leads to bubble formation). After complete digestion of solidifying agent, 50 ml of FeSO₄·7H₂O solution was added to it so as to attain the final concentration of 800mM of FeSO₄·7H₂O in the medium. The plates were poured. After solidification the plates were spreaded with 0.1ml of the MBC and kept for incubation at 30°C till the development of colonies (up to 15 days).

After incubation, presence of cream colored colonies on medium shows the presence of acidophilic heterotrophic iron oxidizers.

**Isolation of chemolithotrophic iron oxidizers, iron oxidizing heterotrophs and non iron oxidizing heterotrophs by using single solid medium (Fe-TSB Medium)**

A Fe-TSB medium was prepared as follows,

Part A:
Ammonium sulfate 0.36g
Magnesium sulfate 0.14g
Tryptone soya broth 0.070g

The components were added in 250 ml capacity Erlenmeyer flask containing 140ml double distilled water. pH was adjusted to 2.3.

Part B: 70 ml double distilled water was taken in 150 ml conical flask and 2.0 g of Agarose (Sisco medium EEO, type I) was added to it. pH was kept neutral.

Part A and B were sterilized by autoclaving at 121°C for 15 min at 15lb pressure.

Part C: 6.9g of Ferrous sulfate heptahydrate was added to 10 ml of double distilled water. pH was adjusted to 2.15- 2.3. This solution has 2.5 Molarity. The solution was filter sterilized by using 0.5 µ cellulose nitrate filter (following aseptic conditions). 1.5 ml solution of C was added to part A. After this solution B was added to that mixture, and plates were poured aseptically. Medium gets solidified within 10-15 minutes. After solidification 0.1 ml of MBC was spreaded on the medium and plates were kept for incubation at 30°C till the development of colonies (up to 15 days).

Development of red/brown colored colonies indicate presence of iron oxidizers, development of white colonies indicate presence of heterotrophic organisms.

**Isolation of acidophilic, non iron oxidizing heterotrophs by using solid medium**

The medium used for isolation of non iron oxidizing heterotrophs was same to Fe-TSB medium except no ferrous sulphate was added and TSB concentration was 0.4 %. After solidification the medium was spreaded with MBC and incubated at 30°C till the development of colonies (up to 15 days). After incubation, development
of small white colored colonies on medium shows the presence of acidophilic non iron oxidizing heterotrophs.

4.17.2 Molecular techniques for identification of acidophiles present in MBC

Identification of MBC was performed by two methods, 
1. Denaturing Gradient Gel Electrophoresis (DGGE)
2. PCR based detection using species specific primers

For performing these methods DNA isolation and amplification of DNA using PCR was done as mentioned below.

DNA isolation method using SIGMA Kit

In this method genomic DNA was isolated using Gen Elute™, Bacterial genomic DNA kit (SIGMA# NA 2110) in the following steps-
Step 1- Resuspension of cell pellet in lysis solution T (180 µl).
Step 2- Treatment with RNase A (20 µl, 20 mg/ml),
Step 3- Incubation at room temperature (25± 3°C) for 2min.
Step 4- Treatment with proteinase K at 55°C for 30 min.
Step 5- Addition of lysis solution C (200 µl).
Step 6- Incubation at 65°C for 1 hr,
Step 7- Addition of ethanol (200 µl),
Step 8- Transfer of the mixture to binding column followed by centrifugation (6500 × g, 2min)
Step 9- Washing of the column with wash solution concentrate.
Step 10- The genomic DNA bound to the column was eluted with elution
solution (200 μl) in a new tube.

**PCR conditions and techniques**

PCR amplification was performed in a master cycler gradient thermocycler (Eppendorf, Germany). PCR mixtures contained (per 20 μl of reaction mixture) equimolar concentration of all the primers (Sigma, USA, 0.2uM) and deoxynucleotides (Sigma USA: dATP, dCTP, dCTP and dGTP, 0.2 mM); 1X Taq polymerase buffer (optimized MgCl₂ concentration) and 1U of Taq polymerase (Banglore Genei India). PCR amplification was performed with this basic format: initial denaturation at 94⁰C for 60s, optimized annealing temperature for 60s and 72⁰C for 60s.

A 20 minute final extension at 72⁰C was performed at the end of cycling steps and reaction mixtures were stored at 4⁰C until further use. Agarose gel electrophoresis and subsequent staining with ethidium bromide were used to verify the presence and size of all amplified products. These PCR reactions were performed under the conditions optimized for the respective primer sets.

### 4.17.2.1 Denaturing Gradient Gel Electrophoresis (DGGE)

1. Sample (80 ml of MBC) was centrifuged at 10,000 rpm for 10 min.

   Supernatant was discarded and cell pellet was washed with 500 μl of saline.

2. Genomic DNA was isolated using GenElute™ Bacterial genomic DNA kit (# NA-2120, SIGMA).

3. PCR amplification of 16S rDNA (1.5 kb) and its internal fragments (600 bp, 400 bp and 200 bp were carried out. Fig.1 shows the amplification of 1.5 kb long 16S rDNA fragment with primers FDD2 and RPP2. (4361, 564 and 125
bp bands can be very faint and may not be clearly visible on the gel (Plate no.).

4. Amplification was also seen in PCR reactions targeting the 600 bp, 400bp and 200bp fragments of 16S rDNA.

5. The PCR amplicons ~ 600 bp and 400 bp were electrophoresed on DGGE gel with a denaturing gradient range of 30 – 50 % (6 % acrylamide) at 130 V for 5 h.

6. Bands resolved on DGGE were excised and eluted in MilliQ water O/N. This eluted DNA was reamplified, purified and sequenced on ABI 3100 sequencer.

**Sequencing of DGGE bands**

The amount of DNA eluted from the DGGE band is not enough for sequencing. So a new PCR amplification for each band is necessary. The PCR conditions are the same as those described above. After cleaning the PCR products, sequencing was performed using the same primers that were used for amplification.

**4.17.2.2 PCR based method with species specific primers**

1. Genomic DNA was used as template for amplification of 16S rDNA fragments using species specific primers. (*Leptosprillum* specific primers were specific for *Leptospirillum* group. Not specific for any particular species of *Leptospirillum*)
2. PCR was carried out in two sets. 1st set of primers used to amplify 16S rDNA fragments. The 2nd set of primers was used to perform a nested PCR in which PCR products of 1st PCR reaction was used as the template (table 2).

**Table 2.** Primers used in PCR mediated identification of Microorganisms using species specific primers.

<table>
<thead>
<tr>
<th>Targeting organism</th>
<th>First round PCR primers</th>
<th>Second round PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acidiphilium</em></td>
<td>Acido-594f</td>
<td>Acido-594f</td>
</tr>
<tr>
<td></td>
<td>Eub-1492r</td>
<td>Acido-1150r</td>
</tr>
<tr>
<td><em>Leptospirillum</em></td>
<td>Eub- 27f</td>
<td>Lepto-176f</td>
</tr>
<tr>
<td></td>
<td>Lepto 679r</td>
<td>Lepto-679r</td>
</tr>
<tr>
<td><em>Sulfobacillus</em></td>
<td>Eub-27f</td>
<td>Sulf-170f</td>
</tr>
<tr>
<td></td>
<td>Sulf-1137r</td>
<td>Sulf-606r</td>
</tr>
<tr>
<td><em>Thiobacillus caldus</em></td>
<td>Cald-460f</td>
<td>Cald-460f</td>
</tr>
<tr>
<td></td>
<td>Eub-1492r</td>
<td>Cald-1475r</td>
</tr>
<tr>
<td><em>Thiobacillus thiooxidans</em></td>
<td>Thio-458f</td>
<td>Thio-458f</td>
</tr>
<tr>
<td></td>
<td>Eub1492r</td>
<td>Thio-1473r</td>
</tr>
<tr>
<td><em>Thiobacillus ferroxidans</em></td>
<td>Ferro-458f</td>
<td>Ferro-458</td>
</tr>
<tr>
<td></td>
<td>Eub-1492r</td>
<td>Ferro-1473</td>
</tr>
</tbody>
</table>

(Durand *et al.*, 1997)