CHAPTER 3

3. MATERIAL & METHODS

3.1 STUDY AREA (Tirap Colliery)

The Makum Coalfield is located in Tinsukia district of Upper Assam. It is about 80 km long and about 5 km wide tract over the Patkai foothills of Himalayas. Coalmine regions are located in between latitudes 17° 23' - 27° 23' N and longitudes 95° 40' - 96° 35' E. The general elevation above minimum sea level (msl) near the plains of river Buri-Dihing is 140 meter rising to 300 to 500 meter on the Patkai Naga Range. The Makum Coalfield consist of six working mines which include two opencast mines namely Tirap and Tikak Colliery & four underground mines viz, Tipong, Ledo, Borgolai and Dilli-Jaipur Colliery.

The Tirap Colliery of the Makum Coalfield is industrially developed since long ago and is well communicated both by roads and railways. The National highway No. 38 runs along the Northern extremity of Coalfield connecting Margherita, Ledo and Lekhapani, the important trading centers of the area. The climate is tropical monsoon. The annual temperature in winter falls up to 4 °C and the maximum summer temperature to 36 °C. The rainy season is confined mainly from June to September with average annual rainfall ranging from 3000 to 4250 mm. The average humidity ranges from 87- 91 percent during wet months.
Regreening of degraded soil of Tirap Colliery of Makum Coalfield of Assam, India

Fig 5. Map of Tinsukia District of Assam, India.

Fig 6. Enlarged view of study area of Tirap Colliery.
3.2 MATERIALS

Higher plants and microorganisms used in the soil remediation study are given below-

(i) Plant materials: *Macroptilium atropurpureum*, Family: Fabacieae
   Local name: Siratro, Dhansa

*(Cajanas cajan*, Family: Fabacieae
   Local name: Arhar

(ii) Entrapped Microbial Consortia (Mixed microbial species) associated with the roots of test plants in the study area.

(iii) Potential microbes: Species of *Rhizobium*, *Pseudomonas*, and *Glomus* selected from the entrapped microflora.

3.3 METHODOLOGIES

3.3.1 SITE SELECTION

Benchmark sites were selected from Tirap Colliery of Makum Coalfield of Tinsukia district of Upper Assam, with or without spars vegetation (Figs. 5 and 6).

3.3.2 SOIL ANALYSIS

Both rhizosphere and non-rhizosphere soil were collected randomly in sterile polythene bags from selected sites and aseptically carried to the laboratory for further investigation. Collected soil samples were sieved through 2 mm screen and air-dried for further study.
The physico-chemical parameters of soil samples were analyzed according to standard method available in the literature are described below. The results were recorded in Table 5 and 6 and Fig 8 respectively.

3.3.2.1 Soil texture

3.3.2.2 Water holding capacity (WHC)

3.3.2.3 Soil moisture content (SMC)

3.3.2.4 pH

3.3.2.5 Conductivity

3.3.2.6 Organic carbon and organic matter

3.3.2.7 Available phosphorus

3.3.2.8 Available nitrogen

3.3.2.1 Soil texture

Soil texture was determined with Bouyoues procedure described by Trivedi et al. (1987). 50 gm properly air-dried and sieved (0.2 mm) soils were taken in a beaker. To this soil, 25 ml of 5% calgon and 400 ml distilled water was added. The suspension was mixed thoroughly by stirring for 15 minutes with the help of an electrically driven high speed stirrer. The contents was then transferred to a 1 litre graded measuring cylinder removing all the adhered soil in
the beaker with the help of stream of distilled water and the volume was made up to 1 litre. The initial reading was taken with the help of soil hydrometer along with the initial temperature reading. The system was allowed to stand for 4 hours and final data was taken. The percentage of clay, silt and total sand was calculated by the following formula.

\[
\text{Clay} \, (\%) = \frac{A \times 100}{S} - 1 \quad (1 \text{ is subtracted as calgon correct})
\]

\[
\text{(Silt + Clay)} \, (\%) = \frac{B \times 100}{S} - 1 \quad (1 \text{ is subtracted as calgon correct})
\]

\[
\text{Silt} \, (\%) = (\text{Silt + Clay}) \, (\%) - \text{Clay} \, (\%)
\]

\[
\text{Total sand} \, (\%) = 100 - (\text{Silt + Clay}) \, (\%)
\]

Where, \( A \) = Final hydrometer (gm litre\(^{-1}\)) reading
\( B \) = Initial hydrometer (gm litre\(^{-1}\)) reading
\( S \) = Weight of the air dried soil (gm)

3.3.2.2 Water Holding Capacity

Water Holding Capacity of the soil samples was measured by the procedure described by Trivedi et al. (1987):
1. Weighed the dry filter paper and again weighed it after saturating with water.

2. Weighed the brass box accurately and the wet filter paper was placed in the brass box and then box was filled with soil sample (without any gravels) tightly and weighed.

3. The soil was saturated with distilled water by keeping it in the silver tray for 24 hrs. After 24 hrs weighed the box with saturated soil. Then the box with saturated soil was kept in the air-dry oven at 105 °C for 24hrs and weighed after 24 hrs.

3.3.2.3 Soil Moisture Content

Soil moisture content was measured by the method described by Trivedi et al. (1987). 10 gm of fresh sieved soil samples was taken in petridishes and kept in oven at 105-110 °C for 24 hrs. Allowed to cool and weighed.

3.3.2.4 pH

The pH of the collected soil samples was determined by standard method (Trivedi et al. 1987) with the help of pH meter Elico LI 120. 5 gm of sieved soil sample was placed in a 250 ml beaker and 25 ml distilled water in the ratio of 1:5 was added to it. The suspension was stirred and within 60 seconds the value was taken with pH meter after the glass electrode was immersed in the freshly shaken suspension.
3.3.2.5 Conductivity

The conductivity of the soil samples was determined by standard method (Trivedi et al. 1987) with the help of conductivity meter Elico CM 180. 5 gm of sieved soil sample was placed in a 250 ml beaker and 25 ml distilled water in the ratio of 1:5 was added to it. The suspension was stirred and allowed to settle for 1 minute and then the value was taken with the help of conductivity meter.

3.3.2.6 Organic carbon and organic matter

Organic carbon was determined by Walkley and Black’s rapid titration method described by Trivedi et al. (1987). This method is based upon the oxidation of carbon by nascent oxygen, which is liberated from potassium dichromate in presence of H$_2$SO$_4$. 1 gm of air dried and sieved (0.2 mm) soil was taken in a dry and cleaned 500 ml flask along with 10 ml of 1 N potassium dichromate (K$_2$ Cr$_2$ O$_7$) [49.04 gm of ( K$_2$ Cr$_2$ O$_7$) was dissolved in distilled water and the volume was made up to 1 litre] and 20 ml of concentrated sulphuric acid (H$_2$SO$_4$). The flask was then left for 30 min. After the reaction was over, the content was diluted with 200 ml of distilled water. To this solution, 10 ml of 85% phosphoric acid (H$_3$PO$_4$) and 1 ml of diphenylamine [0.5 gm diphenylamine was mixed with 100 ml concentrated H$_2$ SO$_4$ and 200 ml distilled water] as an indicator was added. The color was changed to bluish purple. The content was titrated with 1 N ferrous ammonium sulphate [Fe (NH$_4$)$_2$ (SO$_4$)$_2$.6H$_2$O] solution [393.13 gm of Fe (NH$_4$)$_2$ (SO$_4$)$_2$. 6H$_2$O was dissolved in 15 ml concentrated H$_2$SO$_4$ and distilled water simultaneously, to prevent
hydrolysis of the salt and the volume was made up to 1 litre with distilled water] until the blue colour changed to brilliant green. The end point was very sharp in titration. The same procedure was also followed for the blank sample. The organic carbon was determined with the following formula:

$$\text{Organic Carbon (\%) = \left( \frac{V_1 - V_2}{W} \right) \times 0.003 \times 100}$$

$$\text{Organic matter (\%) = \% of organic carbon \times 1.724}$$

Where,

$$V_1 = \text{Volume of 1N potassium dichromate}$$

$$V_2 = \text{Volume of 1 N ferrous ammonium sulphate}$$

$$W = \text{Weight of the soil sample}$$

### 3.3.2.7 Available phosphorus (P)

Dilute acid extraction method was used for measuring available soil phosphorus (P) as proposed by Jackson (1973). The available P was extracted in 0.002 N sulphuric acid ($H_2SO_4$). [A stock solution of 0.1 N $H_2SO_4$ was prepared by diluting concentrated $H_2SO_4$ 360 times (i.e. 2.78 ml concentrated $H_2SO_4$ and the final volume was made up to 1000 ml with distilled water). Volume of this stock solution was diluted 50 times to prepare 0.002 N $H_2SO_4$]. 1 gm of air dried sieved (0.2 mm) soil was taken in a 500 ml conical flask where 200 ml of 0.002N $H_2SO_4$ solution was added and stirred for 30 minutes. It was filtered through whatman No. 1 filter paper. 50 ml aliquot was pipetted out in a 100 ml
volumetric flask and 200 ml of ammonium molybdate [(NH₄)₆ Mo₇ O₂₄] solution and 5 drops of stannous chloride (SnCl₂) reagent was added to the aliquot. Various dilutions (0.1, 0.5, 1.0, 5.0 ppm) were prepared from standard phosphate solution. 50 ml of each dilution was taken and the entire reagent was added as mentioned above. The absorbance of the solution was taken at 690 nm in UV-visible Spectrophotometer, with blank of distilled water with the same amount of chemicals. Reading was taken after 5 minutes. The concentration of P was calculated with the help of standard curve and was calculated with the help of the following formula as mentioned below:

\[ P \text{ in soil (ppm)} = P \text{ in solution (ppm)} \times \frac{\text{Total extraction solution used (ml)}}{\text{Weight of soil sample (gm)}} \]

3.3.2.8 Available nitrogen (N)

Total nitrogen of the soil samples was estimated by Micro-kjeldahl method (Trivedi et al. 1987). 5 gm of air-dried sieved soil sample (0.2 mm) was taken into a digestion flask along with 0.2 gm of digestion catalyst and 10 ml of H₂SO₄. The content was mixed by swirling the flask with care. The digestion catalyst was prepared with 1 gm copper sulphate (Cu₂SO₄. 5H₂O) with 8 gm potassium sulphate (K₂SO₄) and 1 gm selenium (Se) powder. The digestion was commenced at low heat at first to avoid frothing over. The heat was then increased and the digestion was continued until the acid boiled, condensed and the contents turned apple green in colour. The digestion flask was cooled and a
little distilled water was added and the final volume was made up to 100 ml in volumetric flask. It was then filtered through whatman No.1 filter paper. The diluted digested material was distilled by micro-kjeldahl distillation apparatus. 25 ml of the digest was introduced into the micro-kjeldhal distillation apparatus through the side tube by replacing the glass stopper. Thereafter 15 ml of 40% NaOH was added slowly through the funnel to the digest. Outlet of the distillation unit was immersed into the 5 ml of 4% boric acid (H$_3$BO$_3$) and mixed indicator solution. The mixed indicator was prepared with 4% H$_3$BO$_3$ by mixing with 5 ml solution of bromocresol green (0.5%) and methyl red (0.1%) in 2:1 ratio. The mixed indicator turned blue due to dissolution of ammonia. The distillate collected in the boric acid with indicator was titrated with 0.01 N HCl (0.83 ml HCl was added with 100 ml of distilled water), until the blue colour just disappeared and a faint pink colour was developed which indicated the end point. The blank was prepared using all the chemicals except the soil sample and the same procedure of digestion, distillation and titration was followed. The volume of 0.01 N HCl required was recorded and the quantity of nitrogen present in the sample was calculated from the following formula:

\[
\% \text{ of N in soil} = (A - B) \times N \times V \times \frac{1.4}{v \times S}
\]

Where,

- \(A\) = Sample titration reading
- \(B\) = Blank titration reading
- \(N\) = Normality of HCl
V = Total volume of the digest
v = Total volume of the digest distilled
S = Weight of the sample

3.3.3 WATER ANALYSIS

The water samples were collected separately into 1-litre plastic cans from selected sites and aseptically carried to the laboratory and stored in a cold room for further investigations.

3.3.3.1 pH

The water samples (50 ml) were taken in a 250 ml beaker and the pH of collected water samples were measured using pH meter (Elico LI 120).

3.3.3.2 Conductivity (EC)

50 ml of water sample was taken in a 250 ml beaker and the conductivity of the water samples were measured using conductivity meter (Elico CM 180).

3.3.3.3 Sulphate (SO$_4^{2-}$)

The sulphate ion SO$_4^{2-}$ in water samples were measured by Turbidimetric method described by Clesceri et al. (1998). 10 ml of water sample was taken into a 250 ml of conical flask and 20 ml of buffer solution was added to it. The content was mixed in stirring apparatus. A spoonful of BaCl$_2$ crystals
was added to the stirring solution and immediately the time was recorded. The solution was stirred at constant speed for 60 ± 2 seconds. After stirring the solution was poured into absorption cell of photometer and turbidity was measured at 5 ± 0.5 minutes. The concentration of $\text{SO}_4^{2-}$ in water sample was calculated with the help of calibration curve and was calculated with the help of the following formula as mentioned below:

$$\text{mg} \frac{\text{SO}_4^{2-}}{L} = \text{mg SO}_4^{2-} \times \frac{100}{\text{mL sample}}$$

If buffer solution A (Appendix II) was used, sulphate ion concentration was determined from the calibration curve after subtracting sample absorbance before adding $\text{BaCl}_2$.

If buffer solution B (Appendix II) was used, sulphate ion concentration of blank was subtracted from apparent sulphate ion concentration as determined above.

### 3.3.4 HEAVY METAL ESTIMATION OF WATER AND SOIL SAMPLES

For the heavy metal (Cadmium (Cd), Copper (Cu), Lead (Pb) and Zinc (Zn)) analysis the collected water and soil samples were analyzed with the help of Atomic Absorption Spectrophotometer (Varian model spectra 220 FS) by the standard method (Pinta 1975) as described below and the results thus obtained were presented in Table 8 and 9.
Procedure for sample preparation

3.3.4.1 Soil sample

1 gm of air-dried sample was taken in a beaker. 10 ml of Tri acid mixture (Conc. H$_2$SO$_4$, Conc. HNO$_3$ and Conc. HCl in 4: 2: 1 ratio) was added to it. The beaker was then kept on the Hot Plate and the mixture was allowed to evaporate till dryness or changes to white powdery form. Then 10 ml of dil. HCl (1:1) added to it. The solution was filtered through Whatman filter paper. The volume was made up to 50ml by adding distilled water.

The final sample solution (50 ml) was analyzed for the estimation of heavy metals using Atomic Absorption Spectrophotometer (Varian model spectra 220 FS).

3.3.4.2 Water sample

50 ml of water sample was taken in a beaker. 5 ml Conc. HNO$_3$ was added to it. The beaker was then kept on the Hot Plate and the mixture was allowed to evaporate till dryness or changes to white powdery form. The volume was made up to 50 ml by adding distilled water.

The final sample solution (50 ml) was analyzed for the estimation of heavy metals using Atomic Absorption Spectrophotometer (Varian model spectra 220 FS).
### Table. 4. Atomic absorption spectrophotometer (AAS) operating conditions

<table>
<thead>
<tr>
<th>Analytical Conditions</th>
<th>Cd</th>
<th>Cu</th>
<th>Zn</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length (nm)</td>
<td>228.8</td>
<td>324.7</td>
<td>213.9</td>
<td>217.0</td>
</tr>
<tr>
<td>Silt width (nm)</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Operating working range</td>
<td>0.02-3.00</td>
<td>0.03-10</td>
<td>0.01-2</td>
<td>0.1-30</td>
</tr>
<tr>
<td>HC lamp current (mA)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Fuel gas flow rate (L/min)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Air flow rate (L/min)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Type of flame</td>
<td>Air – C₂H₂</td>
<td>Air – C₂H₂</td>
<td>Air – C₂H₂</td>
<td>Air – C₂H₂</td>
</tr>
</tbody>
</table>

#### 3.3.5. MEDIA USED FOR ISOLATION OF MICROORGANISMS

#### 3.3.5.1 Methods and media used for isolation of fungi and bacteria from the soil samples are described below:

- **(a) Fungal media**

  Czapek Dox Agar Medium (Appendix I) was used to isolate fungal species and to maintain fungal cultures. The media was dispensed in 10 ml borosil test
tubes or to borosil conical flasks. Sterilization works was performed at 15 psi pressure for 15 minutes in autoclave.

(b) Bacterial media

Nutrient Agar Medium (Appendix I) was used as basal medium for isolation of bacterial species and to maintain bacterial cultures. The medium was dispensed in 10 ml borosil test tubes or to borosil conical flasks. Sterilization works were performed at 15 psi pressure for 15 minutes in autoclave.

(c) Pseudomonas Agar

Pseudomonas Agar (Appendix I) was used to isolate and maintain the species of bacteria like *Pseudomonas* sp.

(d) Yeast Extract Manitol Agar (YEMA medium)

YEMA medium (Appendix I) containing Congo red was used to isolate and maintain the species of *Rhizobium*.

3.3.5.2 Methods and media used for studying the population dynamics of different types of bacteria in soil

Population dynamics of different types of bacteria in the collected soil samples were studied using specific media for each group using conventional serial dilution method and procedure described by Chatterjee and Chandra (2005). Soil suspension were diluted to $10^{-2}$ to $10^{-7}$ level and suspension was
added to desired medium in separate petriplates. The plates were incubated at 30 ± 0.5 °C in a BOD incubator and the number of colonies were counted (Pelczer et al. 1957, Holt 1984, Collee and Miles 1989 and Lacey 1997). The results were presented in Table 11.

a) Gram- negative bacterial population determination

To assess the population of gram-negative bacteria, soil suspensions were plated on petriplates containing NA medium (Appendix I) containing 0.01 gm of crystal violet, incubated in a BOD incubator at 30 ± 0.5 °C for 72 hours. The bluish-violet colonies were counted and the results were recorded in Table 11.

b) Nitrifying (NH₄⁺ oxidizing) bacterial population determination

The nitrifying (NH₄⁺ oxidizing, NH₄⁺ to NO₂⁻) bacterial populations was determined in Nitrosomonas medium (Winogrodsy’s medium) or ammonium sulphate- agar medium (Appendix I). Soil suspensions were plated on petriplates containing Nitrosomonas medium and were incubated for 11 to 30 days at 30 ± 0.5 °C and flooded with sulfanillic acid reagent. The pink colonies on the petriplates were counted and the results were recorded in Table 11.

c) Denitrifying (NO₃ reducing) bacterial population determination

The denitrifying (NO₃ reducing, NO₃⁻ to NO₂⁻) bacterial populations was determined on Winogrodsy’s medium (replacing ammonium sulphate with potassium nitrate or sodium nitrate from the media). The petriplates containing media were inoculated with soil suspensions and were incubated for 72 hours at
30 ± 0.5 °C and flooded with sulfanillic acid reagent. The pink colonies on the petriplates were counted and the results were recorded in Table 11.

d) Phosphate solubilizing bacterial population determination

The phosphate solubilizing bacteria (PSB) were determined on calcium phosphate agar medium (Appendix I). The petriplates were inoculated with soil suspensions and incubated for 72 hours or more at 30 ± 0.5 °C. The colonies having a clear zone around them were counted and recorded in Table 11.

3.3.6 ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM SOIL AND TEST PLANTS (PRIMARY ENTRAPPED PLANTS)

3.3.6.1 Fungal isolation and identification:

Conventional soil plate method (Warcup 1950, 1955) was used with serial dilution technique to obtain fungal culture from soil as well as from entrapped model. Isolated microorganisms were characterized and identified with cultural characteristics and their microscopic observations using Stereoscopic and compound microscope (Leitz, Germany). For fungal identification spore characters like size, shape, colour, conidial length, structure of mycelia and conidiophores etc. were observed and are compared with literature (Gilman 1995, Barnett & Hunter 1972, Holt J G et al. 1984, Breed et al. 1957, Varma 1998 and Varma et al. 1999) and the results were recorded in Table 14, Figs. 11(a) to 11(g) and Fig. 12.
3.3.6.2 Bacterial isolation and identification:

Conventional soil plate method (Warcup 1950, 1955) was used with serial dilution technique to obtain bacterial culture from soil as well as from entrapped model. Characterization and identification of bacteria was done with conventional microscopic observations and biochemical analysis viz. Methyl Red Test, Acetyl Methyl Carbonyl Tests, Catalase Test, Triple Sugar Iron Agar (H₂S, Glucose, Glucose + Dextrose), Citrate utilization test, Urolytic property test, Gelatin test, NO₃ Reduction test, Starch Hydrolysis test, Indole production test and Congo red test. Identification was done based on standard literature and books (Breed et al. 1957 and Holt J. G. et al. 1984) and the results were recorded in Tables 15, 16 and Figs. 13(a) to 13(d).

3.3.7 ISOLATION, PRESERVATION AND IDENTIFICATION OF A M FUNGI

3.3.7.1 Isolation

Isolation of A M Fungi was done according to wet sieving and detecting methods with graded sieves as used by Gerdemann and Nicolson (1963). For isolation of AM Fungi, 10 gm of soil was suspended in 200 ml of double distilled water and suspension was stirred for 2 minutes until all soil aggregates disperse to leave a uniform suspension. After this, the suspension was kept aside and allowed to settle down the heavier particles. Suspension was decanted through a 710 µm sieve to remove large organic matter and roots. Again 200 ml of double distilled water was added to the suspension and decanted this
suspension through 450, 250, 150 and 45 μm sieves consequently. Different spores of A M Fungi were obtained in different sieves according to their sizes. Each sieve was washed with double distilled water and suspension was allowed to pass through Whatmann filter paper. Finally spores in the filter paper were washed, collected, counted and examined under stereoscopic microscope (40X).

3.3.7.2 Preservation

During counting morphologically similar spores were picked up, surface sterilized with 2% Chloramin T (W/V) + 2% Streptomycin (W/V) and kept in eppendorf tube containing sterile distilled water and preserved in a refrigerator (4±0.5 °C ) for observation, identification and inoculum production. The treatment gave complete sterilization and it was found not detrimental to their viability.

3.3.7.3 Identification

Isolated spores of AM Fungi were studied under microscope by mounting them in slides containing water. Slide were also prepared with 0.01% trypan blue-lactic acid staining solution (875 ml lactic acid + 63 ml Glycerol + 63 ml tap water + 0.1 gm Trypan blue) to study the spore characteristics under the microscope. Spores were studied in respect of their morphological characters particularly the size, shape, color, spore wall characteristics etc. Diagnostic slides were prepared using PVLG and Melzer’s regent (Appendix II), to study their detailed description. Spores were mixed with the mount in a clean microscopic slide with the help of needle. A clean, dry cover slip was then
placed over the mount containing spores with utmost care. The excess water was cleaned off using cotton swab moistened with alcohol. For observing wall characters, some spores were broken by applying light pressure on cover slip. The prepared slides were observed under compound microscope (Olympus) for size, color, shape, wall structure, attached hyphae and form of sporocarp etc. The observations were compared with the species guide provided in the INYAM manual. Identification was done with the help of manual for Identification of V A Mycorrhiza (Schenck & Pervez 1990), other literature in the journals (Gerdemann and Nicolson 1963, Morton 1988) and also from internet. The results obtained were recorded in Table 13, 14 and Figs. 11(h) to 11(k).

3.3.8 INOCULUM PRODUCTION

3.3.8.1 Bacteria

Pure bacterial culture was inoculated to sterilize nutrient broth in 100ml conical flask and kept in BOD incubator at 30 ± 0.5 °C. The broth containing the pure bacterial culture was used as inoculum.

3.3.8.2 Fungi (AM Fungi)

AM Fungi being an obligate parasite cannot be grown for small or large scale production. They can only be grown in a living host. Several practices have been found in literature as proposed by Menge and Johnson (1978), Hayman (1982), Mosse and Thomson (1984), Dalpe and Monreal (2004). However, in this piece of work root organ culture technique as reported by Subba Rao (1999)
was adopted in maize as host plant with some modifications where found necessary. The preserved spores isolated from overburden soil of Tirap Colliery were grown on Maize roots by root culture technique. For the root culture technique, healthy and uniform sized seeds of maize were selected and surface sterilized by dipping the seeds in 0.01% HgCl₂ for 4 min and washed several times with sterile distilled water. The seeds were dried in hot air oven at 26 ± 0.5 °C in sterile condition.

A spore suspension of isolated and preserved AM Fungi was made with a spore concentration of 100 spores, ml⁻¹. Mycorrhization of the sterile soils in the sterile pots (4 kg, pots⁻¹) were done with soil drenched/soil mixture method (Menge and Timmer 1984, Bhowmik and Singh 2004). Two hole (2 cm diameter and 1 cm depth) was made in the autoclaved soil of each pots and 1 ml of spore suspension was placed in each hole followed by sowing of one/two sterilized maize seed. The hole was covered evenly with soil of nearby hole. After 90 days, the plants were harvested and the pot soil was used for mycorrhizal spore isolation and the mass cultured spores were purified and preserved by the method as described in page no. 58 and 59. The roots were harvested, chopped, dried at room temperature and was aseptically stored maintaining the moisture content (10%), temperature (15-30 °C) and pH (in an around 6) of the carrier materials for desired self-life period mostly for 3-6 months.
3.3.8 PHYSIOLOGICAL STUDIES OF BACTERIAL ISOLATES

3.3.8.1 Effect of pH of medium on growth of bacterial isolates

Bacteria grow fairly well over a wide range of pH. In many cases microorganisms itself, as a result of its metabolism, play a major role in setting pH of its environment. Acid producing bacteria and molds tend to grow best at moderately low pH values. Other bacteria raise the pH of their environments and thrive in alkaline condition. To study the effect of pH on growth, bacteria were grown in nutrient broth (5 ml) and incubated aerobically each at following pH 2.5, 4.5, 7.0, 8.5 for 36 hours. Turbidity of the suspensions was measured in Spectrophotometer at 620 nm and the results were presented in Fig. 14(a).

3.3.8.2 Effect of Temperature on Growth of bacterial isolates

Different types of microbes have different requirements of temperature at which they grow. Microorganisms usually show growth within a certain range of maximum and minimum temperatures. The growth of bacterial culture attains the maximum at a certain degree of temperature called the optimum temperature when other conditions remain same. The optimum temperature for growth is normally correlated with temperature of a normal habitat of that particular microorganism. To study the effect of temperature on growth, bacterial isolates were grown in nutrient broth (5 ml) and incubated aerobically each at following temperatures 15, 25, 30, 45 ± 0.5°C for 36 hours. Turbidity of the suspensions
was measured in Spectrophotometer at 620 nm and the results were presented in Fig. 14(b).

3.3.9 EXPERIMENTAL STUDY

3.3.9.1 Entrapment Technique

In entrapment technique, test plants (primary plants) were used for capturing microbial consortia. The test plants consisted of fast growing symbiotic nitrogen fixing local Siratro plant (*Macroptilium atropurpureum*) and Arhar plant (*Cajanas cajan*). On several locations of the collieries at which sparse natural vegetation occurred in the degraded soil, test plants were placed for entrapping native microorganisms. The seeds of the test plants were used for raising seedlings. The roots of the primary plants were heavily packed with normal soil protected by nylon textile and planted in the polluted sites where natural vegetation occurred. Capturing of native microbial consortia with test plants (primary plants) by entrapment technique is diagrammatically represented in Fig. 7.

3.3.9.2 Isolation and Identification of entrapped microorganisms

After contamination of the root system of test plants by the entrapment technique, the test plants (primary entrapped plants) were removed from field and carried to the laboratory for further experiment. They served on one hand to the characterization of the entrapped microorganisms by conventional microbial and biological methods as given earlier and the results were presented in Table...
14, 15 and 16. On the other hand, they were used to inoculate secondary plants, which served to produce material for re-greening polluted / degraded soils and also for maintaining the entrapped microorganisms.

3.3.9.3 **Domestication of secondary plants with entrapped microorganisms** and **production of secondary plants.**

The inoculation of secondary plants from the primary entrapped plants was performed on earthen pots (6 kg capacity) with the test plants, Siratro and Arhar. Surface sterilized seeds of the test plants were used for raising seedlings. The overburden / degraded soil (350 kg approx.) was collected in a seven bags of 50 kg capacity from Tirap Colliery and was carried to the laboratory, IASST, Guwahati. The soil was sterilized to eliminate the resident micro flora of the soil by autoclaving. The pots were thoroughly washed and autoclaved soil was transferred to earthen pots (4 kg, pots$^{-1}$). The surface sterilized seeds (20 seeds, pots$^{-1}$) were then sowed in the periphery of the pots. Once the shooting and rooting is established, primary entrapped plants were planted in the middle of each pots closed to the secondary plantlets. The secondary plants (with entrapped microorganisms) thus obtained were used for the experimental studies using different Treatments (Treatment 1, 2 and 3) for regreening of degraded soil of Tirap Colliery.
3.3.9.4 Inoculation of secondary plants with selected microorganisms

The secondary plants obtained as above were further inoculated with selected potential microorganisms *viz. Pseudomonas* sp., *Rhizobium* sp., and *G. mosseae* (Table 17) directly as inoculum into the soil. These secondary plants inoculated with selected potential microorganisms were used for Treatment study (Treatment 2 and 3).

3.3.10. EXPERIMENTAL STUDY IN POTS

3.3.10.1 Treatment details

- **T<sub>0</sub>** – Control (Secondary plants without native entrapped microbial consortia).
- **T<sub>1</sub>** – Secondary plants (Secondary plants with entrapped microbial consortia).
- **T<sub>2</sub>** – Secondary plants + Potential inoculated microorganisms (Secondary plants with both entrapped microbial consortia and potential inoculated microorganisms).
- **T<sub>3</sub>** – Secondary plants + Inoculated microorganisms + FYM (Farmyard manure) (Secondary plants with entrapped microbial consortia, potential inoculated microorganism and FYM).
Plant cultivars: Variety: Two (2)

\[ V_1: Macroptilium atropurpureum \] (Siratro)

\[ V_2: Cajanas cajan \] (Arhar)

Total Replications: 3 (R_1, R_2 and R_3)

Total treatments: Varieties X Treatments X Replications

\[ 2 \times (3+1) \times 3 = 24 \]

3.3.10.2 Treatment combinations

For variety 1: \( M. \ atropurpureum \) (Siratro)

<table>
<thead>
<tr>
<th>( V_1T_0R_1 )</th>
<th>( V_1T_1R_1 )</th>
<th>( V_1T_2R_1 )</th>
<th>( V_1T_3R_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_1T_0R_2 )</td>
<td>( V_1T_1R_2 )</td>
<td>( V_1T_2R_2 )</td>
<td>( V_1T_3R_2 )</td>
</tr>
<tr>
<td>( V_1T_0R_3 )</td>
<td>( V_1T_1R_3 )</td>
<td>( V_1T_2R_3 )</td>
<td>( V_1T_3R_3 )</td>
</tr>
</tbody>
</table>

For variety 2: \( C. \ cajan \) (Arhar)

<table>
<thead>
<tr>
<th>( V_2T_0R_1 )</th>
<th>( V_2T_1R_1 )</th>
<th>( V_2T_2R_1 )</th>
<th>( V_2T_3R_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_2T_0R_2 )</td>
<td>( V_2T_1R_2 )</td>
<td>( V_2T_2R_2 )</td>
<td>( V_2T_3R_2 )</td>
</tr>
<tr>
<td>( V_2T_0R_3 )</td>
<td>( V_2T_1R_3 )</td>
<td>( V_2T_2R_3 )</td>
<td>( V_2T_3R_3 )</td>
</tr>
</tbody>
</table>
3.3.10.3 Plantation study

Experiments were performed in earthen pots with 2 varieties of test plant viz *M. atropurpureum* (Siratro) and *C. cajan* (Arhar) in the month of late November 2008 to April 2009. Pots were thoroughly washed and filled with overburden soil collected from Tirap Colliery and brought to the laboratory for the experimentation. Ten numbers of one month old secondary entrapped plantletts were transplanted at 1.5 cm depth in each pot containing about 4 kg of over burden soil. Each treatment was repeated three times to maintain the homogeneity among treatments. Survivability of the test plants, growth of test plants, nodules yield of test plant and pods production are the parameters which were studied. Estimation of available P, nitrogen, organic carbon and matter of the rhizosphere soil of the transplanted secondary plants were also studied.

Experiments have been practiced in pots at the experimental field of Biofertilizer unit, life science division of Institute of Advanced Study in Science and Technology (IASST).

3.3.10.4 Monitoring of the transplanted secondary plants

The secondary plants transplanted to the pots were studied for the parameters mentioned below:

1. Survivability of the test plants.
2. Growth of test plants.
3. Pod yield of test plants.

The secondary entrapped plants (one month old seedlings) transplanted to the pots have been monitored for 3 months and the results were recorded in regular manner (15, 30, 45, 60, 75 and 90 Days) for survivability, plant height and pod yield of test plants. However, total numbers of root nodules were counted in fresh roots of each pot of replicate treatments by uprooting them after the completion of 3 months. Estimation of organic carbon and matter, total nitrogen and available P of the rhizosphere soil of the transplanted secondary plants were done as per procedure described by Trivedi et al. (1987) and Jackson (1973). The results obtained from the experiments have been presented in Table 18 to Table 26 and Fig.15 to Fig.20.
Regreening of degraded soil of Tirap Colliery of Makum Coalfield of Assam, India

Chapter 3

Contaminated soil with spontaneous vegetation and adaptation mediating microorganisms

Fig. 7. Schematic representation of Entrapment Technique.